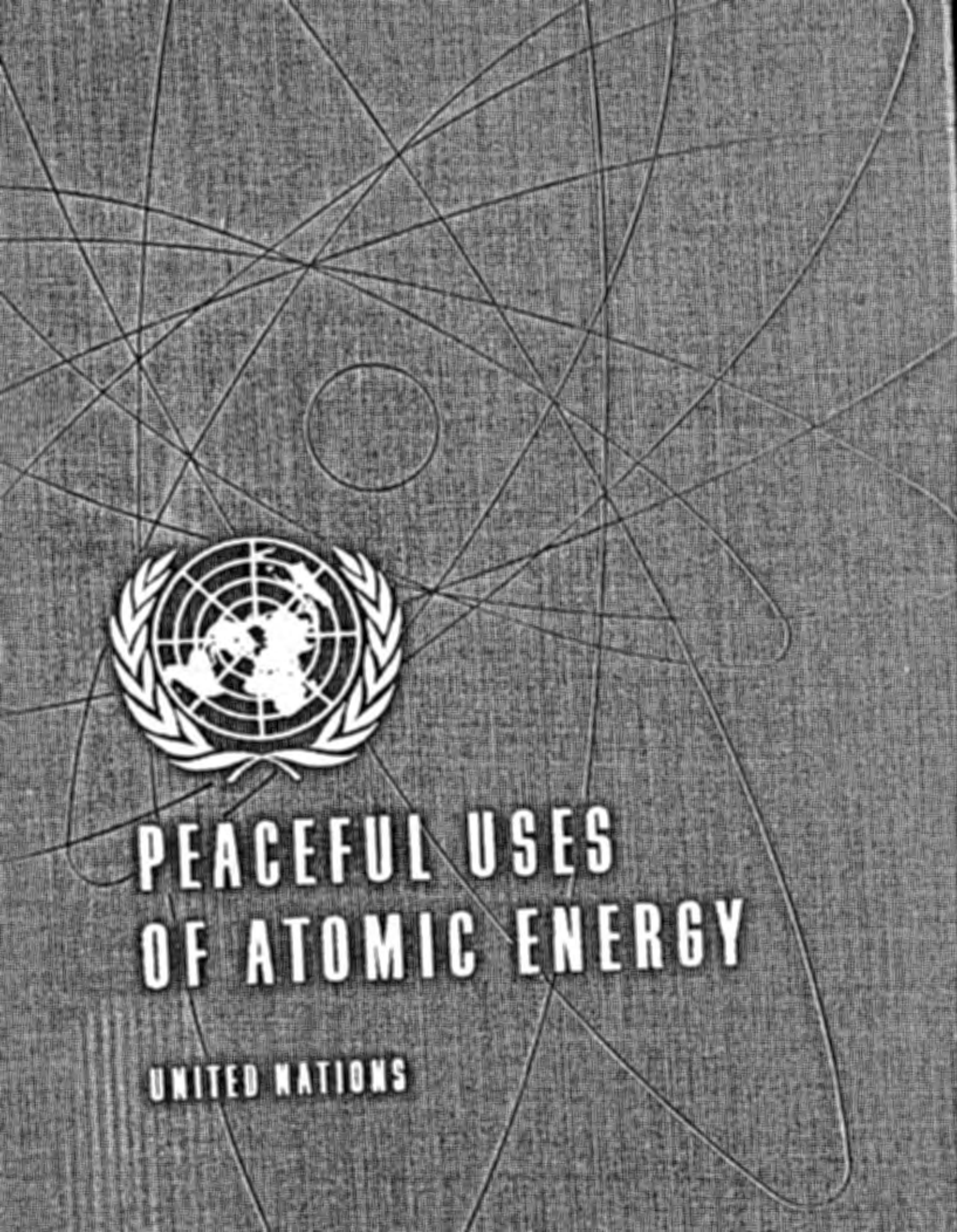

PEACEFUL USES OF ATOMIC ENERGY

UNITED NATIONS

Proceedings of the International Conference on the Peaceful Uses of Atomic Energy

Held in Geneva
8 August - 20 August 1955

Volume 12

Radioactive Isotopes and Ionizing Radiations in Agriculture, Physiology, and Biochemistry

UNITED NATIONS
New York
1956

UNITED NATIONS PUBLICATION

Sales No.: 1956. IX.1. Vol.12

A/CONF.8/12

Price: U.S. $9.00; 63/- stg.; 39.00 Sw. frs.
(or equivalent in other currencies)

PREFACE

The Proceedings of the International Conference on the Peaceful Uses of Atomic Energy are published in a series of 16 volumes, as follows:

Volume Number	Title	Sessions Included
1	The World's Requirements for Energy; The Role of Nuclear Power	2, 3.2, 4.1, 4.2, 5, 24.2.
2	Physics; Research Reactors	6A, 7A, 8A, 9A, 10A.1.
3	Power Reactors	10A.2, 3.1, 11A, 12A, 13A, 14A.
4	Cross Sections Important to Reactor Design	15A, 16A, 17A, 18A.
5	Physics of Reactor Design	19A, 20A, 21A, 22A, 23A.
6	Geology of Uranium and Thorium	6B, 7B.
7	Nuclear Chemistry and the Effects of Irradiation	8B, 9B, 10B, 11B, 12B, 13B.
8	Production Technology of the Materials Used for Nuclear Energy	14B, 15B, 16B, 17B.
9	Reactor Technology and Chemical Processing	7.3, 18B, 19B, 20B, 21B, 22B, 23B.
10	Radioactive Isotopes and Nuclear Radiations in Medicine	7.2 (Med.), 8C, 9C, 10C.
11	Biological Effects of Radiation	6.1, 11C, 12C, 13C.1.
12	Radioactive Isotopes and Ionizing Radiations in Agriculture, Physiology and Biochemistry	7.2 (Agric.), 13C.2, 14C, 15C, 16C.
13	Legal, Administrative, Health and Safety Aspects of Large-Scale Use of Nuclear Energy	4.3, 6.2, 17C, 18C.
14	General Aspects of the Use of Radioactive Isotopes; Dosimetry	7.1, 19C, 20C.
15	Applications of Radioactive Isotopes and Fission Products in Research and Industry	21C, 22C, 23C.
16	Record of the Conference	1, 24.1, 24.3.

These volumes include all the papers submitted to the Geneva Conference, as edited by the Scientific Secretaries. The efforts of the Scientific Secretaries have been directed primarily towards scientific accuracy. Editing for style has been minimal in the interests of early publication. This may be noted especially in the English translations of certain papers submitted in French, Russian and Spanish. In a few instances, the titles of papers have been edited to reflect more accurately the content of those papers.

The editors principally responsible for the preparation of these volumes were: Robert A. Charpie, Donald J. Dewar, André Finkelstein, John Gaunt, Jacob A. Goedkoop, Elwyn O. Hughes, Leonard F. Lamerton, Aleksandar Milojević, Clifford Mosbacher, César A. Sastre, and Brian E. Urquhart.

The verbatim records of the Conference are included in the pertinent volumes. These verbatim records contain the author's corrections and, where necessary for scientific accuracy, the editing changes of the Scientific Secretaries, who have also been responsible for inserting slides, diagrams and sketches at appropriate points. In the record of each session, slides are numbered in numerical order through all presentations. Where the slide duplicates an illustration in the submitted paper, appropriate reference is made and the illustration does not appear in the record of the session.

Volume 16, "The Record of the Conference," includes the complete programme of the Conference, a numerical index of papers and an author's index, the list of delegates, the records of the opening and closing sessions and the complete texts of the evening lectures.

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Session 7.2 (Agriculture)

ISOTOPES IN MEDICINE, BIOLOGY AND AGRICULTURE

LIST OF PAPERS

The Utilization of Radioactive Isotopes in Biology and Agriculture in the USSR

By A. L. Kursanov, USSR

The production of isotopes in the USSR has provided new and broad possibilities for scientific research in biology and agriculture. Artifically produced radioactive isotopes and also stable isotopes have now become available means of research extensively used by scientific workers in our country, who strive to perfect the methods used for nutrition of cultivated plants and to improve their economic qualities. At the present time biologists and agronomists are using radioactive isotopes, mainly as "labelled," or "tagged," atoms. This method, better than any other, permits one to investigate the processes that take place in the soil, the ways in which plants consume nutritive elements, the movement of various compounds in plant tissues, and, finally, the most intimate metabolic reactions in plant cells.

There is no doubt that the application of tagged atoms (as well as any other method, for that matter) calls for the careful appraising of the results obtained. For instance, the extreme sensitivity of the isotopic method sometimes forces the experimenter to forget the principal mass of the substance which he investigates and to consider secondary phenomena, comprising but a small part of the general process. In other cases, biologists have to take into consideration the possibility of direct incorporation of radioactive atoms in the substance, as a result of non-biological isotopic exchange. These phenomena should not be confused with the true course of biological transformations. In spite of certain peculiarities of the tracer method it remains extremely important.

The possibility of directly applying the energy of radioactive disintegration to stimulate, or in some cases to retard, the development of plants, which is done without injury to man, is also of great interest to agriculture. Serious attention is also given to the problem of applying the energy of radioactive disintegration to make more radical changes in the structure and heredity of organisms.

In the Soviet Union, where agriculture is largely mechanized, there are extensive possibilities for a rapid practical application of new methods of introducing fertilizers and cultivating plants, provided that these methods are substantiated by scientific research and checked by practice. At the present time, when extensive measures are being undertaken in our country for the further development of agriculture, there appear extremely wide possibilities for the practical application of new scientific achievements. They can be used on virgin lands, under conditions of irrigated agriculture as well as when cultivated plants are acclimatized in new districts, etc. For this reason, workers in agricultural experimental establishments are greatly interested in the possibility of applying radioactive and stable isotopes to solve the problems facing them.

It has been only recently that radioactive elements have begun to be applied in the practical work of biological and agricultural laboratories. Yet even during this short period of time, isotopic indicators have helped to disclose many new aspects of plant life. Such knowledge makes it possible to improve the nutrition and breeding of agricultural crops. Further study of plants by the application of radioactive elements will, undoubtedly, bring to light many new and unexpected aspects of plant life.

The present paper, which describes some of the more important results obtained in the Soviet Union where plants were studied with the help of labelled atoms, is, therefore, by no means a summary of all that has been achieved. It merely gives a description of the scope and the general direction of these rapidly developing investigations. In our paper we describe only the investigations carried on in the Soviet Union and will not mention investigations undertaken in other countries. The reason for this is our desire to give a fuller account of the application of isotopic tracers in the spheres of biology and agriculture which is undertaken in our own country. In giving this limited description we must not, however, forget that in other countries where the tracer method is applied many important results have already been achieved.

To provide Soviet biologists with information about the successes achieved abroad in the application of isotopes, a special magazine, "The Action of Radiations and the Application of Isotopes in Biology," is published in the USSR. It is a widely circulated journal which is of great assistance in the coordination of our work. Comparison of numerous investigations leads one to the conclusion that scientists of various countries could successfully join

Original language: Russian.

their efforts to solve urgent problems of biology and agriculture by means of radioactive isotopes.

To insure the extensive development of work on the application of radioactive isotopes in the sphere of biology and agriculture it was necessary to construct new apparatus and apply new methods. For example, some workers have suggested the use of instruments which, with the help of $C^{14}O_2$, automatically and accurately record the course of photosynthesis and respiratory metabolism in plants (V. Shirshov, V. Rachinsky and others); simple field methods for physiological experiments with radioactive carbonic acid have been worked out (V. Zholkevitch and others); various dosimeters have been designed which help to determine radioactivity on uninjured plants (S. Tselishchev) and thus make it possible to follow substances tagged by the isotope in the plant organism. The technique of radioautography and microradioautography has been thoroughly elaborated (Y. Mamul and others); this ensures fresh possibilities for visual investigation of the processes occuring in the plant as a whole and in its various tissues.

In their researches Soviet scientists are widely using the plants themselves to obtain preparations of sugar, amino acids, alkaloids, phosphoric ethers and many other substances marked by the isotopes of carbon, sulphur, phosphorus or nitrogen (V. Merenova, A. Kuzin, O. Pavlinova and others). These preparations are successfully applied in physiological observations on the movement and transformation of substances in organisms.

Finally, in more refined investigations, whose aim is the detailed study of separate biochemical reactions, one may apply chemically synthesized compounds marked with carbon or other elements in a strictly defined position (M. Shemyakin, B. Savinov, N. Melnikov, K. Bokarev, V. Baskakov and others).

Root nutrition is the most easily controlled aspect of the physiological development of plants. For this reason many scientists, in applying radioactive phosphorus, calcium, sulphur and other elements, have concentrated their attention on the problems of distribution and transformation of nutritive substances in the soil and on their assimilation by plants. By the application of radioactive phosphorus it was possible to change the previously existing opinion that only 10–12 per cent of phosphoric fertilizers were assimilated by plants. This opinion was formed as a result of the comparison of the general amount of phosphorus present in the yields of plants grown on fertilized and non-fertilized soils; however, no distinction was made between the phosphorus present in the soil itself and the phosphorus introduced with the fertilizer. But the experiments conducted by A. Sokolov demonstrated that when phosphate fertilizers marked by, say, P^{32} of the double superphosphate are introduced into the soil, wheat and other plants first of all assimilate the phosphorus of fertilizers, to the extent of 48–68 per cent. At the same time the amount of phosphorus assimilated from the soil itself is reduced. These results prove that plants assimilate much larger quantities of phosphorus fertilizer than previously supposed and they compel us to look for ways of increasing the percentage of phosphates assimilated by plants directly from the soil.

Another problem of vital importance for agriculture that can now be easily solved is the rational distribution of fertilizers in the soil to ensure a most rapid and complete assimilation of fertilizers by the roots of plants. This problem is particularly important for the granulated phosphoric fertilizers which are lately being widely applied in the USSR. If we place the granules of fertilizers labelled with radioactive phosphorus into different sections of the soil, we can easily see that the isotope appears in the leaves only 15–20 minutes after the contact of the rootlet with the source of P^{32}. This makes it possible, by observing the appearance of the first signs of radioactivity in the leaf lamina, to establish exactly the moment when the roots come in contact with the fertilizers. And by watching further increases in radioactivity we can also determine the rate at which a given fertilizer is assimilated.

For instance, employing this method in his experiments with oats, E. Ratner showed that if phosphorus labelled with the isotope is introduced into the soil and placed 3–4 cm below the seeds, "contact" between the roots and the fertilizer occurs in only 2–3 days after the germination of the seeds; but when the granules are displaced 5–6 cm sideways or placed much deeper than the seeds this "contact" is postponed to 3–4 weeks. Consequently, the beginning of phosphorus assimilation, which is so vital for young plants, is also postponed.

Similarly, at the present time radioactive phosphorus (P^{32}) is being used to test the efficiency of various methods of mechanized fertilization in planting cotton in the fields of the Tajik SSR (I. Antipov-Karatayev and I. Lipkind), in the Uzbek SSR (F. Reshetnikov) and in other districts of Central Asia. Undoubtedly, in the future isotopes will be of still greater importance when the various methods of mechanized fertilization are appraised.

By extensively employing P^{32} in their field experiments V. Klechkovsky and N. Kashirkina were able to study in every detail how different plants assimilate phosphorus fertilizers in the presence of other elements with varying soil humidity, liming and other conditions. It is quite obvious that such information can successfully be used to improve the methods of nutrition of agricultural crops. Many experimental stations in the Soviet Union are already extensively using these possibilities. Suffice it to say that in 1954 dozens of experimental stations used superphosphate marked with P^{32} in their field experiments.

The introduction into the soil of radioactive isotopes of several elements can also be utilized to observe the distribution of the root system, which was previously done by laborious and far from perfect methods of digging out roots and freeing them from earth. By introducing fertilizers tagged with radioactive isotopes into various soil strata, the researcher can easily observe the distribution of roots in different strata, without disturbing the plant structure, by simply watching for the appearance of radioactivity in the plant's leaves. In this way it is possible to study the influence of soil cultivation, of methods and time of irrigation, and of temperature and other factors on the development of root systems in plants. Such information is sometimes necessary for the proper nutrition of plants under specific conditions.

The application of radioactive phosphorus (P^{32}) to the study of the absorbing function of roots has permitted investigators to discover in this process, which seemed to be so well known, some new and interesting features which provide a theoretical basis for the application of granulated fertilizers. By experimenting with spring wheat, E. Ratner and his assistants were able to show that when a granule of phosphrus comes in contact with a single rootlet comprising only 4–5 per cent of the whole root system, the absorbing function of such a rootlet immediately becomes 20 or even 30 times stronger than ordinarily: as a result, one rootlet is capable of satisfying to a considerable degree the whole plant's requirement in this element.

Thus we no longer consider the root system to be a uniformly acting mechanism which sends water and nutritive substances to the above-ground portions of plants. The root system is a highly labile organ whose activity in different sections is rapidly changing under the influence of nutritive substances and the requirements of the plant. These peculiarities of the roots enable them to utilize easily the local deposits of nutritive substances: in particular, granulated fertilizers.

Radioactive isotopes have made it possible to study more closely the role played by soil microorganisms in the nutrition of plants. For instance, employing vitamin B_1 marked with radioactive carbon, G. Shavlovsky showed that this vitamin, as well as certain other vitamins of the B-group which microbes form in the soil, are easily absorbed by the roots of buckwheat and enhance the general development of plants. At the same time, isotopic sulphur helped to reveal that plant roots also utilize the amino acids methionine and cysteine, which microorganisms excrete into the soil.

In certain periods of their development, for example, setting of fruits, plants often become incapable of absorbing phosphorus and other nutritive elements through their roots. Yet the processes of movement and accumulation of plastic substances, which predominate at this given period, still require new stocks of nutritive salts and could be intensified if these salts easily penetrated into the plant.

Agricultural science has already solved this problem by applying the so-called non-root nutrition, which includes spraying, dusting, and sometimes even fumigation of the above-ground parts of the plants with the required nutritive substances. For example, in many parts of the USSR, non-root feeding by phosphorus raised the yields of sugar beet (Yakushkin) and of cotton (Uchevatkin); non-root nutrition with ammonium salts also raised the yields of cabbage and other vegetables, particularly in the north where low temperatures of the soil prevent normal penetration of nitrogen through the roots (Dadykin).

The application of labelled atoms in this case too resulted in substantiating this useful undertaking and in bringing order to it. For instance, when superphosphate, marked with P^{32}, was utilized as a non-root nutrient, it became possible to observe how the fertilizer penetrated through the leaves into the plant and how it was distributed in its tissues.

Radioautographs reproduce a clear picture of this distribution. On the basis of these documentary pictures, the agricultural workers can easily check the efficiency of nutrition and determine more exactly when such feeding should be done and how much nutrient should be applied.

Utilizing radioactive carbon (C^{14}), A. Kursanov, A. Kuzin, N. Krukova and co-workers discovered a new function of the root system, which consists in the following: the roots absorb carbon dioxide from the soil and bring it to the leaves and other green portions of the plant. It turned out that for synthesis of sugars and other products of assimilation, carbon dioxide of the soil can be utilized on a par with the carbon dioxide absorbed from the air, provided there is light. This fact has not only theoretical, but practical value as well. It proves the important role humus and the microbiological processes going on in the soil play in supplying carbonic acid to the plants. It also warns against the one-sided understanding of the tasks and possibilities of utilizing mineral fertilizers.

If we use plants labelled with radioactive carbon as green manure and introduce them into the soil, we can observe the degradation of organic residue by the rate at which $C^{14}O_2$ is liberated. Such observations are of great practical importance. They enable one to determine the intensity with which humus-forming processes take place in the soil under the influence of its cultivation, humidity, temperature and other factors. At the same time, when we grow plants in the soil containing radioactive organic residues, we are able to directly observe how they absorb and utilize various organic substances as well as the carbonic acid of decaying remains (V. Merenova).

Obviously, the relative role of carbonic acid received by the plant through its roots in the carbon nutrition of the plants as a whole may vary greatly; at present time this problem is being studied under field conditions.

The application of radioactive carbon (C^{14}) together with other up-to-date methods enabled us to study this new phenomenon in great detail and to determine its significance for plant life. It was shown that sugars formed in the leaves when CO_2 was assimilated from the air move downwards along the phloem and, on reaching the roots, penetrate into their thinnest and most active branchings. The speed of this descending movement, determined by means of radioactive carbon, is in the case of the sugar beet about 70 cm per hour.

In the roots, sugars undergo glycolytic disintegration with the accompanying formation of pyruvic acid. It is this acid which, by means of a special enzyme, incorporate the carbonic acid of the soil. The latter, in the form of carboxyl, combines with the pyruvic acid and transforms it into oxaloacetic acid which is easily reduced and transformed into malic acid, the first comparatively stable compound containing carbonic acid of the soil. Later on, as a result of mutual transformations of acids, malic acid is partly transformed into citric, ketoglutaric and other acids.

It is necessary to say that when CO_2 is introduced into organic acids in the capacity of the carboxyl group, we do not observe any practical increase in the free energy of the substance and, consequently, we cannot consider β-carboxylization as such to be equivalent to carbon dioxide nutrition of plants. But organic acids, formed in the roots and containing the carbonic acid of the soil, move upward and penetrate into the green fruit, growing apices and leaf laminae. The speed of this ascending movement, even in the case of herbaceous plants, is about 3 cm per minute or 2 metres per hour. Therefore it takes very little time for the carbonic acid of the soil to reach the assimilating tissues where it can be reduced in the process of photosynthesis, forming carbohydrates, proteins and other products rich in energy.

Some of the sugars thus formed are, in their turn, translocated to the roots where, during the process of glycolytic disintegration, they are transformed into pyruvic acid and accept from the soil fresh portions of CO_2 in order to transfer them to the leaves. Such is the principal cycle along which the process develops. But in addition to this, there are a number of subsidiary phenomena which condition the dependence of carbon dioxide nutrition in roots on other aspects of the physiological activity of plants.

By employing isotopes and chromatographic analysis, A. Kursanov, O. Tuyeva, A. Vereshchagin, I. Kolosov and S. Ukhina have succeeded in discovering a new and important function of the roots, which consists in the initial synthesis of a number (about 14) of amino acids. These amino acids are utilized in the root itself as well as in other portions of the plant where they participate in metabolism and the synthesis of new proteins. So it turned out that besides its absorbing function, which has been drawing the attention of scientists and practical workers for a long time, the root system fulfills another very important role connected with the protein metabolism of the entire plant.

At first it was considered that this aspect in the activity of the root system was an independent function having no connection with the absorbing function of the roots, but later on it was discovered that this is exactly the mechanism by which roots assimilate ammonium nitrate from the soil.

In this process a very important role belongs to the carbonic acid of the soil. This acid brings about the carboxylization of pyruvic acid and some other products which are initial acceptors of the nitrate of ammonium fertilizers.

At the same time it has been proved that for the functioning of this system the roots should be well provided with phosphorus, since phosphoric acid participates in a number of stages of the glycolytic process as well as in the cycle of di- and tricarboxylic acids. Therefore, when there is a shortage of phosphorus the roots lose, as a rule, the ability of accumulating organic acids (A. Vereshchagin) and, consequently, of fixing soil carbonic acid (O. Kulayeva). All this inevitably results in difficulties of nutrition of plants.

Such is the picture of one of the most important aspects of plant nutrition which we have at the present time and which it did not take long to discover by means of labelled atoms. In our opinion, this is convincing proof of the broad possibilities which the application of radioactive and non-radioactive isotopes opens up to biologists.

Our conceptions of the velocity of the processes taking place in plants have also been modified. The exact course of some of these processes has now been determined, thanks to the application of tracers; we have been compelled to reject the opinion that various processes occurring in the plant proceed very slowly. For example, F. Turchin, who used heavy nitrogen (N^{15}) which he introduced into the soil in the form of ammonium sulphate, showed that proteins of all the organs of the plant are constantly renewed and that this renewal proceeds at such a pace that a protein particle of vegetating rye sometimes lives no more than a few hours. Even the nitrogen of chlorophyll, forming the very centre of this pigment molecule, is renewed so rapidly in actively vegetating plants that sometimes in the course of 2–3 days more than half of it is already replaced.

Labelled atoms enable one to approach experimentally the problem of biosynthesis of chlorophyll

and carotene in plants. Thus, in the Byelorussian Republic, T. Godnev and A. Shlik, utilizing in their experiments glycocol labelled in carboxyl with C^{14}, and also radioactive glucose, obtained new evidence on the course of chlorophyll, phytol and carotene synthesis in plants.

Now with the use of radioactive isotopes it is very easy to study in detail the movement of plastic substances in the plant, and to determine not only the direction and speed of this movement but also the composition of the moving products. If we bear in mind that proper nutrition of various portions of the plant and the accumulation in them of valuable nutritive elements depend precisely on this process, then we shall understand the great importance for biology and agriculture of the direct observations on the flow of plastic substances which have been made possible only with the aid of radioactive elements.

Employing this method, M. Turkina, E. Vyskrebentseva, and N. Pristupa, working in our laboratory, demonstrated that substances move in plants more quickly than was heretofore believed. For instance, the products of photosynthesis travel from the leaves to the roots of the sugar beet, pumpkin and some other plants at the rate of 70–100 cm per hour and even more. Somewhat slower is the movement of assimilants towards fruit and growing shoots, but even in this direction the organic substances usually make not less than 40–60 cm per hour. Therefore, in the majority of agricultural crops, it takes no more than 20–40 minutes for the products of photosynthesis to reach the growing points and organs of accumulation.

Finally, according to A. Akhromeiko's experiments, water, especially in some of the trees, moves along the xylem at 6–8 or even more metres per hour.

Labelled atoms, therefore, have shown that in plants, despite their outward immobility, physiological processes proceed at a very rapid rate. Consequently agricultural workers should take this fact into consideration so as to co-ordinate their methods of tending plants and the "rates" of plant activity.

Radioactive carbon (C^{14}) is used to study the composition of substances moving in the plant.

Thus, experiments conducted in our laboratory with sugar beet, pumpkin, cotton, and other crops, demonstrated that along the conducting tissue usually moves a mixture consisting of saccharose, glucose, fructose, hexophosphoric ethers, organic acids and amino-acids, with saccharose and hexophosphoric ethers forming the greater part of this mixture. In addition, they move faster than the other components and this leads to the conclusion that they are the basic mobile substances in plants.

However, the composition of the nutritive mixture moving in the conducting tissue differs in various plants and may change depending on the stage of development or under the influence of light and mineral nutrition.

All these data show that, using radioactive carbon as an observation method, we can approach the problem of arbitrarily changing the direction and composition of the plastic substances that move in the plant; this will provide agriculture in the nearest future with some new and still unused possibilities.

However, V. Zholkevich, who utilized the tracer method on the irrigated lands of the Trans-Volga, has already proved that assimilants, formed in wheat leaves from labelled carbonic acid, travel to the seeds far more rapidly in irrigated plants than in plants suffering from water shortage. With the aid of radioactive carbon dioxide, it was also shown that when potatoes are cultivated under the conditions of the extreme north, the products of CO_2 assimilation mainly travel from the leaves to the stems and growing apices, which results in abundant stems and leaves. The long day and low temperature of the soil caused a slow and retarded flow of nutritive substances to the tubers, and this was the reason for comparatively low content of starch in the local potatoes (Z. Zhurbitsky). At the present time agricultural workers of the north are searching for methods to combat this negative phenomenon, employing isotopic indicators to appraise the efficiency of the measures they undertake.

Radioactive isotopes enable one to observe not only the movement of organic substances but also the way nutritive salts move and are redistributed in the plant. In this respect interesting experiments are conducted in the Ukrainian SSR with fruit trees. By means of P^{32} and Ca^{45} it was possible to study in detail the picture of the distribution and redistribution of these elements in the development of trees (N. Lubinsky and K. Garnaga). Similar experiments are conducted on other woody plants, such as scoompia (*Cotynus coggygria Scop.*), eucommia (*Eucommia ulmoides*) and others (S. Slukhai). These experiments also deserve special attention, particularly if we bear in mind that our information on the mineral requirements of woody plants is far from complete.

Special chemical preparations, such as 2–4 dichlorophenoxyacetic acid, methyl ether of α-naphthylacetic acid, 4-iodophenoxyacetic acid, etc., have become widely applied in practical farming as a means of influencing growth and formation processes in plants. The biological aspect of this peculiar influence has been studied lately thanks to the utilization of labelled atoms.

Thus, for instance, if we introduce into a tomato plant a solution of 4-iodophenoxyacetic acid, marked with radioactive iodine (I^{131}), we can exactly determine its localization in plant tissues after some time has passed. The greatest part of the iodine preparation is concentrated in the flowers and fruit, thus creating a peculiar polarization: from leaves, roots and other portions of the plant nutritive substances travel to these organs ensuring their

intensive growth and accumulation of nutritive products (Y. Rakitin).

But the isotopic method not only enables one to observe the movement and distribution of substances in the plant: it also helps to observe the course of further transformations.

Thus, A. Krylov, Y. Rakitin and N. Melnikov utilized as a growth stimulant the methyl ether of α-naphthylacetic acid marked in carboxyl or in other positions by radioactive carbon. They discovered that while this substance intensifies growth, the plant at the same time always strives to rid itself of this influence by splitting off carboxyl groups and by further rearrangement of the molecule of the physiologically active compound. In this way, the radioactive isotopes enable us to approach the solution of a problem which is of great importance to biology and agriculture—the detoxification of alien substances in the body of plants.

In a similar manner we can also observe the normal course of metabolic processes. For instance, A. Prokofiev, introducing a solution of saccharose marked with radioactive carbon (C^{14}) into the leaves of kok-saghyz, discovered that radioactive rubber very shortly appears in the latex of the plant. In this way it was proved once and for all that rubber originates from carbohydrates. This experiment is interesting from another point of view also, for it provides an explanation of the accumulation of rubber in kok-saghyz after harvesting. Practical workers have been interested in this problem for quite some time.

Developing these experiments on kok-saghyz, B. Savinov and his assistants utilized d-fructose, d-glucose, levulinic, pyruvic, and acetic acids marked with radioactive carbon in definite positions. In this way they were able to study more closely the processes of sugar transformation leading to rubber formation.

The isotopic method has also considerably modified our former ideas on the synthesis of saccharose in sugar beet. Utilizing C^{14}-labelled carbon dioxide and glucose and fructose also labelled with radioactive carbon, A. Kursanov and O. Pavlinova demonstrated that the tissues of the root and shortened stem of the sugar beet, which were formerly considered to be the organs capable of intensive synthesis of saccharose, actually cannot form this biose from glucose or fructose.

Further experiments prove that in sugar beet the synthesis of saccharose takes place mainly in the leaves where this biose is easily formed as the first free sugar of photosynthesis, or where it may be formed as a secondary product from glucose and fructose. From the leaves saccharose rapidly travels along the conducting tissues to reach the roots. This conclusion compels agronomists and selectionists striving to raise the sugar content in the industrial varieties of sugar beet, to pay more serious attention to the development of the leaf-system and to the processes occurring in it, while previous investigations underestimated the role played by the leaf system in the synthesis of saccharose.

Another example of successful application of the isotope method in plant physiology is furnished by interesting experiments conducted by V. Pontovich and A. Prokofiev. By means of C^{14}-labelled carbon dioxide they discovered that in the ripening fruits of poppy there occurs a metabolic cycle which is of great significance for the normal development of seeds. This process begins in the following manner: as a result of intensive respiration of young seeds, large portions of carbon dioxide are evolved into the core of the fruit. However, this gas is not accumulated here but diffuses to the green surface of the fruit walls where it is quickly reduced in the process of photosynthesis, forming sugar and other nutritive products which, through placenta, are transmitted from the walls back to the growing seeds. Thus, in the green poppy fruit this peculiar type of turnover ensures a quick removal of superfluous carbonic acid from its core, maintains a high concentration of oxygen necessary for the synthesis of fat, and results in the restoration of organic substances which the seeds use in respiration.

The tracer method opens especially broad possibilities for scientists engaged in investigating photosynthesis. Utilizing isotopes, Soviet scientists have achieved a number of successes in this sphere. The discovery of photolysis of water, made almost simultaneously in the Soviet Union (Vinogradov and R. Teis) and in some other countries, is particularly important. It enables one to understand the mechanism of photoreduction of carbonic acid.

At the present time we are approaching the solution of the problem of primary organic substances formed in the process of photosynthesis. The utilization of labelled carbon dioxide in combination with paper chromatography ensures an extreme sensitivity of such experiments and enables one to detect labelled products of photosynthesis only 0.5 seconds after irradiation. Thus, N. Doman has recently discovered two new substances of low molecular weight whose nature is now under investigation. For the time being we know only that the first of these substances does not contain phosphorus and, consequently, is not phosphoglyceric acid. These and many other investigations conducted by means of radioactive and non-radioactive elements are preliminary to the decisive step in the direction of disclosing the mystery of photosynthesis and of mastering this process, a step which probably will take place in the near future.

Since we cannot consider in detail a number of such investigations in this paper, we shall take up only the question of the direct products of photosynthesis. A. Nichiporovich, N. Voskresenskaya, T. Andreyeva, and others, utilizing radioactive

carbon (C^{14}) in the form of carbon dioxide and heavy nitrogen (N^{15}) in the form of an ammonium salt, demonstrated that not only carbohydrates but proteins as well are the direct products of photosynthesis in the leaves of plants. The composition of the photosynthetic products may vary considerably under the influence of the species of the plant and its age, as well as its environmental conditions. This process is most strongly influenced by the spectral composition of light and its intensity. These factors, in combination with mineral nutrition, can considerably change the composition of the primary products formed in the leaves. Thus, carbohydrates are synthesized mainly in the red and yellow parts of the spectrum, while proteins are formed under the influence of blue light. Besides its fundamental significance for biology, this fact also provides practical possibilities to influence the development and properties of plants by changing the composition of the primary products of photosynthesis which are being formed. There is every ground for the practical utilization of this discovery under hothouse conditions where it is possible to use sources of lighting with varying spectral composition and intensity which enables the agronomist to control not only the quantitative aspect of photosynthesis but the qualitative as well.

We have already said that the present paper does not describe all the new data in the sphere of metabolism and plant nutrition obtained in the USSR by applying the tracer technique. It is our opinion, however, that this paper proves that application of the achievements of nuclear physics for studying plant life opens great possibilities for the development of biology and agriculture.

But even at the present time, when we are only beginning to discover the practical possibilities of radioactive isotopes as a method of studying plant life, we can state with satisfaction that in the USSR this method has already seriously influenced the development and improvement of practical farming methods.

For instance, it was by means of radioactive isotopes that the problem of the effective application of granulated fertilizers was finally solved: this at once ensured wide application of this method of plant nutrition, and it is now successfully used on thousands of large farms in the Soviet Union.

Radioactive elements helped to bring about a similarly radical change in the attitude to non-root nutrition, whose expediency was for a long time disputed. At the present time the non-root nutrition is successfully practised in the USSR on thousands of hectares. This method is applied to those crops and in those periods of vegetation when non-root feeding can give the greatest effect required.

Radioactive isotopes have also demonstrated the possibility of a more economical and at the same time more effective utilization of fertilizers for feeding plants. This resulted in a number of rational changes in soil cultivation, in reconstruction and improvement of sowing machines, and in other measures that are being rapidly introduced in practical farming in our country.

But all this does not exhaust those great possibilities that are opened before biologists and agronomists utilizing atomic energy for the aims of peaceful construction. Employing radioactive isotopes, we can now more profoundly study the laws of plant life, and it is our duty to show to the agricultural workers ways and means of obtaining abundant and stable yields, ways of securing a prosperous and tranquil existence for all.

The Uses of Atomic Energy in Food and Agriculture*

INTRODUCTION—THE WORLD FOOD PROBLEM

The present world population of about 2.5 thousand million is growing at the rate of nearly one and a half per cent each year. Every day nearly 100,000 additional hungry mouths appear at the breakfast table, and twenty-five years hence it is expected that we shall be 3.5 to 4 thousand million in number. Evidently one of the many major problems facing us, probably basically the most important for the future welfare of mankind, is how we shall be able to provide food, clothing and shelter for the world's peoples at the ever higher standards that they so rightly expect.

The neo-Malthusians believe that we are engaged in a losing battle and that it will not be possible for world resources to meet requirements. FAO believes that technically it is possible to achieve the necessary increases in production of foodstuffs and basic raw materials to meet world needs for the foreseeable future, and has recommended to governments that they aim at increases in production one to two per cent greater than their anticipated growth in population. For many countries this will mean production increases of three to four per cent a year. This admittedly will do little more than maintain the *status quo* but even so the social, political and administrative problems involved will be of immense magnitude. Their solution alone, quite apart from the further urgent need to make a real improvement in the inadequate subsistence levels which at present characterize so many parts of the world, will require determination and fortitude on the part of governments. Although freedom from want must depend essentially upon the efforts made by each country on its own behalf, success in achieving this is of such general concern to all peoples that these national efforts should be supplemented by pooling the knowledge and experience of all countries on an international basis for the common good. In doing so, it is imperative that all possible advantage be taken of the contributions to increased production that might come from scientific and technical advances in other fields. Amongst those advances the development of atomic energy is of great significance for agriculture and related forest and fishery industries.

From the technical standpoint the solution of the problems of providing more of the primary necessities of life can come in three main ways. The easiest method of increasing supplies is by taking adequate measures to reduce the heavy losses which at present occur in all segments and stages of production, storage and distribution. Secondly, the productivity of land now under cultivation could be much increased and the current utilization of our fisheries and forestry resources intensified through development of improved technical methods. Finally, supplies could be increased by developing new areas and resources, but this is the most difficult course as those areas and resources most easily developed are already being utilized.

The ways in which atomic energy can help in feeding, clothing and housing the world's growing population will be considered in relation to these three main ways of improving production and making better use of our natural resources.

THE SIGNIFICANCE OF ATOMIC ENERGY FOR FOOD AND AGRICULTURE

When relatively inexpensive power from nuclear reactors becomes generally available it will undoubtedly make a substantial impact on agriculture, if only by cheapening costs of production and distribution, improving conditions of work, and making modern conveniences and comforts more widely available in rural communities. This is particularly true in areas where other sources of power are deficient. These and other, at present more speculative, developments must, however, await the widespread application of nuclear power in industry, and its possible contributions will be discussed a little more fully later. At present and for the immediate future the radioactive isotopes and radiation that have become available as by-products of nuclear reaction are of greater importance to agriculture.

It was at one time thought that radiation and radioactive isotopes might prove of direct value in the stimulation of plant growth. To date the only stimulatory effects in the irradiated generation which have been observed in extensive investigations conducted in the United States of America, the United Kingdom and Canada have been those associated with damage to the plant, much as growth is stimulated, in a sense, by the use of the pruning knife, and there has been complete failure to substantiate the earlier hope for useful stimulation.

Neither radiation nor radioisotopes therefore can make any direct contribution to increased production in the sense that an application of fertilizer leads to increased crop yields. Their contribution to food and agriculture is indirect, but nevertheless of immense potential. The value of radiations lies partly in their ability to induce inherited changes in the germ plasm and partly in their sterilizing effects on biological

* A survey by The Food and Agriculture Organization of the United Nations.

tissues. The ability to induce mutations is being used in plant-breeding programs and in other ways, whilst the sterilizing effects have promising applications in food preservation and pest control. The value of radioisotopes in agriculture arises from the ease and accuracy with which they can be identified and measured in extremely minute amounts, which makes it possible to use them as highly refined research tools in so-called tracer studies in a wide range of nutritional, metabolic, developmental and pathological investigations in plants, animals and man. In this way they are giving information which at the present time could be obtained in no other way or only at much greater expense in terms of time and money. Thus, by giving a clearer insight into basic biological processes that have hitherto been obscure, the use of radioisotopes in tracer studies is already leading to greater efficiency and economy in the production and utilization of agricultural products. Bearing in mind that it was only the unfettered scientific investigation of the nature of the atom—the pursuit of knowledge for its own sake—that made nuclear energy available to mankind, the potential value of the contributions which radioactive tracer studies can make to food and agriculture may similarly be almost unlimited.

APPLICATIONS OF RADIOISOTOPES IN AGRICULTURE AND FORESTRY

Food Preservation and Storage

Potential food supplies and other agricultural products are subject to heavy losses in all phases of production, distribution and storage through fungal and bacterial infection and the ravages of insect pests, and it is by combatting these that the most immediate and spectacular improvements in supplies can be achieved. No valid estimate of total world losses is possible, but they are undoubtedly of immense magnitude. A very conservative estimate that has been brought forward for losses in stored grain, largely from the depredations of weevils and other insect pests, is ten per cent, but the losses are undoubtedly much greater in the hot and humid areas of the world, where the figure of twenty-five to fifty per cent loss of harvested cereals and pulses which has been estimated for Central America is probably generally applicable in most of the less-advanced countries. In addition, perishable foodstuffs such as fruits, vegetables, meat and fish are particularly subject to spoilage in distribution and storage. Similarly the deterioration resulting from fungal infection and the attacks of various insect pests is of major concern in timber utilization.

Evidently there is great scope for the adoption of control measures, and these can be applied relatively easily at a cost which is usually but a small fraction of the value of the returns. Already radiations and radioactive isotopes show promise of making important contributions to the development of improved control measures which would markedly improve the supply situation. Thus the destructive power of radiation has been used to eliminate insect infestations in grain and cereal products at costs which compare favourably with those of the more conventional procedures such as fumigation. The sprouting of potatoes has been successfully inhibited as a result of the effects of radiation on the enzyme system, thus permitting transportation under less stringent conditions than are usually required and extending storage life by many months. Much attention is also being given to the possibility of food preservation through cold sterilization by irradiation at normal temperatures. Whilst full success has not yet been attained, significant improvements in the keeping quality of meat and meat products have been achieved without off-flavours or colour changes arising from detrimental side reactions. In some countries trichinosis is an important health problem and it has been shown that the irradiation of pork can kill or sterilize the trichinae, thus rendering infected meat safe for human consumption.

Evidently radiation may have a very useful field of application in food processing and preservation, although much more exploratory work must be done to evaluate its full potentialities. One of the most attractive features of this application is the wide scope it offers for the useful employment of the radioactive residues arising as a by-product of the operation of nuclear reactors.

Reducing Losses in Growing Crops

Every year a large part of the potential harvest is destroyed by diseases and pests which attack the growing crop. It has been said that even in such relatively developed countries as the United Kingdom the work of over 51,000 skilled farm workers is lost each year, and that in the United States of America losses caused by insects, weeds, and plant diseases account for losses in farm output equivalent to thirteen thousand million dollars per year. Most of these losses could be avoided by the timely application of control measures, and radioisotopes are proving to be exceptional tools in studies which are leading to the development of improved materials and methods for safeguarding our harvests. Adequate control of a destructive insect, for instance, generally requires a thorough knowledge of its life cycle and habits, and tagging insects with radioisotopes provides a much more efficient means of determining their flight range, migrating routes and overwintering habits than methods such as painting which were previously used. In Canada cobalt-60 has been used to label wireworms, making it possible to follow their underground meanderings, while in both the United States of America and Canada the flight and overwintering habits of a number of forest insects are under investigation, using similar techniques. These studies will undoubtedly be important factors in better control.

Extremely effective insecticides have become available in recent years but one of the problems associated with their continued use is that insects frequently

build up resistance to these poisons. Studies with insecticides labelled with a radioisotope permit comparison of uptake and metabolism by normal insects and by those that have developed resistance. This work may be a step in determining the nature of resistance to the poisons, concerning which so far little is known except that resistant insects take up the poisons just as susceptible ones but do not react to it in the same way.

Similarly, radioactive tags are playing an important part in the development of fungicides, insecticides and weed killers, for use either as direct applications or as systemic poisons. These latter are substances which are applied, usually through the soil, and taken up by the plant without harm to it but are toxic to pests feeding on the plant. It is important to know that such substances are not altered in use to products harmful to man or animals. For instance the weed killer 2-4-D when used to kill weeds in the bean field is readily absorbed by the bean plant also and has been found by the use of labelled material to be distributed throughout the plant together with at least two additional products derived from 2-4-D. It is obviously important in the case of edible plants to be able to trace such compounds and their metabolic products because of their possible effects on the consumer, animal or man. The use of radioactive labels is playing an important part in the development of safe materials and methods.

Breeding Improved Crop Varieties

Although control of crop pests and diseases by chemicals can be extremely effective, their use involves additional expenditure and usually requires considerable care. Undoubtedly the most satisfactory safeguard, and one that is particularly applicable in the under-developed areas, is the growing of crop varieties resistant to the prevalent pests and diseases. The plant breeder has long been engaged in developing such varieties by well-known conventional methods involving selection and hybridization, and has achieved marked success, but this is a never-ending task, for existing varieties do not satisfy all needs and new pests and diseases or new forms of old ones are constantly appearing. In his task of developing disease and pest-resistant varieties, as well as other improved types with better agronomic characteristics and higher productivity, the plant breeder is already being greatly assisted by the new potent sources of radiation which have recently become available, and their use in this connection may well ultimately prove to be one of the most significant contributions of atomic energy to agriculture. It has long been known that radiations cause heritable mutations in plants and animals. With the advent of atomic energy further kinds of radiations and more potent sources have become freely available for experimental use, and in many countries extensive programs have recently been initiated for crop improvement through irradiation, with a view to accelerating the normal rate of mutation and so increasing the variability available to the plant breeder for selection.

Radiations are applied either in the early flowering stages in order to affect the developing gametes or to seeds in which somatic mutations are first induced, and such affected cells may subsequently give rise to germinal tissue, in which case the mutations are transmitted to later generations. Although as in the case of spontaneously appearing mutations the vast majority are deleterious, desirable types occur in low proportion and have been selected in a wide variety of crops. Improved types that have been obtained include higher-yielding or disease-resistant strains of cereals and other crops such as peanuts, stiff-strawed types of cereals resistant to lodging, types better adapted to mechanical harvesting on account of particular size or shape characteristics, types with extended or reduced maturity period, and types with changed ecological requirements, for instance in relation to higher or lower rainfall or soil fertility. An example of a particularly significant outcome of work of this type may be cited from Canada where amongst some twenty barley mutants produced by irradiation and now under field trial are some maturing sufficiently early to extend the area in which barley might be grown in that country.

Plants such as some orchard crops which are normally propagated vegetatively are also receiving radiation treatment for the induction of bud mutations. Mutants have similarly been induced in algae and in this way types have been obtained which are adapted to high temperatures and thus more suitable for growth in mass culture. Such types may one day become an important source of food and industrial raw materials. In forestry increasing emphasis is being given to the breeding of fast-growing types and varieties resistant to pests and diseases, and radiation may play an important role in the production of such improved strains of trees.

In the case of disease control the ability of radiations to induce mutations in the disease-producing organism itself may also prove to be a tool of exceptional value. Such pathogens as the rusts that affect wheat and oats or the smut attacking maize undergo spontaneous mutations in nature at a rate rapid enough to cause constant trouble for the crop breeder. If he develops, for example, a wheat resistant to black stem rust, he may find that within a comparatively short time a new mutated form of the rust organism appears to which his variety is no longer resistant as it was to the original form. Current work has shown that radiation will produce new races of such disease organisms with increased virulence. By developing these new races artificially and under controlled conditions, the breeder may be able to anticipate the resistance requirements of his crop and to breed adequate resistance prior to the appearance of new strains of the pathogens in the field.

Increasing Productivity through Improved Practices

In addition to the adoption of adequate measures for the control of plant and animal diseases and pests

and the use of higher yielding crop varieties developed by the plant breeder, the productivity of land now under cultivation can be increased by the adoption of a wide range of improved methods of crop and animal husbandry. Here again through tracer studies using radioisotopes, atomic energy is making notable contributions to the development of improved techniques through the advancement of fundamental knowledge in animal and plant nutrition and physiology and a better understanding of the complex relationships among animal, plant, soil, water and sunlight on which man is dependent for his existence. The study of such a dynamic biological system is normally particularly difficult and the tracer technique has immeasurably facilitated such investigations. In many cases it provides the only practical approach to the solution of a problem and without it further progress in certain directions would at this time have been impossible.

Soil Fertility

The major limiting factors in the productivity of land now in cultivation are the inherent yielding potential of the crops and livestock on them and the amount of nutrients available to enable those plants and animals to produce to their capacity. Contrary to general belief, some of the most productive soils in the world were originally of rather poor fertility and have been brought to their present capabilities by man's careful tending of the soil and the development of various principles of good husbandry, which through the ages have built up the fertility of the soil. The potentialities for increasing production through adoption of those principles on a wider scale are very great. As just one example, the yield of rice in Japan is about four times that of India's one ton per hectare. Much, though admittedly not all, of this increased productivity is due to the much greater use of inorganic fertilizers in Japan, and evidence from experiments indicates that through the use of only a moderate dressing of 30 kg of nitrogen per hectare, with other plant nutrients where necessary, India's annual rice production of about 35 million tons could be raised by more than 10 million tons, which would mean much to the welfare of the population and to the economy of the country. However, the present limited use of fertilizers in such countries is almost entirely a question of economics, so that it is essential that the best use is made of the natural fertility of the soil, and that added fertilizers are employed in such a way as to ensure the fullest possible return from them. Here tracer studies with isotopes in many countries are providing a wealth of fundamental information and practical hints. Radioisotopes of most of the important plant nutrients are now available and they have opened up new avenues of attack on important problems of soil fertility.

The fact that phosphorus is to so large a degree retained by soils in forms that are not readily available for use by plants is one of the major concerns of soil science. Advances in the study of this problem are being made through such work as that in the United States of America with phosphorus-32 and calcium-45 on the factors influencing the fixation of phosphorus in calcareous soils. Canadian workers, who were among the first to point out the advantages of the tracer technique in soil fertility and plant nutrition problems, have shown that the usual procedure for evaluating the contribution of a phosphorus fertilizer by comparing the total phosphorus absorbed by the plant from fertilized and unfertilized plots could be misleading. Before the use of tagged material it was thought that the increased uptake from the fertilized plot all came from the fertilizer. Through the use of radioactive phosphorus it has been found that when the fertilizer is applied, the plant takes up additional phosphorus from the soil itself as well as from the added nutrient.

Rice, the basic food of nearly one-half of the world's population, is typically grown under irrigation, in submerged soil. In Japan the radioisotopes of phosphorus, sulphur and iron are being employed in preliminary investigations of the chemistry of such submerged soils, which is quite different from that of ordinary soils, and valuable information is to be expected on the factors affecting the uptake and translocation of these nutrients by rice. Similar work on the determination of the phosphorus fertility status of tropical and submerged soils is under way in India. Other investigations are giving information on the most economic source of specific nutrients and the best placement and time of application of added fertilizers, particularly in relation to the growth period when the plant can best use them and their placing in relation to the main feeding zones of the roots. In this connection a good deal of attention is being given to investigation of the characteristic rooting habits of different plants through use of labelled nutrients, and this ability to define the zones from which particular crops obtain the major portion of their nutrients and moisture should make a valuable contribution to better agricultural practices.

Soil Moisture, Drainage and Irrigation Problems

Many other soil problems are amenable to attack by this new tool. It makes possible, for instance, rapid determination of the moisture content and the density of soils, a matter of interest alike to the agronomist, the soil conservationist, and the engineer. Moisture content of the soil may be estimated by a method dependent on the degree of neutron scattering by hydrogen atoms contained in the soil water. A similar method involving gamma rays instead of neutrons is used in measuring the density or degree of compaction of the soil. One application of these methods in the United States of America is the study of the effects of tillage and harvesting machinery in compacting the soil.

A different application to soil problems is the addition of phosphorus-32 or rubidium-86 to surface waters such as streams or ponds to determine the rate and direction of drainage into the soil, and to irrigation waters to find out whether they reach the

farthest points of the field as well as the nearest points—that is, to evaluate the efficiency of distribution of the water in the soil. Radioisotopes are also being used, for instance in Japan, to detect leakage in irrigation dams and to survey supplies of underground water, and tritium might have a particularly valuable application in large scale research in hydrology, for example over entire watersheds.

Plant Nutrition and Metabolism

In plant nutrition the basic mechanisms involved are the uptake of nutrient elements, their transport through cell membranes, and their subsequent translocation or movement throughout the plant. Such mechanisms are of course basic also in the case of animals. Investigation of these fundamental mechanisms through the use of a considerable variety of radioactive elements is under way in many countries. Fuller knowledge of the phenomena involved is essential to the understanding of plant nutrition and growth, and discoveries that were hitherto impossible in this field are now being made. Among other practical aspects the mechanism of transport plays an important part in the utilization of growth-affecting or growth-regulating factors—the so-called plant hormones which are used to regulate development in crops, particularly in horticulture—and in the utilization of such compounds as the previously mentioned 2-4-D which are used as weed killers, and the systemic insecticides which are administered to the pest by way of the host plant. An interesting example of the place of such translocation studies in relation to practical problems is the work on mistletoe in Australia, where this parasite is a serious menace of eucalyptus. In work directed towards its control, the isotopes of cobalt, iron and zinc are being used to obtain information on the efficiency of movement of toxic compounds from the host-tree to the parasite.

Many of the plant hormones and weed killers are used as leaf applications, and essential nutrients may also be applied to aerial parts of the plant as well as by way of the soil. Thus under certain conditions, especially with trees, temporary deficiencies of such vital elements as iron and zinc may occur, and these can often most readily be corrected by spray applications either in the dormant stage or when in leaf. Radioisotopes have been particularly helpful in demonstrating that some plants can absorb such nutrients efficiently through the foliage and that nutrients so absorbed are rapidly translocated throughout the plant. This principle is already being applied rather widely in practice, urea for instance being used fairly extensively as a leaf spray by fruit and vegetable growers in the United States of America. Additional information on the absorption and translocation of such substances is needed and through the use of urea labelled with carbon-14 it has already been found that crops differ markedly in their ability to utilize this compound as a source of nitrogen, cucumbers using it more than four times as fast, for instance, as do cherries and potatoes. In the case of strawberries, calcium is readily absorbed, but it has been found that it is not translocated into daughter plants and hence foliar feeding as a major source of calcium is not adequate in this plant.

In Puerto Rico studies of the formation of *Hevea* rubber are being made in tissue cultures, using possible chemical precursors of rubber tagged with carbon-14. Such investigations should lead to increased knowledge of the basic reactions and mechanisms involved in the secretion of gums and resins and will contribute in the long run to more efficient production of these economically important materials.

Radioisotopes offer a means of determining the rate of movement of water in plants and rather direct evidence of the structural pathways involved. Canadian forests are suffering from a die-back in birch, and radioactive phosphorus and rubidium have been used in the current investigation of the disease. These isotopes have shown that the normal narrow-band and upward spiral path of solution movement is broken in the affected tree and replaced near the dying region by a confused irregular pattern. Although no answer to the die-back is as yet forthcoming, it may be anticipated that the new techniques will play their part in the ultimate solution.

Well in the forefront amongst the contributions of radioisotopes to the advancement of fundamental knowledge of plant nutrition and metabolism has been the remarkable progress made through their use in the elucidation of the highly complex mechanism of photosynthesis, the process by which green plants use the sun's energy in the formation, from air and water, of compounds essential to life. Using radioactive carbon, the early principal pathways of this element have been established, and subsidiary pathways and the specific biochemical reactions involved in carbohydrate synthesis are being investigated.

The efficiency of energy conversion by photosynthesis is low: probably not more than one per cent of the total available energy in the sunlight falling on a green leaf is effectively used. This low value arises at least in part from the fact that the process is limited somehow by the plant itself. Investigations are now under way with the aim of identifying the compound or compounds that limit the photochemical reaction. Inefficient though this process is, so far in earth's history it has been the primary source of all food used by men and animals to sustain life, and the source of all our fuels including wood and the fossil fuels, coal and oil—those to which we now refer as conventional fuels in contradistinction to the nuclear fuels of the atomic age.

The remarkable advances that are being made in the understanding of photosynthesis may well lead to important methods of increasing the efficiency of this conversion of energy from the sun into chemical compounds. How much this might contribute to the enlarging of the world's food supply is at this stage a matter of speculation, but the work so far seems to hold much promise.

Another field of investigation of major importance concerns the part played by enzymes in life processes. More detailed knowledge about them is necessary if we are to have a true understanding of the metabolism and the synthesis of organic products. In this field earlier British work on cell metabolism is being paralleled by Canadian studies on the role of enzymes in the synthesis of sugars and amino-acids in the living organism, while in the United States of America fundamental investigations are under way in the basic mechanisms and the factors influencing reaction rates and the dynamic equilibria characteristic of living cells.

Animal Husbandry

Tracer studies are also giving much impetus to investigations on basic problems involved in animal production. As in the case of plants, understanding of enzyme action and other metabolic processes is of prime importance in animal nutrition. Some of the more fundamental investigations concern the amino-acids, which are combined in complex ways to make the proteins of the body. Among the amino-acids essential in the animal diet are two that contain sulphur, methionine and cystine. It is known that cystine can partly replace methionine in animal metabolism. Cows, sheep, goats and other ruminants have the power of synthesizing these amino-acids from inorganic sulphur through the action of micro-organisms in the rumen, or first stomach. It had been thought that non-ruminants, which do not have multiple stomachs, could not carry out this process within their own bodies. But recent work with inorganic radioactive sulphate in the diet of poultry has shown, through the recovery of radioactive cystine, that non-ruminants can also synthesize at least part of the cystine they require, which in turn can partly replace methionine; and hence that inorganic sulphate should be considered an important mineral nutrient for poultry, and for swine also, as well as for cattle and sheep.

Studies on the biochemistry of lactation provide another example of the effective use of tracer techniques in metabolic studies. In such work in the United Kingdom radioactive carbon and tritium, an isotope of hydrogen, have been used to label dietary components. Isolation of the milk constituents and determination of radioactivity indicate directly which milk components are derived from the labelled source and permit some well-founded speculation as to the metabolic route involved. In a typical experiment, sodium acetate labelled with radiocarbon was administered intravenously to a lactating goat. Analysis of the fatty acids in the milk clearly indicated that the synthesis of acids from the acetate took place in the udder itself, not earlier. Such studies, evaluating dietary components and elucidating the steps by which they pass into the sugars, fats (lipids), proteins, and other milk constituents, should lead to more efficient feeding and management for production.

Radioisotopes are especially effective in studying the efficiency of food utilization because it is possible with this technique to arrive at more accurate values than are obtainable by the usual methods employed in food balance studies, which permit only the determination of the total intake and loss of nutrients from the body. In materials found in the waste it has not hitherto been possible to differentiate those that passed through the intestinal tract without being absorbed from those absorbed and subsequently excreted. The use of tagged nutrients permits ready identification of the source of the excreted materials and in such investigations in animals it has been possible, for instance, to distinguish endogenous (body) and exogenous (feed) calcium and phosphorus.

As an example, the value of alfalfa as a source of phosphorus for lambs had long been considered, on the basis of the usual evaluation procedures, to be of the order of twenty per cent; that is, only a fifth of the phosphorus in the alfalfa was utilized by the body. The tracer technique has shown that the phosphorus from alfalfa is actually far more effectively absorbed, about ninety per cent being utilized. The low value previously estimated was due to the fact that phosphorus was being rapidly involved in the body chemistry, and the large amount excreted was not phosphorus from the feed but phosphorus that had been absorbed earlier, used, and returned to the intestinal tract. Studies of this nature on food utilization have been extended to the egg, the foetus and the milk, permitting evaluation of the relative contribution of the diet to each.

All of this newer knowledge contributed by radioisotopes work on the metabolism and nutrition of animals is of major concern for the world food problem since with rising standards of living there is a demand for a higher proportion of animal proteins in the diet.

Just as in the case of crop production, advances in the control of animal pests and diseases and the development of improved breeds also constitute important methods of increasing animal production. In the case of animals, however, experimentation is usually more costly and time-consuming than with plants with the result that, with some important exceptions, the utilization of radioisotopes and radiation has not progressed so rapidly. Thus while irradiation for inducing mutations in plants is being quite widely used for crop improvement, this technique has not as yet been employed to the same extent with livestock nor has it yielded comparable results. Irradiation has also been shown to induce mutations in fungi causing crop diseases, and these mutants are being used in experiments to develop greater resistance to those diseases. It may be expected that this kind of work with animal disease-causing organisms will have comparable important practical applications. Thus variants of reduced virulence developed by radiation may prove to be of value for use as vaccines in the production of immunity to commonly occurring more virulent forms of the pathogen, and irradiation may also be of help in developing polyvalent vaccines. These are

possibilities which, though so far untested, may have great significance, since vaccines constitute the most effective way of combatting the many widespread animal diseases which cause losses of millions of animals throughout the world.

A somewhat different use of radiation has been made within a limited area in the case of at least one insect pest of livestock, the screw-worm fly (*Callitroga americana* C. and P.), which is responsible for damage to the cattle industry in the United States of America to the extent of some twenty million dollars annually in killed or crippled animals and damaged hides. An ingenious method of control has been successfully demonstrated by the United States Department of Agriculture in cooperation with the Dutch authorities on the island of Curaçao in the West Indies. Large numbers of male flies were bred in the laboratory, sterilized by radiation from radioactive cobalt, and released in numbers far in excess of the normal population of males in the area so that females were much more likely to encounter a sterilized male than a fertile one. The female fly mates only once in her lifetime and hence there was no possibility that she would subsequently produce fertile eggs. This unique undertaking was carried out after a close study of the life cycle of the insect, the determination of the susceptible developmental stage and dosage requirements, and careful estimation of the screw-worm fly population of the area. The possibility of eradicating other insects by radiation undoubtedly merits further attention following such fruitful results with the screw-worm fly.

APPLICATIONS OF RADIOISOTOPES IN FISHERIES

The world's fisheries at present contribute over twenty six million tons of high-quality food. Although this represents but a small proportion of the animal protein consumed by man, fish is an important element of the diet of many peoples, especially in certain countries in the tropics where proteins of animal origin may otherwise be almost absent from the diet. Up to the present, however, the potentialities of the ocean and inland waters as sources of food have been utilized to only a relatively limited extent, but with the application of modern scientific and technological advances revolutionary developments in their exploitation may be anticipated.

As in agriculture and forestry, radioactive isotopes have many uses in fisheries research and their employment will accelerate the conclusion of certain investigations which are essential for the evaluation of fishery resources and the understanding of those resources that will permit efficient exploitation. Probably the most important application has been the use of carbon-14 in estimating the productivity of ocean waters. Carbon-labelled sodium acetate has been added to water samples taken at various depths and the samples incubated under controlled conditions. The growth of the phytoplankton or plant life under these measured conditions gives an estimate of the basic productivity of the water from which the samples were taken. The use of this method is rapidly being extended and will contribute significantly to the achievement of a realistic estimate of the basic productivity throughout the seas. The importance of this contribution will be clear since the production from the plant life in the oceans determines the quantity of organic material in subsequent links of the food chains and more particularly in those links that furnish material of economic value to man.

Applications similar to this have been made in fresh-water studies. Radioactive phosphorus has been used in several investigations in fresh-water lakes to study the efficacy of mixing, or the distribution of the phosphorus throughout the water and to the flora and fauna. Recent experiments in Canada have indicated that the turnover of phosphorus in lake waters is much more rapid than had previously been estimated, and further that the rapid turnover under natural conditions appears to be caused primarily by bacteria. This clearly indicates the need for further work since it had not hitherto been thought that planktonic bacteria play an active part in the phosphorus cycle. On the contrary, the primary organisms involved were considered to be algae, which are highly important in the food cycle of fishes. What contribution, if any, the bacteria make to productivity in these waters is therefore unknown, and it is possible that their high consumption of phosphorus may adversely affect the growth of algae. The importance of radioisotopes in this connection is that they will accelerate the analysis of the ecological systems in inland waters and add precision to the available methods. It may be emphasized that this is especially important in inland waters where the opportunities for human intervention to modify ecological systems are much greater than in marine systems.

Radioactive tags have also been used in fisheries work for tracing and measuring more massive movements of material. For example, they have been employed in studies of the movement and population density of fish, where they have some advantages over the usual marking methods. There is also the possibility that radioactive tags might be used for following the movements of oceanic currents and for measuring water transport in marine systems. Evidently radioisotopes can play at least as great a part in increasing the productivity of our fisheries resources as they can in agriculture and forestry.

APPLICATIONS IN HUMAN NUTRITION

Reference has already been made to the particular value of radioisotopes in analysing the movements of specific nutrients in a dynamic biological system and the important part that such tracer studies can play in elucidating problems of animal nutrition and metabolism. Much of the knowledge concerning human nutrition and metabolism is gained from animal studies and therefore much of what has been discussed earlier with reference to animal nutrition applies equally in the field of human nutrition. Thus work in progress on the metabolism of amino-acids

and proteins in mammals is especially significant in connection with certain deficiency diseases such as *kwashiorkor* in man. Current studies also include work on the absorption and excretion of fat and cholesterol, which are considered important factors in arteriosclerosis, a type of hardening of the arteries.

Use of the tracer technique with human beings is rather limited because of understandable reluctance to submit them to chronic internal exposure to radiation; but such studies are being made in the United Kingdom, the United States of America, and France on fat and cholesterol metabolism under normal and abnormal conditions. A limited amount of work has also been done with human beings in the field of amino-acid and protein metabolism, using carbon-14 and radioisotopes of suphur and iodine. In connection with mineral nutrition, work has been carried out in the United States of America on the metabolism of calcium in young male children.

An excellent example of international co-operation in the study of an important health problem was one involving the use of the tracer technique in an investigation of iodine metabolism in areas of endemic goiter in Argentina, where with the help of radioactive iodine Argentinian and American doctors make a complete study of uptake and metabolism under conditions of minimum iodine supply. Although the work did not result in any startling new discoveries, it was possible to observe the patterns of iodine metabolism, the changes imposed by deficiency, and the adaptation of the body to deficiency. In addition this investigation contributed new knowledge of the dynamics of iodine transfer within the body.

It is evident that tracer studies can make valuable contributions to a better understanding of the physiology of human nutrition. This in turn will affect the pattern of utilization of available food supplies and help in the development of adequate balanced diets which are so important for the maintenance of health and efficiency.

FUTURE OUTLOOK

Research

It is obvious from the foregoing discussion of past and present work that radiations and radioisotopes will play an increasingly important part in research and development in agriculture and related fields. In fact, the vast field of potentialities of the peaceful uses of atomic energy was hardly been touched, and it may be said that the applications of radioisotopes and radiation to problems of concern to agriculture are limited only by the imagination and ingenuity of the investigators.

The adaptability of radioisotopes to such a wide range of research arises from the fact that they may be quantitatively determined in such minute amounts. As we have noted, this makes it possible to introduce an identifiable component into a complex system such as the soil, a plant, or an animal, and follow its path to determine its fate in the dynamic system. The techniques involved, however, require carefully trained personnel and a considerable investment in laboratory facilities and instrumentation. Because of these limitations it would be well, for the immediate future at least, to confine the use of this tool in general to those problems in which it is the only applicable method, or to cases in which the desired information may be obtained by the use of isotope techniques with a marked saving in time and effort.

The supply of the more important radioisotopes is now generally adequate for present needs and the rate of progress in research in the immediate future will be determined largely by the number of investigators with the necessary training and experimental facilities. Hence, it will be necessary to make arrangements as rapidly as possible for the training of investigators in the handling of radioactive materials and the research techniques involved. The governments of those countries with experience in atomic energy are already generously making their training facilities available to the nationals of other countries, and the Food and Agriculture Organization of the United Nations, like other international agencies, is prepared to assist in facilitating arrangements on request and hopes from time to time to be able to award fellowships for training and research in the applications of radioisotopes to problems in agriculture and related fields. To make the best use of the available knowledge, skill and facilities, it may be desirable to develop co-operative programs of research on the more important problems of general interest. Many of the fundamental investigations using radioactive isotopes can be pursued at any centers with adequate facilities irrespective of location, but studies of the applicability of the results in specific regions must often be undertaken locally, and here again it is likely that progress will be accelerated through co-operation between neighbouring countries.

The encouragement of promising long-term investigations might well be a suitable function of the projected atomic energy agency in consultation with the appropriate specialized agencies of the United Nations, which have much experience in stimulating international cooperative investigations where such procedures can lead to more effective use of relatively restricted resources in trained manpower and of facilities.

Power Aspects

Little reference has so far been made to the potential benefits to agriculture and related industries that may be expected from the more abundant supplies of cheaper electric power that will eventually become available from atomic energy developments, other than to indicate in a broad way the general lowering of costs of production and distribution, and the improvement in the conditions of work and in domestic amenities for rural populations that would result. With the reservation that such benefits must undoubtedly await the widespread application of nuclear power in industry, mention may appropriately be made of some of the further though admittedly still speculative developments that might be possible in

agriculture, forestry and fisheries. Although at this stage any discussion of these possibilities may seem to be purely visionary, some reasonable speculation appears to be fully justifiable in view of the spectacular technological advances in the past few decades that have revolutionized the human way of life in less than the span of a generation. Already an atomic-powered submarine is in existence and in some quarters atomic-powered locomotives and aircraft are said to be only a few years away. Much, therefore, of what appears to be fantastic today may be a commonplace of tomorrow.

It has already been pointed out that whilst the reduction of losses due to diseases, pests and spoilage, and the adoption of various improved techniques which lead to more efficient exploitation of areas already cultivated are the most immediately promising ways of increasing production, attention should also be given to the benefits that might arise through bringing new areas into cultivation, although this is a more difficult and expensive undertaking. Probably the greatest contribution in this direction would be through the provision of irrigation facilities, particularly in the desert and semi-arid areas of the world. Many areas suitable for such development exist in North and South America, Africa, Central Asia and North China, to name but a few. There are also areas where drainage and reclamation of marshlands and deltas, especially in the tropics, could render land fit for cultivation, though there are fewer opportunities for this type of action than in the case of irrigation. The availability of more abundant and cheaper power from atomic reactors might well make possible the development of such irrigation and drainage projects. In addition work is now under way on the partial de-salting of water, and abundant and cheaper power would make it possible to do this on a scale adequate for irrigation. Such applications would be of particular significance to the under-developed countries, especially where conventional power sources are deficient.

As in the case of agriculture, in forestry more abundant and cheaper power would have far-reaching implications. It would affect not only the primary, often small, rural forest industries as well as the larger ones such as the manufacture of pulp and paper, but also forest policy and management in general. For instance at the present time about one-half of the world's harvest of wood is used for fuel, which forms a particularly important source of energy in rural areas. Past experience has shown that where a more convenient, and sometimes more efficient, source of power is available, the use of wood as fuel drops very rapidly. This may be of advantage or disadvantage to good forest management, according to the circumstances. In areas where wood is scarce and the forests badly abused by destructive harvesting to meet the needs of the population, as in some Mediterranean and Asian countries, the replacement of wood by an alternative source of power would greatly facilitate the much-needed reconstruction of an adequate forest cover. On the other hand in some regions the use of wood as fuel is often an important factor in good silviculture, offering the sole or one of the few markets for small and rough assortments that would otherwise be unmarketable. This shows the complexity of the problems involved and, particularly in view of the length of time needed to plan and execute changes in forest management practices and policies, explains the unusually keen interest of forestry in the long-time changes in power sources and costs that may be affected by atomic energy developments.

The availability of cheaper power would also make possible the more efficient exploitation of large forest areas and would have a particularly marked effect on the practicability of establishing economically sound and integrated wood industries in the remote and lesser-developed parts of the world. Looking even further afield, it might play a most important part in opening up some of the world's last untapped forestry resources in the tropical areas, particularly in South America and Africa.

Similarly atomic power could be of considerable significance to the fishing industry, where the development of nuclear power units of a size suitable for installation in ships would be of particular interest for possible use in mother and factory vessels of fishing and whaling fleets operating over long periods at great distances from their bases, especially in the Antarctic, since they might in various ways lead to substantial reductions in operating costs.

This discussion of the possible implications of cheaper and more abundant power for agriculture, forestry and fisheries is by no means intended to be exhaustive but rather to give in broad outline an indication of what atomic energy developments could mean to these industries. Agriculture is perhaps the most conservative of our arts, but when modern methods of farming and the multitude of highly technical services provided by an up-to-date department of agriculture are compared with primitive nomadic and pastoral systems of food production, it is obvious that the farmer is ever ready to adopt improved methods which increase the efficiency of his usage of land, water, capital and labour. It is therefore reassuring to know that this newest of our scientific advances, atomic energy, can contribute in so many ways to man's oldest industry and thus open up the way to improved methods of feeding, clothing and housing the world's ever-growing population.

Record of Proceedings of Session 7.2 (Agriculture)

THURSDAY MORNING, 11 AUGUST 1955

Chairman: General B. C. de Mattos (Brazil)
Scientific Secretaries: Messrs. L. F. Lamerton, A. Finkelstein and I. D. Rojanski

PROGRAMME

P/618 The utilization of radioactive isotopes in biology and agriculture in the USSR ..... A. L. Kursanov

P/780 The uses of atomic energy in food and agriculture ... Food and Agriculture Organization

DISCUSSION

Mr. A. L. KURSANOV (USSR) presented paper P/618.

Mr. R. A. SILOW (Food and Agriculture Organization) presented paper P/780, as follows: I am speaking on behalf of the Food and Agriculture Organization of the United Nations. I propose to discuss in general terms the contributions that atomic energy can make to better nutrition and higher standards of living for the peoples of the world, through improvements in food and agricultural production.

The problems involved are of immense magnitude, for the world's population is growing so rapidly that it is likely that within even the next quarter century we shall be about 50 per cent more in number than we are at present. The neo-Malthusians believe that we are engaged in a losing battle, but FAO considers, on technical grounds, that the necessary increases in food and agricultural production can be achieved, and has recommended to governments that they should aim at increases in production 1–2 per cent greater than their anticipated growth in population. For many countries, this will mean actual production increases of 3–4 per cent a year. This will not be an easy task for governments, but success is of such general concern to all peoples that national efforts should be supplemented by pooling the technical knowledge and experience of all countries on an international basis for the common good.

In this connexion, the exchange of information that is taking place at this Conference will be of great value, for atomic energy can contribute much to agricultural and food production.

There are three main ways of providing more of the primary necessities of life. The first, and easiest, way is to reduce the heavy losses which now occur in production, storage and distribution. The second is to increase the productivity of land and other resources now in use, through the application of improved technical methods. The third is to develop new areas and resources. Atomic energy can contribute to each of these ways of increasing supplies. Some of the potential contributions are mentioned briefly in the FAO paper (P/780), and many will be dealt with in much greater detail in the papers to be presented by others in the technical sessions.

First, there is atomic power itself to be considered. This could without doubt affect agriculture in many ways, but it is something for the future and must await the widespread application of atomic power, in industry in the first place. Cheaper and abundant power could make a substantial impact on agriculture, not only by generally cheapening costs of production, improving conditions of work and making modern conveniences and comforts more widely available to rural workers, but also through other, less conventional, possibilities.

Any discussion of these must at this stage be purely speculative, but in view of the spectacular technological advances of the past few decades—as, for instance, in air travel and in radio and television—some attempt to assess the contributions that might be expected from atomic power seem fully justified. These contributions would be of particular value for the opening up of new areas for cultivation.

In this connexion, there is the possibility of extending irrigation facilities, and related to this is the possibility of partially, or even completely, desalting saline water on a scale adequate for irrigation. Such applications could revolutionize the development of the semi-arid and desert areas of the world. There is also the possibility of draining swampy but potentially productive areas, particularly in the tropics. Similarly, cheaper power would make possible the more efficient exploitation of large forest areas in remote parts. A word of warning, however, seems desirable here. Opportunities for such applications are particularly common in areas which are underdeveloped, but in such areas developments of this nature will not and cannot occur overnight.

Turning from such speculations concerning atomic power, there can be no doubt whatsoever that the radioactive isotopes and radiation that have become available as by-products of nuclear reaction are of great significance to agriculture. It was at one time thought that these might prove of direct value in the stimulation of plant growth, but extensive investigations in several countries have failed to substantiate

such hopes. Neither radiation nor radioisotopes appear to be capable of direct contributions to increased production in the sense that an application of fertilizer leads to increased yields. Their contribution to food and agriculture is indirect, but nevertheless of immense potential. The ability of radiations to induce inherited mutations is being used extensively in plant breeding programmes and in other ways, whilst their sterilizing effects have promising applications in food preservation and pest control. The value of radioisotopes lies in their use as highly refined research tools in tracer studies in a wide range of nutritional, metabolic, developmental and pathological investigations in plants, animals and man. In this way they are giving information obtainable in no other way, or only at much greater cost in time or money.

By giving a clearer insight into basic biological processes that have hitherto been obscure, the use of radioisotopes in tracer studies is leading to the development if improved techniques and to greater efficiency and economy in the production and utilization of agricultural products. Bearing in mind that it was only unfettered scientific investigation of the nature of the atom, the pursuit of knowledge for its own sake, that made nuclear energy available to mankind, the contributions which radioactive tracer studies can make to agricultural production through research may similarly be almost unlimited in magnitude. In the time available it is possible to do no more than mention a few of those contributions.

Potential food supplies and other agricultural products are subject to heavy losses through fungal and bacterial action and the ravages of insect pests, and it is by combatting these that the most immediate and spectacular improvements in supplies can be achieved.

The use of radiation to kill insect pests in stored grain and to extend the storage life of perishable foodstuffs such as fruit, vegetables, meat and fish can do much to reduce wastage in storage and distribution.

Tracer studies are leading to better knowledge of the pests and diseases that attack crops, and are playing an important part in the development of more effective insecticides and fungicides.

Contributions to increased productivity include the development of higher yielding varieties of crops by irradiation, whilst tracer studies are leading to the development of improved methods of crop and animal husbandry. Thus the present limited use of fertilizers in many countries is almost entirely a question of economics, and tracer studies are showing how fertilizers should be employed in order to ensure the fullest possible return from them.

Through the use of radioisotopes remarkable progress has been made in the elucidation of the mechanism of photosynthesis which may well lead to more efficient methods of converting energy from the sun into chemical compounds. How much this might contribute to the enlarging of the world's food supplies is at this stage a matter of speculation, but the work so far seems to hold much promise.

Tracer studies are also giving much impetus to investigations of the basic problems involved in animal production. They have been used, for instance, in fundamental investigations concerning protein-synthesis in the animal body, and the biochemistry of lactation, and they have been very effectively employed in studying the efficiency of food utilization in animals. An example of a particularly ingenious application of radiation to the control of insect pests in livestock is provided by a successful demonstration in a limited area of the possibility of eradicating the screw-worm fly which causes losses in the United States of America to the extent of some $20 million annually in killed or crippled animals or damaged hides.

Although the major part of the world's surface consists of water, the oceans and inland waters have been exploited only to a limited extent, and provide less than 2 per cent of the world's food supplies. Nevertheless, fish is an important element in the diet of many peoples. Radioactive isotopes have many uses in fisheries research. They are providing information essential for the evaluation of fisheries sources and leading to a better understanding of the factors involved in ocean and fresh-water productivity that will permit more efficient exploitation of our water resources.

Finally, radioisotopes have important applications in the field of human nutrition. Much of such knowledge is gained from animal studies. Thus work in progress on the metabolism of amino acids and proteins in mammals is of special significance in connexion with certain deficiency diseases such as the protein deficiency syndrome known in Africa as *kwashiorkor*.

Use of the tracer technique with human beings has naturally been rather limited up to the present, but some work has already been done in connexion with fat and protein metabolism and mineral nutrition. It is clear that tracer studies can make valuable contributions to a better understanding of the physiology of human nutrition, especially when more suitable isotopes become available. This in turn will affect the way in which available food supplies are utilized, and thus will help in the development of adequate balanced diets, which are so important for the maintenance of health and efficiency.

From this brief survey of biological and agricultural applications, it is evident that atomic energy can contribute in many ways to the better feeding, clothing and housing of the world's ever-growing population. Some of the previous speakers in this Conference have already pointed out that shortage of food rather than lack of power may well be the limiting factor in human progress in the relatively near future. This survey shows that this need not be so; indeed, in the long run the contribution that atomic energy can make to increased production and to the more effective

utilization of agricultural products may turn out to be just as important for our welfare as the more obvious and more spectacular contribution that it makes as a source of industrial power.

DISCUSSION OF P/618 and P/780

Mr. R. S. CALDECOTT (USA): My question is addressed to Mr. Kursanov: I wonder whether you were able to determine that carbon dioxide entered the plant from the soil and that it did not enter as some other sort of carbonaceous compound.

Mr. KURSANOV (USSR): In our first experiments we placed the whole plant by the roots in a vessel so arranged that there could be no exchange of gas in the air surrounding the root and that this air could not penetrate to the upper parts of the plant. We used small concentrations of carbonic acid or of a carbonate for this purpose. The possibility of the carbon dioxide passing through in any other way was excluded in these experiments. We then analysed the roots and the other parts of the plants. Later, the carbon dioxide continued its passage. By appropriate treatment of the roots, we established the presence of carbonic acid in the form of carboxyl groups in the organic acids.

Mr. S. MITSUI (Japan): I should like to ask Mr. Kursanov two questions. The first one is: do you think it is promising to promote plant growth by internal irradiation from radioisotopes: in other words, by the absorbed radioisotopes? My second question is: do you have any experience in the USSR on the use of radioisotopes for the fundamental research work or applied experimentation on soil erosion control?

Mr. KURSANOV (USSR): I think that in a number of cases internal irradiation may affect the rate of growth of plants. Quite a large number of experiments of this kind have been carried out in the Soviet Union, and Professor Kuzin has compiled a special report on them which has been made available to this Conference. It seems to me, however, that internal irradiation has so far not given us any results which can be reproduced with absolute certainty. While stimulation can be observed in some cases, the method does not prove quite effective in others. I think that this experience offers possibilities for further research and requires a thorough preliminary study of metabolic changes.

To reply to the second question: Soviet scientists have begun to use radioactive isotopes in the study of soil erosion, but, so far as I know, only recently. I cannot, at this moment, give the results of any completed studies.

Mr. J. W. T. SPINKS (Canada): I was much interested in the work described by Mr. Kursanov on the utilization of radioactive isotopes in the USSR. Would the speaker care to tell us the extent to which universities participate in this work, and, secondly, what administrative machinery is used to give overall direction to the program in the USSR?

Mr. KURSANOV (USSR): The universities are participating in the work on the utilization of isotopes, along with many other scholastic, scientific, and experimental centres of the Soviet Union. The normal procedure is followed in the use of radioisotopes for biological work; they are obtained in the same way as other reagents and are applied as appropriate by institutions engaged in scientific research in the various fields.

Mr. I. REIFER (Poland): My question is meant for Mr. Kursanov. Has any work been done with radioactive isotopes in the biosynthesis of alkaloids in plant tissues, such as the synthesis of opium alkaloids? I feel that tracers would solve the problem of whether the alkaloids are or are not end products of the metabolism of nitrogenous compounds.

Mr. KURSANOV (USSR): You are quite right. Tracers, of course, open up great possibilities for the biological study of the synthesis of secondary substances, and in particular of alkaloids. You are interested in certain special alkaloids. Using radioactive carbon, Ilyin has just recently done some very interesting work in Moscow on the synthesis of alkaloids of the nicotine groups in the roots of tobacco and makhorka (*Nicotiana rustica*). These experiments showed in particular that alkaloids, as clearly demonstrated by the tests of Shmuk and others, are synthesized in these plants and that carbonic acid absorbed by the roots from the soil is used in this synthesis.

Mr. E. C. WASSINK (The Netherlands): I should like to comment on a statement in Mr. Silow's printed text. The statement that not more than one per cent of the total available energy in the sunlight falling on a green leaf is effectively used by photosynthesis seems true for various crop plants as far as the entire growth period is concerned. There are indications, however, that in certain short phases of the growth the yield may be significantly higher. Moreover, special experiments have shown that the entire photosynthetic chain runs with a much higher efficiency at lowed light intensities, both in higher plants and in green algae. The process as such, and especially the photochemical reaction, does not appear to be particularly inefficient, but external limitations, for example, carbon dioxide and water supply, and mineral nutrition, may well interfere with obtaining maximum yields under various natural conditions.

Session 13C.2

RADIATION-INDUCED GENETIC CHANGES AND CROP IMPROVEMENT

LIST OF PAPERS

The Contribution of Radiation Genetics to Crop Improvement

By W. R. Singleton, C. F. Konzak, S. Shapiro and A. H. Sparrow,* USA

Radiation genetics had its origin in the researches of Muller and Stadler almost thirty years ago. They demonstrated that by means of X-rays it was possible to induce genetic changes in animals and plants. These researches really opened a new era not only for fundamental genetics but for the practical plant and animal breeder.

Before, the breeder was limited tu the spontaneous mutations that had occurred at some time in the material with which he was working, and since in most crops there was an abundance of spontaneous mutations the plant breeder was able, by the application of genetics, to do a creditable job of plant breeding. Rapid advances were made follnwing the rediscovery of Mendel's law in 1900 by Correns, DeVries and Tschermak.

However, the discovery that mutations could be induced by radiation gave added impetus to plant breeding efforts. No longer was the plant breeder limited to mutations that occurred naturally. He could make them occur. Whereas a particular mutant might appear only once in a hundred years, by radiation it was possible to increase the frequency as much as a hundredfold, making it possible to induce desired mutants perhaps in one year.

Hence, evolution can be accelerated. This does not mean that the plant breeder's problems are all solved. It merely means that he can have a wealth of material from which to select. In his original paper, "Artificial Transmutation of the Gene," Muller[4] made the statement, "Similarly, for the practical breeder, it is hoped the method will ultimately prove useful."

The method has proved useful. Its usefulness was demonstrated first in Sweden through the brilliant researches of Nilsson-Ehle, Gustafsson and their associates, who demonstrated in cereals that higher yields, stiffer straw and earlier ripening types could be produced by radiation. Considerably later, its usefulness was demonstrated in the United States shortly after World War II in a comprehensive experiment by Dr. W. C. Gregory at the North Carolina Experiment Station.[1] He began by X-raying one hundred pounds of peanuts, *Arachis hypogaea* L., through the facilities at the Oak Ridge National Laboratory. He observed 975,000 X_2 plants and from these was able to select types that were superior in yield, better adapted to mechanical harvesting, and resistant to a serious leaf spot disease.

The peanut breeding project of Gregory demonstrated the wisdom of large populations in a radiation breeding program. Since the rate of mutations, even under radiation, is only a few per cent and since relatively few of the induced mutants will have any practical application, it is imperative to work with large numbers.

Dr. Gregory's experiment also demonstrated that it is possible to induce mutations in a rather stable organism. The peanut has a fairly low spontaneous natural mutation rate, but mutations were abundant following radiation. Also the peanut, being self-pollinated, is not subject to natural crossing whereby new germ plasm can be incorporated. Hence, radiation has provided about the only feasible way of introducing new germ plasm into this rather stable organism and thereby increasing the genetic reservoir from which the plant breeder can extract new and desired types.

Radiation genetics research at the Brookhaven Laboratory has been concerned with obtaining information regarding the effectiveness of the different types of radiations in producing mutations and also how the altering of various conditions including extra and intracellular environment may change the effectiveness of radiation.

The effects of chronic gamma radiation on growing plants have been investigated. This was made possible by placing a Co^{60} source in the center of a field with plants grown in concentric circles[8] (Fig. 1). This type of radiation is effective in producing endosperm mutations in maize, with mutations increasing more

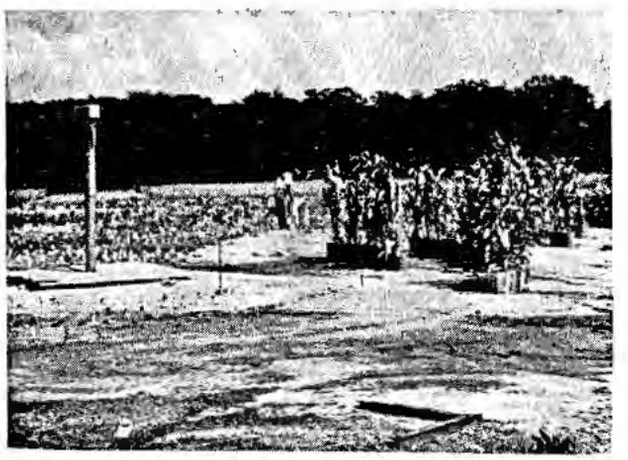

Figure 1. Gamma radiation field at Brookhaven Laboratory. Plants grow around Co^{60} source in center

* Brookhaven National Laboratory.

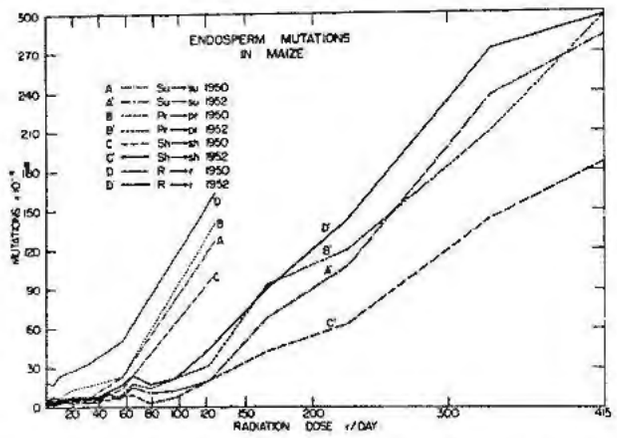

Figure 2. Endosperm mutations in maize as affected by chronic gamma radiation

sharply than the radiation dose, giving a curvilinear relationship (Fig. 2).[5] Also it has been found that there are extreme differences in sensitivity at different stages in the meiotic cycle (Fig. 3).[6]

This last finding suggests a different approach to the use of gamma radiation for the production of mutations, which can be produced as readily by giving a high exposure of one day's duration, as by exposure to a lower dose during a longer period. Sufficient radiation for mutation production can be obtained by placing plants fairly close to a small source.

With this in mind a Co^{60} gamma-radiation machine has been designed by the Nuclear Engineering Department in cooperation with the Biology Department (Fig. 4). This machine will house a 200-curie source and it is estimated it can be produced for less than $5000, thus making chronic gamma radiation available to more research workers in other locations throughout the world.[3]

Although the sensitive period during the meiotic cycle for other plants has not been determined, it seems reasonable to assume that a period analogous to that of corn might be expected. If such proves to be the case it will be possible to induce mutations in the developing gametes by exposing growing plants for a short period to a Co^{60} machine similar to the one described above. For plants in which it is difficult to obtain quantities of pollen for treatment perhaps such a technique would be best to produce mutations in the gametes. Such mutations would give rise to a whole plant carrying the mutant gene. This is in contrast to mutations induced in the somatic tissue, including seeds, in which the mutant affects only a part of the plant. This will be discussed more fully under somatic mutations.

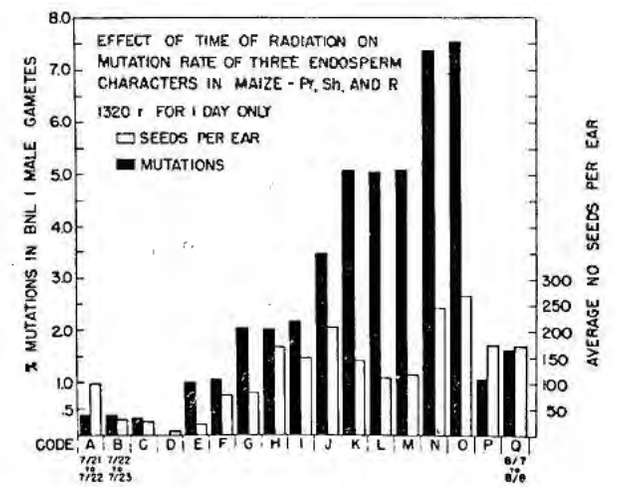

Figure 3. Late stages in pollen development (*N* and *O*) more susceptible to mutations by radiation than meiosis (*D*) or pre-meiotic stages, (*A*—*C*)

With a plant like maize, also some of the forest trees, where it is possible to obtain readily quantities of pollen, and where mass pollinations can be made easily, it is feasible to induce mutations by exposing the mature pollen to such radiations as ultraviolet light, X-rays and either slow or fast neutrons. It has been found that slow neutrons are comparatively efficient in producing mutations in maize pollen. Mutation rates in excess of 4% per gene for endosperm characters have been obtained, with a maximum not yet reached. Different genes react differently. Mutations of the Sh_1 gene reach the maximum at a much lower dose than the other genes used, *Pr*, *R* and *Su*. Mutation rates in excess of 3% per gene have been obtained with X-rays and ultraviolet.

SEED IRRADIATIONS

Technically mutations produced by radiation of seeds are somatic mutations (to be discussed later). However seed irradiation is quite distinct from the radiation of growing plants for the production of somatic mutations and hence will be discussed separately.

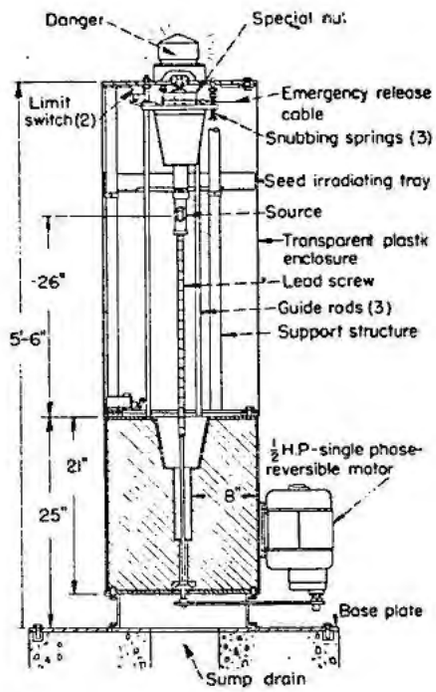

Figure 4. Co^{60} radiation machine with 200-curie source for irradiating plant material

Mutations can be produced in abundance by X-rays or by slow or fast neutrons. Neutrons are considered more efficient since a more uniform effect is produced regardless of the physiological conditions of the seed.

Extensive oat irradiation experiments have shown that it is possible to produce resistance to stem rust (*Puccinia graminis avenae*) in oats (*Avena sativa* L.) by exposure of the seeds to thermal (slow) neutrons.[2] There were 48 cases of induced resistance (Fig. 5) among plants grown from treated seed at a rate in excess of 10%.

In another radiation experiment seeds of the Tama variety of oats, which is susceptible to Victoria blight caused by *Helminthosporium sativum* var *victoriae*, were irradiated with thermal neutrons. From 642 R_2 progenies 10 mutations were found (Fig. 6). Seeds were also X-rayed (25,000 r). In this experiment 3 mutations were recovered from 122 progenies. No mutations were observed in 140 control progenies.

These experiments demonstrated also that it was possible to obtain resistance for blight while retaining a type of resistance to another pathogen, crown rust, *Puccinia coronata avenae*. Rust-reaction classes varying from type 1 (resistant) to type 4 (fully susceptible) have been observed in blight-resistant mutant types. The rust-reaction type of the original variety may not have been recovered but there is hope that it may be.

The result of these studies suggest that the use of mutagenic agents may make possible certain combinations of hereditary characteristics which are difficult to produce by hybridization methods and which may not yet have been found in nature. For example, resistance to certain races of crown rust is closely associated with blight susceptibility in oat varieties of Victoria parentage. This association has not been broken in regular genetic studies. However, the preliminary results reported here indicate that it may be possible with radiation to break this association and recover types not yet produced.

Figure 5. Induced resistant mutant (right) compared with parental susceptible variety, Mohawk

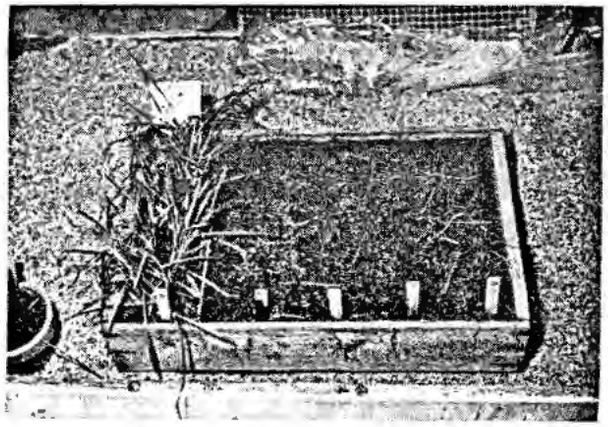

Figure 6. Oat variety normally resistant to *Helmintosporium* (left) and few resistant mutations produced by radiating susceptible variety

These preliminary experiments with inducing disease resistance in oats have been confirmed by other workers in a number of cereals and other crops. When it is realized that plant diseases cost the farmers in the United States alone an estimated 3 billion dollars a year, the importance of induced disease resistance is clear.

There is some evidence that resistance to *Helminthosporium turcicum* has been induced in the Wisconsin-16 inbred of maize. In 1953 pollen was irradiated in the thermal column of the nuclear reactor. Following radiation the pollen was used to sib-pollinate (mixed pollen placed on silks of the same inbred) plants of the W16 line. These were selfed in 1954 and tested for resistance to *H. turcicum* in the winter of 54–55 in southern Florida. Fifteen of the 83 progenies (18%) showed increased resistance. Further tests are necessary to determine whether the resistance was actually induced or was segregating. Additional tests are being conducted, also experiments using inbred lines susceptible to *Helminthosporium carbonum*. It is expected that these trials may provide definite information regarding the feasibility of inducing disease resistance in maize.

SOMATIC MUTATIONS

Mutations in the somatic, or body, cells of the plant, in contrast to the mutations induced in the germ cells, are of considerable interest and economic importance. Many of our varieties of horticultural plants, such as the pink dogwood, the Golden Delicious apples, and others, have arisen as somatic

mutations. In carnations the William Sim or Red variety has given rise, by somatic mutation, to a number of others, notably White Sim, Harvest Moon, "Mamie" and Tetra Red. These derivatives of the William Sim variety, along with William Sim constitute a fairly large percentage of all carnations grown.

Since it was found feasible to induce gametic mutations so readily, the question naturally arose whether we could induce somatic mutations. These could then be propagated asexually and new varieties established immediately.

As this appeared to be a project with practical applications it seemed wise to enlist the aid of the agricultural experiment stations which are interested in new varieties for their respective areas. A conference was called at the Brookhaven Laboratory. Representatives of the experiment stations and universities in the northeastern part of the United States were invited. They came and were enthusiastic. A cooperative project was born.

The original gamma-radiation field was enlarged and the source increased to 1800 curies, enabling the field to accommodate a larger number of plants at areas of effective doses. Eight universities and experiment stations are growing a wide variety of horticultural crops in the field. An idea of how the "somatic" part of the field looks can be obtained from Fig. 7.

Although it is too soon to expect results from most of the slower growing woody plants, positive results have been obtained with some of the herbaceous plants such as *Antirrhinum majus* L., in which tetraploid sectors were induced (Fig. 8). *Dahlia pinnata* Cav. and *Nicotiana Sanderae* Sander have produced mutations involving at least a whole flower. Other flowers showing smaller somatic mutations have been tabulated by Sparrow.[7]

In carnations (*Dianthus caryophyllus* L.) several propagatable somatic mutations have been produced. Eleven of 87 cuttings from plants of White Sim receiving the highest doses of radiation up to 340 r/day produced branches that had all red flowers (Fig. 9). The only two plants removed from the 340 r/day row later developed branches with all red flowers. Cuttings of the red flowered type have produced plants with only red flowers.

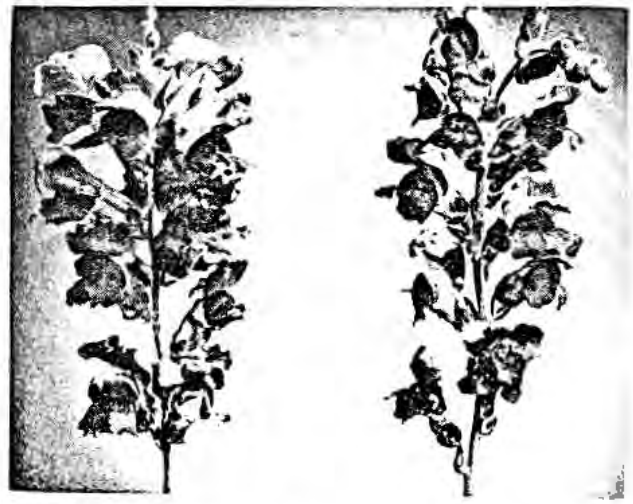

Figure 8. Normal *Antirrhinum* (right) compared with induced tetraploid (4N) (left,) with larger flowers

Graphs showing the dose response relationships for somatic mutations are found in Figs. 10–13. It is not possible to state definitely whether there is a threshold in each case since in some cases the control rate is below the effects produced by the low level

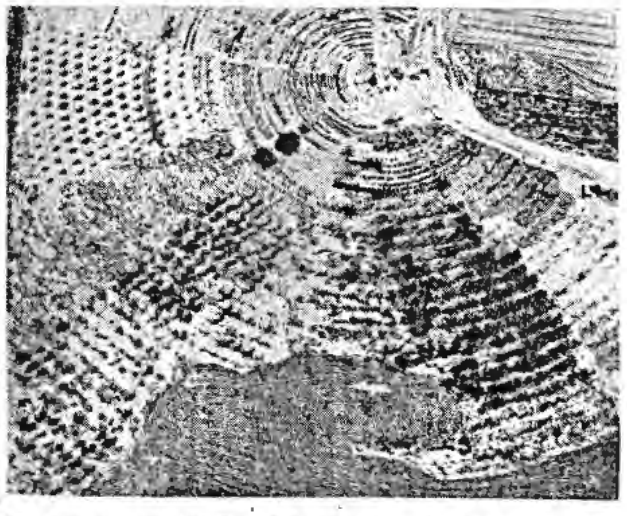

Figure 7. Gamma radiation field showing orchard trees in somatic mutation program

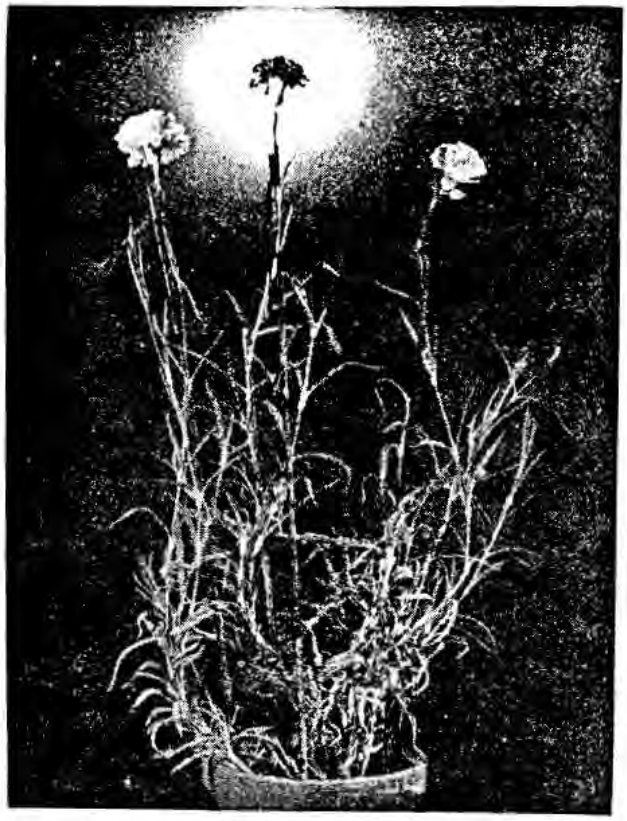

Figure 9. Branch with red flowers induced by radiating White Sim carnation

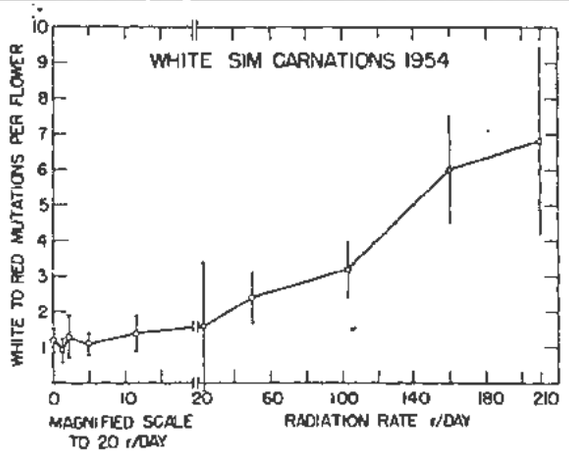

Figure 10. Dose response of somatic mutations in White Sim carnations

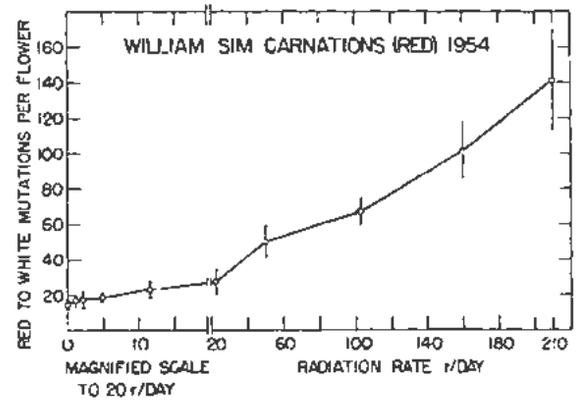

Figure 11. Dose response of somatic mutations in William Sim carnations

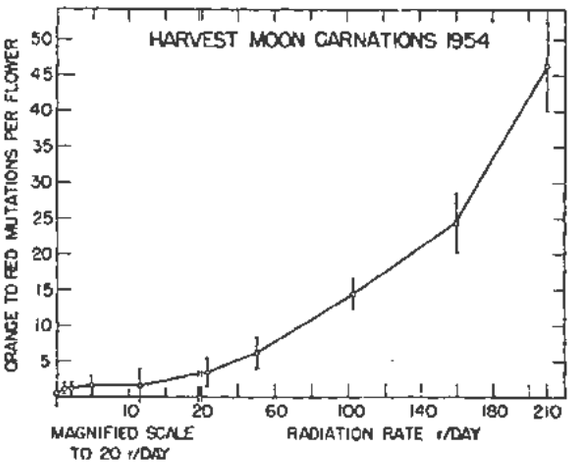

Figure 12. Dose response of somatic mutations in Harvest Moon carnations

radiation effects. This is definitely true of the *Antirrhinum* where the control is significantly below the "plateau" of low level radiation effects. Two of the carnation graphs, for William Sim and White Sim, do not differ significantly from a straight line relationship, while the Harvest Moon graph seems curvilinear.

Extensive somatic mutation data were obtained with the P^{RR}/p gene in maize (Fig. 14). More than

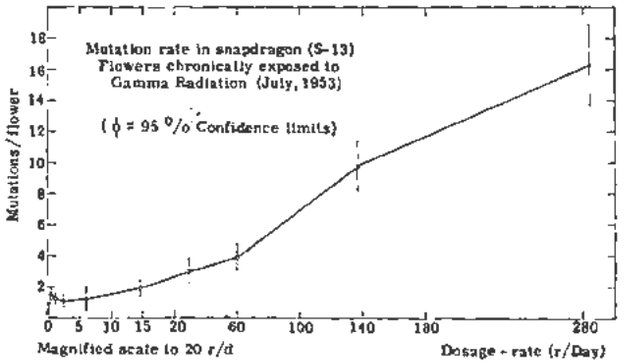

Figure 13. Dose response of somatic mutations in snapdragon (*Antirrhinum*)

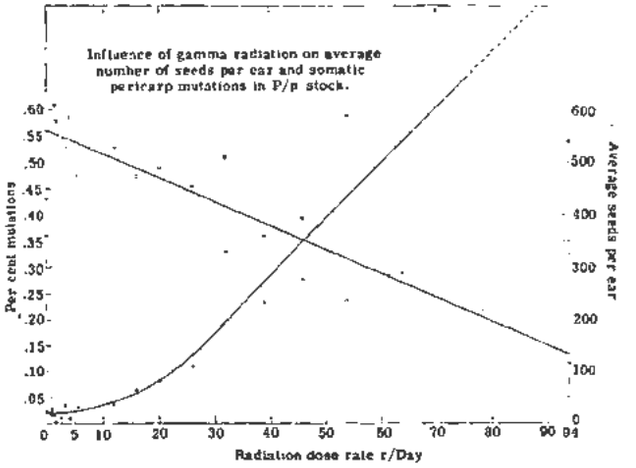

Figure 14. Dose response of somatic mutations in P p stock of maize, and reduction in seed set as a result of radiation

a million kernels were observed. Most of the somatic changes were rather small, producing in many instances a very fine streak. However, for the graph presented here only those mutations which were greater than trace were scored as mutations. In a few instances somatic mutations involved a whole kernel or a cluster of kernels. These were comparatively rare. Most mutations were small, indicating initiation rather late in the life of the ear. From the lower doses of radiation there is not a linear relationship between dose of radiation and mutations. Below 5 r/day there was no apparent effect of the radiation.

It was also observed that radiation injured the developing ear. From less than one to approximately 100 r/day, there was an inverse linear relationship between radiation received and number of seeds per ear (Fig. 14).

COOPERATIVE RESEARCH

It was mentioned earlier that the gamma field is used cooperatively by research workers in eight different experiment stations and universities. These workers grow their material in the field, which is maintained by the Brookhaven Laboratory. Any resulting new varieties become the property of the investigator who takes full responsibility for taking all notes on the material growing in the field.

In addition to this cooperative project other facilities unique to the Brookhaven Laboratory are made available to other investigators. One such facility is the thermal column of the nuclear reactor for treating seeds and scions. Also material is X-rayed for those not having access to an X-ray machine. To date, radiations have been made on more than 60 crop plants for well over 100 investigators located at many places throughout the United States and Canada. It is too early to expect positive results from each of these investigators. However, we are pleased to report that workers in Minnesota are able to report positive results of disease resistance induced in plants. That work is being reported to this conference by Dr. W. M. Myers.[9]

Radiation as a tool in plant breeding has made a modest beginning. We feel that each succeeding year will show progress. It is impossible to envision the eventual good that may arise from the use of radiation in plant breeding; and we thoroughly agree with the late Enrico Fermi who said, "I believe truthfully, that the conquest of atomic energy may be widely used to produce not destruction, but an age of plenty for the human race."

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The Production of Beneficial New Hereditary Traits by Means of Ionizing Radiation

By L. Ehrenberg, I. Granhall and Å. Gustafsson, Sweden

Ionizing radiations, irrespective of mode of production and of properties, readily induce hereditary changes (mutations), which are stable and lead to new traits manifested also in following generations. Truly induced changes of genes and chromosomes were first described by H. J. Muller.[8] This discovery has influenced genetic research immensely. For a long period of time induced changes were considered by most workers to lead to a break-down of the hereditary material, the induced mutations thus being exclusively "harmful", this word taken in the sense that the individuals, mutants, manifesting the new property, would be, if not entirely monstrous or lethalized, at least distinctly inferior to the original type as regards viability. It is a fact indeed, that most of the induced mutations decrease viability in the homozygous state, i.e., when the mutated gene is present in the double dose, and that many mutations lethal when homozygous show a more or less detrimental effect even in the heterozygous state. But such a type of behaviour prevails in spontaneous mutations, too.

Nevertheless, recent studies have shown that the induction of the mutations is an important tool in plant breeding. Relative to several hundred "harmful" hereditary changes there are one or two raising production in the homozygous state or, in some other respect than production, implying a "positive" alteration. (We here define a positive mutation of an agricultural species as a hereditary change in some way beneficial to man's interests and well-adapted to his very peculiar type of "ecological niche".) The spontaneous mutation rate is so low that the time, labour, and experimental area required for the collection of positive mutations, naturally arisen, would be unreasonably great. Since, by the application of ionizing radiations, the mutation rate can be raised several thousand times, such a collection is now made possible and can be adopted as part of the breeding routine, without any special expansion of ordinary experimental stations.

In this connection it ought to be pointed out that many mutations, which from the traditional point of view should be regarded as deleterious, since they cause lethal effects when homozygous, will increase viability when heterozygous. In organisms like barley (Gustafsson 1946, 1947, 1954) or *Antirrhinum*[10] these "beneficial lethals" are not uncommon, but their occurrence has been described also in *Drosophila*,[7] even for the X-chromosome.[9] In fact, Gustafsson concluded that lethal factors play an important role in the population dynamics of cross-fertilizing organisms. This does not contradict the views, eloquently vindicated by Muller, about the eugenic hazards of an uncontrolled use or rather misuse of ionizing radiations in human activities. Chromosome rearrangements of various kinds, as well as long deficiencies and duplications of the chromosome material, are relatively more abundant when applying ionizing radiations than is the case in nature, and many gross chromosome alterations show conspicuous effects of a deleterious kind even when heterozygous.

The principles for the induction of positive mutations have now been worked out in a large number of agricultural species. The Swedish group treating the subject has chiefly concentrated on barley, which is a diploid self-fertilizing organism, where the mutations are easily identified, and on wheat which is a polyploid, but still a self-fertilizing organism suitable for mutation analysis. In barley, in striking contrast to wheat, there is a wide range of morphological variation in the mutants, from small, scarcely perceptible changes to most profound alterations in type, even breaking the morphological frame of the variety or species. In spite of their greatly altered appearance, many mutants are surprisingly productive. This is for instance the case with the "erectoid" mutants in barley, about 75 cases of which are now being studied. The erectoid mutation takes place, as far as we know, by changes in 16–20 different gene loci. Many of these mutants display features, valuable from an agricultural point of view. The grain (and straw) production approaches or equals that of the mother strain; in some cases the mutant is even superior by a few per cent. At the same time erectoid mutants distinctly surpass the mother as regards stiffness of straw. Some are earlier, possess higher protein contents, etc. In addition, the erectoids are, more so than the mother line, able to utilize very high nitrogen dressings, a desired property in to-day's farming. Other mutants, e.g., the so-called bright-greens, behave quite contrary in this respect; they thrive better in a meagre soil. On the other hand, they appear to be more resistant towards certain fungi than the respective mother strains.

The changed response to nitrogen dressing is one example of an altered reaction norm, involving the sudden registration of new ecological requirements by induced mutation. A new "ecotype" or race has arisen. As previously mentioned, ionizing radiations induce frequent chromosomal rearrangements (sometimes these are beneficial when homozygous, detrimental when heterozygous, in contrast to lethals) leading to deviating chromosome configurations detectable in cell division. Now and then the erectoid mutations originate simultaneously with such chromosome rearrangements. This causes the formation of more or less pronounced sterility barriers in the crosses between mutants and mother strain. In fact, by one stroke and simultaneously, we may induce all the essential characters distinguishing species in nature: (1) the origin of a sterility barrier, (2) a drastic change in morphology and anatomy, (3) an altered ecological response, and (4) a new karyotype detectable microscopically (Gustafsson 1954).

It has been definitely shown, in barley and other experimental plants, that induced mutation can increase the yielding capacity of a variety, or leave this capacity intact and improve upon special characters of importance in agriculture (apart from the stiffness of straw or the response to an increased nitrogen dressing) as regards earliness, protein or oil content, baking quality, malting properties, fibre strength, and grain size, in cereals as well as in peas, lupines, flax, mustard, tomatoes, etc.

A few examples of interest will be given: In Sweden, where the yellow sweet lupine (*Lupinus luteus*) is cultivated north of its centre of adaptation, it has been considered especially important to breed for an increased earliness. This was found to be difficult with the traditional methods of breeding (hybridization and selection). After X-irradiation of seeds a greater variation in the actual property was obtained, and selection for earliness became successful, without any significant decrease of the yielding capacity (Tedin 1954). A few X-ray varieties are at present released into the market: the Primex white mustard of Svalöf (Andersson and Olsson 1954), which by an increase of grain yield as well as oil content surpasses the mother strain, in oil yield per area about 7 per cent; the "Strålärt" (Ray Pea) of Weibullsholm, and the "Schäfers Universal" beans, *Phaseolus* (cf. Knapp[6]). Numerous rather promising mutants in barley, wheat, peas, lupine are being tested in large-scale experimentation; some are in fact included in the official Swedish state trials.

The majority of high-productive mutants, especially of species represented by very high-bred and specialized varieties, like barley and wheat, are not suited for direct marketing. The basic use of induced mutations therefore consists in the building up of a new variability, similar to that occurring in the old-time varieties of the species, but based on the highest-yielding modern varieties. The subsequent crossing of these mutants with one another or with other varieties will contribute to the gradual improvement of cultivated plants and may effect this with a considerable gain of time.

In fruit trees spontaneous somatic mutations (bud sports), which can be propagated vegetatively, have played a great role in the production of new market varieties. Out of 143 apple varieties with known parentage, marketed in the USA and Canada during the last thirty years, no less than one fourth have originated as bud sports (Granhall, 1954), and in pears, peaches, plums, and cherries about ten per cent have originated in such a manner. At the Balsgård Fruit Breeding Institute, Sweden, techniques have therefore been worked out to treat scions or seedlings with different ionizing radiations, in order to induce bud sports. The investigations carried out have presented valuable information, in so far as mutations regarding colour and shape of fruits as well as ripening time have been obtained (Granhall).

In barley, the effects of different ionizing radiations are extensively being compared, partly as a first step in investigating the methods of how to direct and control the mutation process, i.e., to influence the distribution of mutation types in a special direction. These studies have so far revealed that, when seeds are irradiated, the total mutation frequency increases more rapidly with linear energy transfer (ion density*) than does the lethalizing action of the radiation (Ehrenberg and Nybom 1954). The lethalizing action of fast neutrons, produced by (d,n) reactions in cyclotrons (ion density: 400–800 ion pairs/μ) or in the pile centre (1000-3000 ion pairs/μ), is about 20 times that of 175-kv X-rays ($\sim$100 ion pairs/μ) or Co^{60} γ-rays (8 ion pairs/μ). Since, however, then mutagenic efficiency is about 40 times greater, about twice as many mutations are obtained, at the point of 50 per cent survival.

As to the distribution of various mutation types it has been shown (Ehrenberg and Nybom, 1954) that lethal mutants, deficient in chlorophyll and other plastid-borne pigments, are relatively more common after neutron irradiation, and that consequently the vital mutants, i.e., those of practical interest, are commoner after irradiation at a low ion density: twice as many mutants, relatively seen, being vital after X-ray treatment as after neutron treatment. With a corresponding survival of the irradiated generation the proportion of mutants of practical interest will therefore be about the same after treatment with X-rays and γ-rays on one hand and fast neutrons on the other.

Although the material is still rather scant, it can be stated with a high degree of probability that, within the group of vital mutants in barley, the relative frequency of erectoid mutations increases with increasing linear energy transfer of the radiation, the frequency in the 1953 experiments being about 25 per cent after irradiation with X-rays, 50

* Expressed as ion pairs/μ, i.e., the average number along the linear particle path in tissue of unit density and assuming the absorption of 32.5 ev to correspond to the formation of one ion pair.

per cent for cyclotron-produced neutrons, and nearly 100 per cent after irradiation in the pile. Data from the 1954 experiments further evidence this difference in relative efficiency.

Within the group of chlorophyll-deficient mutations several lethal types can be recognized [3], viz., a colourless *albina*, a light green or yellow-green *viridis*, and a yellow *xantha*, which are the most common ones, and in addition several rare types. Each group of mutants is realized by changes in a great many genes. The relative frequencies of these types are seemingly identical when X-rays with 100 ion pairs/μ or neutrons ionizing at 400–800 ion pairs/μ are applied. When ion density is varied within a wider range, it becomes evident, however, that the relative frequency of rare chlorophyll mutations, treated as a group, increases with increasing ion density.[1,2]

In addition it has been shown that a variation of the irradiation conditions, other than ion density, may provoke displacements of the types of chlorophyll-deficient mutations. The *viridis* type, which occurs in combination with a high sterility of the irradiated generation,[3] is thus more common under conditions favouring a high sterility (Ehrenberg, 1955). About 22 per cent *viridis* are obtained when moist seeds are irradiated with low doses ($\leqslant$ 5000 r), whereas the frequency amounts to about 62 per cent when dry seeds are irradiated with high doses. The latter very high frequency approaches what is obtained when chlorophyll mutations are induced by means of the chemicals, mustard gas and nitrogen mustard (Mackey 1954).

Summing up we may state:

1. The inducing of mutations by means of ionizing radiations has given and can give hereditary changes of high production capacity in agricultural plants. If the mutants, although high-productive, are not suited for direct marketing, they form a source of variation valuable for the normal continued breeding.

2. It has been proved, in principle, that the spectrum of mutation types can be intentionally displaced in different directions. This is achieved, for instance, by a variation of the ion density, from the minimum obtained with Co^{60} γ-rays or P^{32} β-rays, via X-rays and neutrons of different origin to densely ionizing α-rays, but also by a variation of the irradiation conditions or by the application of mutagenically active chemicals. In this way also the origin of positive, directly valuable mutations can be controlled. These results form a first step in our attempts to learn how to influence selectively the mutation of individual genes.

Most agricultural species, even high-bred ones like barley, wheat or corn, are still rather old-fashioned as to morphology, anatomy and karyology, in spite of fifty years or more of intense breeding work. They need to be reconstructed in agreement with the requirements of modern agriculture, facilitating a high mechanization and an intense fertilizing of the soil, in this way increasing yield and quality but decreasing labour and costs of cultivation. Recent studies by Wettstein (1954, unpublished) indicate that the construction of the straw of wheat and barley ought to be fundamentally repatterned, with regard to internode number and internode length as well as cross-section area of the nodes and internodes, before the varieties can be considered fully adapted to high nitrogen dressings and intense mechanization. With regard to internode structure, barley should be changed towards wheat; with regard to cross-section area, wheat, in its turn, should be remade to resemble barley. These changes can be effected by induced mutation, possibly more easily than by hybridization of different varieties and subsequent selection. Similarly, in corn, the mutual development of vegetative and generative parts ought possibly to he readjusted in such a way as to decrease the vegetative system and to increase, relatively speaking, grain production, without an unnecessary waste of nutrients and photosynthetic materials. Finally, the karyotype of some species, like barley, seems to be rather old-fashioned, remaining on an ancient status. The modernization of the karyotype may render possible a better coordination of genes and mutations in the continued breeding program of the future.

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A full account of the mutation studies carried out by the Swedish group of research workers is given in a series of articles, "Mutation Research in Plants", in *Acta Agriculturae Scandinavica*, Vol. *IV*:3, where the articles cited and not listed below can be found. Further work on the mechanism of action of ionizing radiations in plant seeds is summarized by Ehrenberg in *Svensk Kemisk Tidskrift* Vol. *67*, 1955, pp. 207-222.

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Studies on Genetic Effects of Chronic Gamma Radiation in Plants

By Knut Mikaelsen,* Norway

Most studies on biological effects of ionizing radiation have been made after acute exposures to radiation over short periods of time. Treatments of seeds or pollen have been the methods most frequently used to induce genetic effects in plants. Much work on radiation-induced mutations in plants has been reported since the early work of Stadler.[16, 17]

More recently other methods have been demonstrated to produce genetic effects in plants. At Brookhaven National Laboratory in the USA Sparrow and Singleton [15] installed a Co^{60} source in a field, and plants grown around it were exposed to continuous or chronic gamma radiation during growth and development. Granhall, Ehrenberg and Borenius [1] have reported that experiments with chronic gamma irradiation have also been started in Sweden.

Although Stadler [17] claimed that radiation-induced mutations are deleterious to the plants, some optimism about a possible application of artificial mutations as a method in plant breeding has appeared. A considerable amount of work has been carried out and some positive results have been obtained. A special issue of Acta Agriculturae Scandinavica [18] has been devoted to the subject of mutation research in plants and the most important works in the field are reviewed and discussed in it. For further information we refer to that publication.

In Norway, mutation research was started in 1948 and a great number of mutants have been produced in barley by different treatments of ionizing radiations. A few mutants with valuable agronomic characteristics can be mentioned. We have mutants with all degrees of stiffer and shorter straws. Early ripening mutants have also appeared frequently. One extremely early mutant may deserve some attention. This mutant has, for the last 4 years, matured 3 weeks earlier than the mother strain. Unfortunately, however, the mutant has weaker straw and gives a lower yield. The same may also be true for other mutants carrying certain favourable characteristics. Therefore, several crosses are being carried out to find out if the mutant genes may be of more advantage in new combinations.

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Although some advantageous mutations are known, the majority of radiation-induced mutations are deleterious. In our experiments we have learned that it is necessary to study a very large amount of material in order to find the infrequent positive mutations. Thus, for plant breeding purposes, it is of great importance to work out methods by which not only the total mutation frequency but also the frequency of advantageous mutations can be increased. This problem has received much attention in our studies. Different acute treatments of seeds by X-rays, gamma rays and neutrons are being carried out. Since 1953, experiments with chronic or continuous radiation during growth have also become possible and new methods are being tried in our attempt to make ionizing radiation a useful tool in plant breeding. Some results of these chronic radiation experiments will be presented.

THE RADIATION FIELD

The arrangement of our radiation field is in principle similar to the radiation field at Brookhaven National Laboratory in the USA.[15] Our radiation source is located in a metal tube mounted on the end of a lead cylinder 20 cm long. The lead cylinder slides inside an aluminium pipe. By a cable which is attached to the lead cylinder and a windlass 60 m from the source, it is possible to move the source up and down. The source can be lowered 1 m below surface. In this location the source is so shielded by the lead cylinder and a concrete block that negligible radiation can be measured on the surface. This safety precaution makes it possible to enter the field for planting, cultivating, taking notes, collecting material, etc., without exposing oneself to radiation. When the source is in operation, it is raised 70 cm above the surface.

The plants are ground in concentric circles or arcs around the source (Fig. 1). Radiation doses vary with distance from the source. In 1953, a 12-curie Ir^{192} isotope was operating in the field. Because of its relatively short half-life of 74.5 days, Ir^{192} was inconvenient as radiation source and was considered as a preliminary arrangement. It was replaced in 1954 with a 25-curie Co^{60} source. In the relatively small radiation field of 1953 more thorough studies

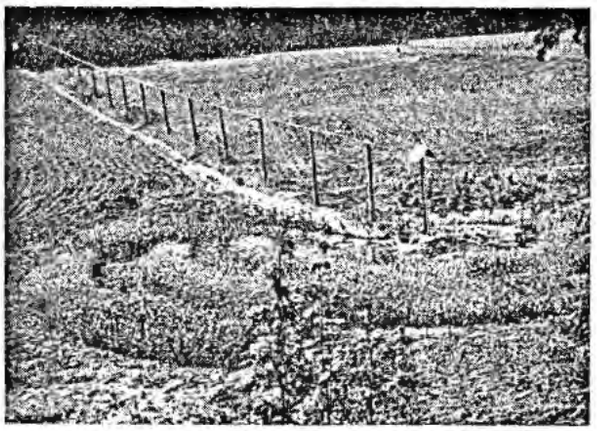

Figure 1. The radiation field. Plants grow in circles around the radiation source

were made only on barley and *Tradescantia paludosa*, while other species such as oats, wheat, lupines, onions and cabbage were grown only for observation. In 1954, the field was considerably extended and included barley, oats, wheat, *Tradescantia*, potatoes, sweet corn, blackberries, tomatoes, beans, strawberries, roses and other horticultural plants.

EFFECTS OF CHRONIC GAMMA IRRADIATIONS

As indicated by Sparrow and Singleton,[15] the tolerance of difference species to chronic gamma irradiation varies. In our material, *Tradescantia paludosa* proves to be very radiosensitive while barley seems to be one of the most radioresistent species tested. Physiological effects of radiation on growth and fertility are usually very pronounced at heavier doses or dose rates[9], and can be easily demonstrated in the field. Genetic effects can usually be verified only by more elaborate studies and analysis.

Effects on Chromosome Aberrations in *Tradescantia paludosa*

The cytological effects of chronic gamma irradiation which are presented by Sparrow and Singleton[15] indicate that the percentage of micronuclei in microspores of *Tradescantia paludosa* increases linearly with dose rates (r/day) and total dose given. The results also indicate that the type of aberrations represented by micronuclei are more dependent on the amount of radiation received per day than upon the total dose.

The effects on chromosomes of chronic gamma irradiation are also reported by Mikaelsen[7,8] in studies of the anaphase of root-tip cells of *Tradescantia paludosa*. He found that frequencies of acentric fragments and of bridges are both linearly related to dose rates (r/day) and to total doses. The experiments seemed also to indicate that the dose rate was a more important factor in the yield of aberrations than the total dose given. This finding seems to deviate from the usual concepts of dose relationship and a comparison between an acute and a more chronic radiation exposure would be of interest.

The following experiments have been carried out: Cuttings of *Tradescantia paludosa* (Clone B2-2) were used as experimental material. Part of the material was exposed to 12.5, 25, 50, 100 and 200 r in a few minutes as an acute treatment, while other cuttings were exposed to the same doses over 24- and 48-hour periods to give chronic exposure during the process of mitosis. Root tips were fixed in alcohol/acetic acid (3:1) 24 hours and 48 hours after the start of the experiment. Slides were prepared using the acetocarmine smear technique. Both acentric fragments and bridges were scored at anaphase and the results are summarized in Tables I and II. The figures are based on an analysis of 300–400 anaphase cells, except for the heavier doses of 200 r where fewer anaphase cells were found after all the acute exposures and in the 48-hour chronic series.

Table I. Number of Acentric Fragments per 100 Cells after Acute and Chronic Exposures to Gamma Radiation in Root Tips of *Tradescantia paludosa*

Total dose, r	After 24 hours		After 48 hours		Acute, from 2–168 hours after irradiation
	Acute	Chronic	Acute	Chronic	
12.5	1.6	6.8	1.0	–	2.3
25	4.1	15.7	2.4	9.1	5.0
50	3.2	29.3	12.1	16.9	9.9
100	50.0	59.7	45.7	35.3	30.0
200	117.2	–	50.0	76.1	93.7

The results indicate that the yield of chromosome aberrations after doses from 12.5 to 100 r are greater when the radiation dose is given over 24- or 48-hour exposure periods than when the exposure time is only a few minutes. At a dose of 200 r where the data are based on approximately hundred cells only, no difference can be demonstrated.

An exact comparison between the two radiation treatments is difficult because the sensitivity of chromosome varies during mitosis. During chronic radiation over the period used in this experiment, all stages of mitosis are exposed to radiation, while

Table II. Number of Bridges per 100 Anaphase Cells after Acute and Chronic Gamma Irradiation in Root Tips of *Tradescantia paludosa*

Total dose, r	After 24 hours		After 48 hours		Acute, from 2–168 hours after irradiation
	Acute	Chronic	Acute	Chronic	
12.5	0	1.3	3.2	–	0.9
25	0.6	2.3	1.4	1.9	1.8
50	0	5.3	0.8	3.1	3.0
100	4.6	5.1	0	5.3	5.6
200	13.8	–	14.3	12.0	24.2

analyzed cells in the acute series were all in a particular stage during the radiation exposure. In the column to the right in Tables I and II the sum of all analyses from the following periods after the acute exposures are listed: 2, 6, 12, 18, 24, 36, 48, 72, 96, 120, 144 and 168 hours. Thus, cells in all stages of mitosis and delayed dividing cells are then included in the analysis and the total radiation damage of the acute exposures should have been analyzed. The frequencies are still lower than after chronic treatments at the moderate doses of gamma irradiation. The same tendency can be demonstrated if the data only from 2–24 hours and from 2–48 hours are added together.

Effects on Chlorophyll Mutations in Barley

The most striking effect of ionizing radiation in plants is the segregating of chlorophyll mutants in the progenies of the irradiated plants (Stadler,[16, 17] Gustafsson,[4, 5] and many others). Since chlorophyll mutation is the most frequent type of mutation in irradiated barley plants, analysis of chlorophyll mutants should be an important means of evaluating the genetic effects of radiation treatments.

After radiation exposures of seeds, the segregation of chlorophyll mutants appears in the second generation (the progeny of the irradiated plants). After chronic radiation of growing plants, the segregation of chlorophyll mutants appears in the third generation. The second generation of plants from chronic radiation treatments and the first generation of plants from acute treatments of seeds have many characteristics in common. The appearance of chlorophyll mutants is as rare as the spontaneous rate by which those mutations occur. During the late fall of 1954 and winter of 1955, analysis of chlorophyll mutants in the third generation of barley material grown in the gamma field in 1953 was carried out (Fig. 2). A wide range of types of chlorophyll mutations have been defined and described.[5] Detailed analyses of the different types were carried out, but will not be presented in this communication because more material is accumulating.

In Table III are tabulated the results of the analysis of chlorophyll mutations in the Norwegian barley variety, *Domen*. It appears that the relatively radioresistent barley has a considerable chlorophyll mutation frequency of 0.8 per 100 spikes at a dose rate of approximately 5 r/day and hence a total dose of approximately 280 r. The mutation rate increases proportionally with dose. The increase is slower between 5 and 25 r/day than between 25 and 56 r/day. No conclusions about mutation-dose relationship can be reached on the basis of these data, but it is tempting to point out that the results seem to be in good agreement with the results obtained by Sparrow and Singleton[15] on mutation in some particular loci in maize exposed to chronic gamma radiation. A gradual increase was obtained between 5 and 32–57 r/day. Above 57 r/day, a more marked increase was obtained.

Table III. Frequencies of Chlorophyll Mutations in Progenies from Barley Plants Treated with Gamma Irradiation for 56 Days during Growth

Dose rate, r/day	*Total dose, r*	*No. of spike progenies*	*No. of mutations*	*No. of mutations per 100 spikes*
0	0	0	0	0
± 5	± 280	1186	10	0.8
± 13	± 700	1121	15	1.3
± 20	± 1100	1100	9	1.6
± 25	± 1400	315	8	2.5
± 31	± 1700	376	18	4.8
± 41	± 2300	83	6	7.2
± 56	± 3700	84	8	9.5

It is surprising that these relatively small total doses of gamma radiation have produced such large numbers of chlorophyll mutations. Acute treatments of gamma rays on seeds would certainly not have produced these mutation rates at these doses. No convincing gamma-irradiated material are available for comparison. X-ray material of *Domen* variety from two independent experiments may be used for comparison and as a measure of the effectiveness of the chronic irradiation. The X-ray doses are given to dormant seeds. In Table IV the chlorophyll mutation frequencies are tabulated from those two experiments in 1951 and 1952, respectively.

From the seeds treated with 20,000 r and 25,000 r only very few plants survived and the mutation rates of those doses are not reliable. It is striking that a considerably larger dose of X-rays given to the

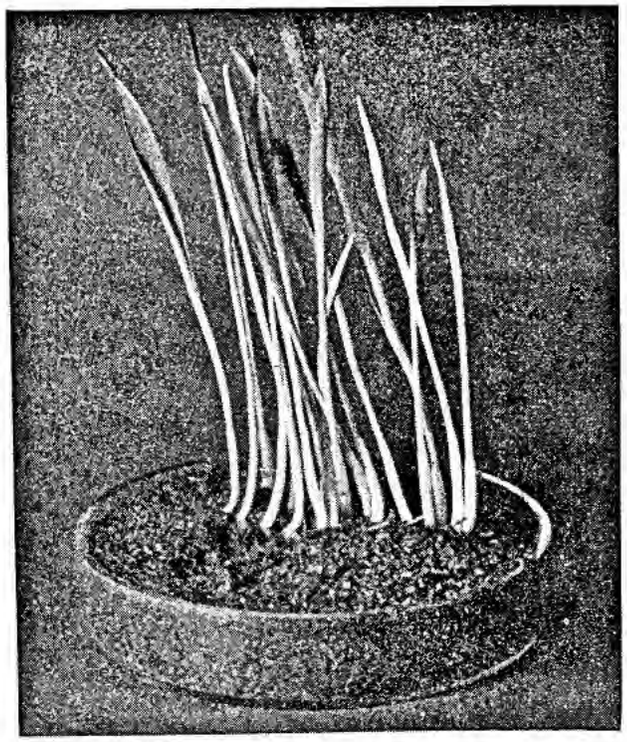

Figure 2. Segregating of chlorophyll mutants in a spike of barley

Table IV. Frequencies of Chlorophyll Mutations in Barley after Acute X-Irradiation of Seeds

Dose	*5000 r*	*7500 r*	*10,000 r*	*15,000 r*	*20,000 r*	*25,000 r*
1951	–	–	2.7	6.7	(5.6)	(25.0)
1952	1.2	4.5	4.0	6.8	(34.8)	–

dormant seeds is necessary to produce the same effects as the more sparsely ionizing gamma rays given to the growing plants.

Effects on Somatic Mutations

In vegetatively propagated plants, such as many fruit trees and some horticultural plants, bud mutations have played an important role in the production of new varieties and of genotypic variation. Such changes appearing in somatic tissue are considered as somatic mutations, which can be of great importance if they can be propagated vegetatively. Sparrow [13, 14] has studied such somatic changes appearing after acute and chronic radiations in a number of flowering plants.

In our experiments *Tradescantia paludosa* and different strains of carnations were chosen as experimental material. *Tradescantia paludosa* (Clone B2-2) was grown in the gamma field for 56 days in 1953 and the plants were exposed to chronic gamma radiation of dose rates from 5.6 r/day to 76.4 r/day. Studies on the effects of radiation were confined to changes in flower morphology and colour. By a thorough study of the flowers of the normal non-irradiated plants, it appeared that among 465 flowers studied, 12.7% carried one or more abnormal flower characters (Table V).

The frequency of abnormal flowers increased remarkably after the radiation exposures. At an average dose rate of 5.6 r/day, throughout the experimental period, the percentage of abnormal flowers increased to 72.5. At dose rates between 5.6 r/day and 18.2 r/day the percentage of abnormal flowers varied between 72.5 and 94.9.

The changes in flower parts refer to changes in number, shape and colour of sepals, petals, stamens and pistil. A detailed description and analysis of the abnormalities will be given elsewhere, since the investigations are not finished. In Fig. 3 some changes in sepals are illustrated. Beside the normal flower with three sepals, flowers with two and four sepals are found. Intermediate situations also appear as, for example, small accessory sepals and leaves which were partly petal and sepal. Similar changes were observed in number of petals (Fig. 4). The larger number of petals was sometimes accompanied by a reduction in number of stamens, indicating that floral parts had developed into petals instead of stamens. A larger number of stamens had developed partly into petals. Changes in shape of petal (Fig. 4) appeared also frequently as well as white chimaeras of different size and shapes on the blue petals. Shorter stamens and pistil were also common abnormalities in the flowers. When the plants were exposed to a higher dose of 37.2 r/day flower development was entirely inhibited and no flowers appeared (Table V). The inflorescences were after a while overgrown by clusters of modified leaves (Fig. 5). The development of plants receiving dose rates of 54.1 and 76.4 r/day (Table V) was completely inhibited and the plants finally died.

Cuttings were taken from the plants exposed to dose rates of 7.7 to 18.2 r/day. Most of the plants derived from these cuttings reverted to normal growth pattern. Some plants still produced high percentages of abnormal flowers.

Some of the cuttings from the irradiated plants have not reverted to normal growth and seem so far to be constant in their new characters. The fact that most cuttings taken from irradiated plants showed normal growth patterns may indicate that the abnormalities are mainly due to physiological effects

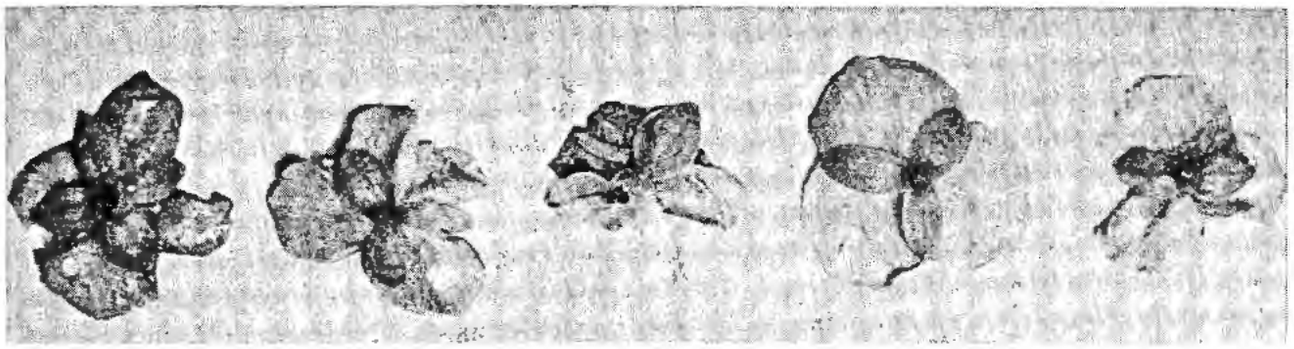

Figure 3. Changes in sepals of *Tradescantia paludosa* after chronic gamma radiation for 56 days. Second from right is a normal flower. Flowers with 2 (right) and 4 (left) sepals are found

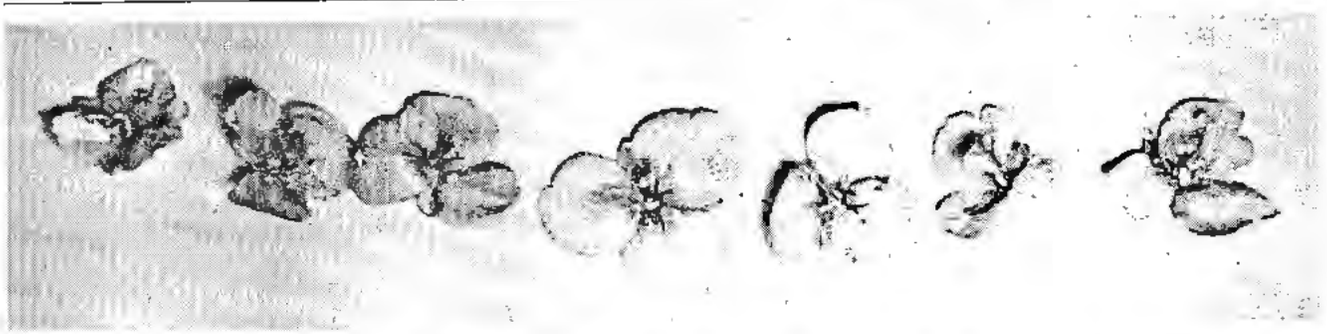

Figure 4. Chronic radiation-induced changes in petals and stamens of *Tradescantia paludosa* flowers. Normal flower in the middle

Figure 5. Flower heads of *Tradescantia paludosa*: left, normal,non-irradiated plant. Centre and right, heads from plants irradiated with 37.2 r/day for 56 days. The inflorescences are overgrown by clusters or modified leaves

of irradiation. The cuttings which have not reverted to normal growth, however, indicate that cytogenetic effects are also involved. Gunckel *et al.*,[2, 3] who have done a thorough study of the variations in vegetative and floral morphology of *Tradescantia paludosa*, claim that the changes they describe can not be ascribed to cytogenetic effects, but are considered as physiological, because cuttings from those plants revert to normal growth pattern. Therefore, it may be argued whether or not the changes observed should be defined as somatic mutations. The question may also be regarded as solely a matter of definition.

Table V. Effects on Flower Structures in Tradescantia paludosa after Exposures to Chronic Gamma Irradiation from Ir^{192} for 56 Days

Average dose rate, r/day	*Total No. of flowers*	*No. of normal flowers*	*No. of abnormal flowers*	*Per cent abnormal flowers*
0	465	406	59	12.7
5.6	51	14	37	72.5
6.8	92	21	71	77.2
7.7	126	9	117	92.9
8.9	93	20	73	78.5
10.3	84	16	68	81.0
12.1	39	2	37	94.9
14.4	78	12	66	84.6
18.2	43	3	40	93.0
37.2	0			
54.1	0			
76.4	0			

In 1954 somatic mutations were also studied in carnations. Plants from the strain, William Sim, with red flowers and a spontaneous mutant strain from this, White William Sim, were grown in the gamma field and were exposed to different dose rates of gamma rays. No quantitative analysis was carried out on this material, but observations on somatic changes in petal colours were recorded. In the red flowers white chimaeras of different size and shape (Fig. 6) appeared frequently in the irradiated plants. In the white flowers of the other strain similar red chimaeras were observed. These changes are true somatic mutations. New shoots derived from a mutated cell may contain completely the mutant characteristics and by vegetative propagation give rise to a mutant clone.

SUMMARY

Ir^{192} and Co^{60} have been installed in a field where different plant species are exposed to chronic gamma irradiation during growth and development. The installation and method of operation of sources of approximately 12 and 25 curies is described.

Figure 6. Somatic mutations causing white spots on petals in red carnation plants exposed to chronic gamma irradiation

The effects of chronic irradiation on chromosome aberrations have been studied in root tips of *Tradescantia paludosa* over a period of 24 and 48 hours. The results are compared with the results of acute exposure of the same doses given during a few minutes. The yield of chromosome aberrations was larger in the chronic series than in the acute at doses from 12.5 to 100 r.

The genetic effect of chronic gamma irradiation is studied on chlorophyll mutations in barley. The mutation rates increased with radiation dose. The dose relationship seems to be non-linear. Compared with X-irradiation of barley seeds, relatively small doses of chronic gamma radiation during growth produced pronounced frequencies of chlorophyll mutations.

Variability in flower characters and abnormal development of flower parts were noted in 12.7 per cent of the inflorescences on non-irradiated plants of *Tradescantia paludosa*. At a dose rate of 5.6 r/day of chronic gamma irradiation for 56 days, the percentage of abnormal flowers increased to 72.5. At dose rates between 5.6 and 18.2 r/day, the percentage of abnormal flowers varied from 72.5 to 94.5. At a dose rate of 37.2 r/day, the development of flowers was suppressed and the inflorescences were overgrown by clusters of modified leaves. At dose rates of 54.1 r/day and 76.4 r/day the development of flower heads was completely inhibited and no growth whatsoever was observed.

For the study of somatic mutations, the plants were propagated vegetatively. Cuttings from the irradiated plants reverted easily to normal growth, but some cuttings retained the mutant character of the mother plant.

Somatic mutations were also studied in a white and red strain of carnations.

This paper is published as Report No. 10 from the Institute of Genetics and Plant Breeding (Director: Professor dr. H. Wexelsen). The Agricultural College of Norway, Vollebekk, Norway. The studies are supported with grants from the Norwegian Research Council for Science and the Humanities.

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Ionizing Radiations as a Tool for Plant Breeders

By Richard S. Caldecott,* USA

The most readily available and effective mutagenic agents are high energy radiations which initiate changes in biological systems either directly or indirectly through ionizations. Prior to the advent of atomic fission most of the radiations used in biological studies were electromagnetic. However, there is presently a great deal of interest in the biological effects of high energy particle radiations. Some of this interest has been directed toward determining the relative mutagenic effectiveness of radiations with different specific ionizations.

For maximum utilization of the various sources of radiation in plant breeding, basic information must be catalogued on the relative kinds and frequencies of genetic and physiological phenomena that result from irradiation. To collate such information adequately, all of the factors which are known to modify the sensitivity of one species of radiation must be investigated to determine their influence on the other kinds of radiations. At present it seems that only through such studies will it be possible to obtain detailed information on the spectrum of genetic events that may prove useful to the plant breeder.

The principal objective of the present studies has been to determine what, if any, biological differences that are related to ion density could be detected in the growth and cytology of the barley plant. The writer would like to point out that space limitations have placed severe restrictions on the presentation of all the data pertinent to this subject. It is expected, however, that detailed reports on most of the unpublished data cited herein soon will appear in the literature.

Because of their availability, X-rays, 2-Mev electrons, thermal neutrons, fast neutrons, and to a lesser extent, cobalt-60 gamma rays were employed as radiation sources. The X-rays were generated with a G. E. Maxitron apparatus operated at 250 kvp and 30 ma, and were filtered through 1 mm of aluminum and the beryllium window of the tube. The 2-mev electrons were obtained from a Van de Graaff electrostatic generator.

Thermal neutron bombardments were conducted in the thermal column of the Brookhaven National Laboratory's nuclear reactor. The fast neutrons were obtained from the fission of U^{235}. Details of the irradiation procedures, radiation purity, and the spectrum of energies from each radiation source have been presented elsewhere.[1] It is significant that in our test organism, the barley seed, 98% of the radiation from thermal neutrons resulted from protons and alpha particles through capture reactions with nitrogen and boron.

It is recognized that, as we used them in these studies, a distribution of ion densities occurred with all these radiations. However, with X-rays, 2-Mev electrons, and gamma rays, a relatively large fraction of the energy loss occurred at a low specific ionization. On the other hand, with the neutron sources of radiation a relatively large fraction occurred at a high specific ionization. These studies then, are concerned with comparing the biological effectiveness of radiations which have, for the most part, a high specific ionization with radiations which have a low specific ionization.

PHYSIOLOGICAL AND CYTOGENETIC EFFECTS OF RADIATIONS

General Effects of Ion Density

Seedling Studies

Earlier studies have shown that when barley seeds were subjected to a dose of X-rays sufficient to reduce seedling growth materially, the seeds were not uniformly injured and the distribution of seedling heights about the mean was skewed. It was of considerable interest, therefore, when we found that seedlings from seeds treated with any dose of thermal neutrons gave a normal distribution of heights about the mean irrespective of any reduction in average seedling height resulting from the treatment.[2]

All efforts to explain the lack of uniformity with X-rays, either on the geometrical distribution of the X-ray beam and/or of the seed, failed. It was decided that this lack of uniformity must result from the physical nature of the radiation. From this inference, and from the fact that, through capture in the barley seed, thermal neutrons indirectly have a much higher specific ionization than X-rays, it seemed reasonable to assume that the difference may result from the spatial distribution of ion pairs in the irradiated material. In order to check this reasoning, and to preclude the possibility that the observed phenomena resulted from some experimental idio-

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syncracy, it was decided that the results should be compared with two different sources of radiation which had specific ionizations comparable to X-rays and thermal neutrons. Two-Mev electrons and fast neutrons were selected for this purpose.

Dormant seeds were subjected to X-rays, 2-Mev electrons, thermal neutrons and fast neutrons and then analyzed to determine the distribution of seedling heights from a number of doses. To elucidate the difference between the two types of radiations (those with a high as compared to a low specific ionization), doses of each radiation which caused a comparable average inhibition of seedling growth were graphed (Fig. 1). This figure lucidly illustrates the similarity between the curves obtained with thermal and fast neutrons on the one hand and between X-rays and 2-Mev electrons on the other. It seemed logical to conclude from these data that the differences observed were related to the physical characteristics of the radiations and, probably, to their specific ionization (cf. MacKey[4,5]).

Cytogenetic Studies

In one study which compared the effects of X-rays and thermal neutrons, treated barley seeds were planted in the field and permitted to produce mature plants. Cytogenetic specimens were taken at the pollen mother cell stage and the frequency of chromosomal interchanges as a function of dose was determined. In addition, counts of plant survival were made for all treatments before the plants were harvested for seedling mutation studies (cf. Caldecott *et al.*[2]).

By plotting the interchange frequency as a function of survival it was clearly demonstrated that more genetic interchanges could be obtained per unit survival with thermal neutrons than with X-rays (Fig. 2). The same was later shown to be true for seedling mutations. Preliminary studies conducted in the greenhouse indicated that differences of the same order of magnitude were obtained when seeds treated with gamma rays from cobalt-60 were compared with seeds treated with fast neutrons (cf. MacKey[4,5]).

Detailed comparisons of the kinds and relative frequencies of mutations induced with X-rays and thermal neutrons have failed to demonstrate any major differences (Table I), even though a higher frequency of any given type of change per unit survival is obtained with thermal neutrons than with X-rays. The same was shown to be true for the types and frequencies of interchanges. This indicates that densely ionizing radiations should be of more value to plant breeders than sparsely ionizing radiations because they give him a better probability of inducing the genetic changes which he seeks.

INFLUENCE OF HYDRATION

Through a combination of storing seeds in atmospheres with different vapor pressures and actually steeping them in water, a wide range of embryo water contents was obtained. By subjectings seeds with different water content levels to different types of ionizing radiations it was then possible to demonstrate pronounced differences in sensitivity modifica-

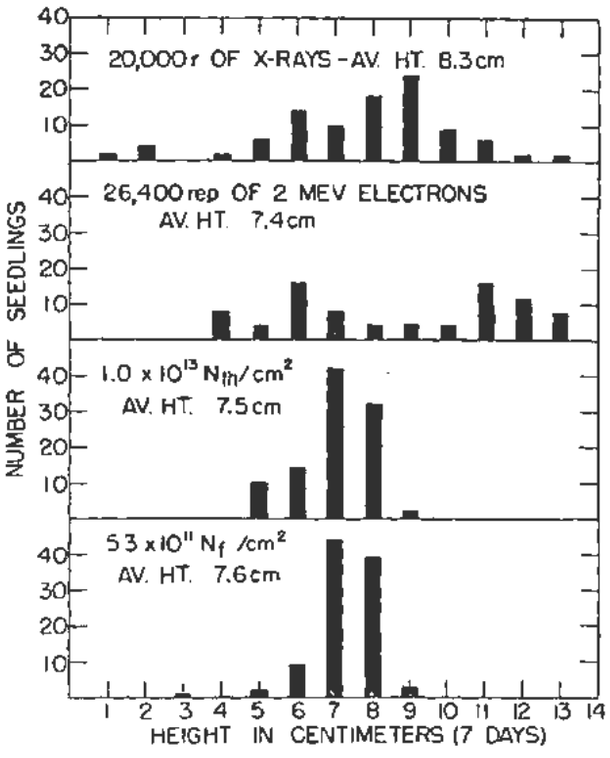

Figure 1. Frequency distribution of seedling heights from doses of X rays, 2-Mev electrons, thermal neutrons and fast neutrons that reduce the average height to a comparable degree

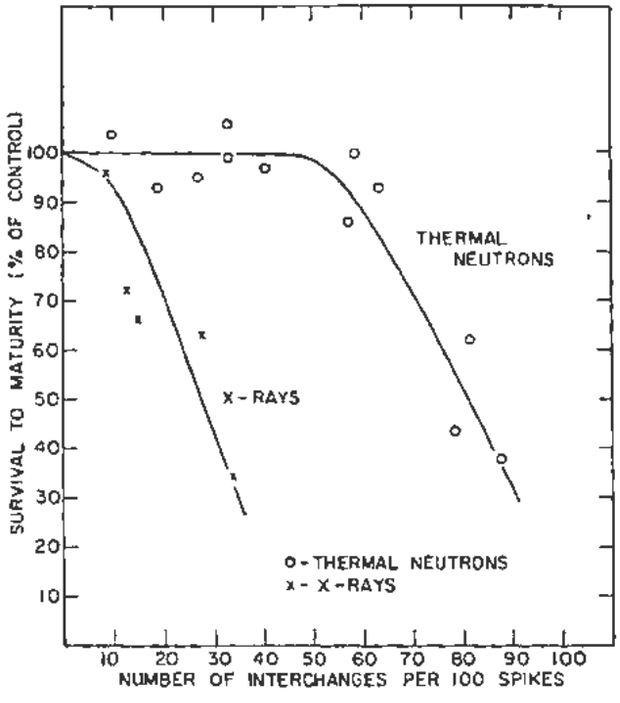

Figure 2. Relation of survival to interchange frequency in seeds of barley treated with X-rays and thermal neutrons

Table I. Frequencies of Types of Seedling Mutants Induced in Dormant Seeds of Barley Irradiated with X-rays and Thermal Neutrons

Mutant types	*Treatment*				*Total*	
	X-rays		*Thermal neutrons*			
	No.	*%*	*No.*	*%*	*No.*	*%*
White	387	56.0	779	56.8	1166	56.5
Yellow	102	14.8	140	10.2	242	11.7
Yellow green	114	16.5	237	17.3	351	17.0
Virescent	10	1.4	42	3.1	52	2.5
Striped white and yellow	17	2.5	36	2.6	53	2.6
Banded shrivel	20	2.9	52	3.8	72	3.5
Others	41	5.9	86	6.3	127	6.2
Total	691		1372		1063	

tion. These differences are of particular significance to the plant breeder because they give him an indication of the variables that must be controlled when seeds are irradiated if he is to be assured of a mature plant population to study.

Effect of Vapor Pressure

Seedling Studies

Dormant barley seeds were stored over solutions with different vapor pressures (Table II) until they reached weight equilibrium. At this time the water contents of the embryo ranged from about 4% to approximately 16% of their dry weight. Seeds representing each water content level were subjected to one of a range of doses of X-rays and then germinated and grown for 7 days in petri dishes after which time seedling height measurements were made (Fig. 3). The data demonstrate that the sensitivity of the seeds decreased as the water content of the embryo increased from 4% to about 8%. At this upper point a plateau was reached and apparently added increments of water between about 8% and about 16% gave little further modification of radiosensitivity. Efforts to obtain similar results with both fast and thermal neutrons were essentially negative although some experiments indicated a very slight sensitivity modification (unpublished).

These studies point to the fact that less rigid controls over seed storage conditions would be required to predict a given response when seeds are subjected to neutrons than when they are treated with X-rays. This is of particular consequence to plant breeders because they often experience difficulty in predicting biologically effective X-ray doses from year to year, and a readily available neutron source for treating their material would largely alleviate this difficulty.

Cytogenetic Studies

A sample of the seeds having the embryo water contents listed in Table II were subjected to 20,000 r of X-rays and then sacrificed for cytological analyses by removing the shoot tip of the germinating seed during the first cycle of cell divisions.

Cells at anaphase from embryos of all water content levels were scored to determine the frequencies of normal cells and those that had chromosomal bridges (Fig. 4). As the water content of the embryo increased from about 4% to about 8%, the frequency of detectable aberrancies decreased. At the upper level of hydration, a plateau was reached which apparently was not subject to modification by further addition of water to the embryo up to 16%. A comparison of

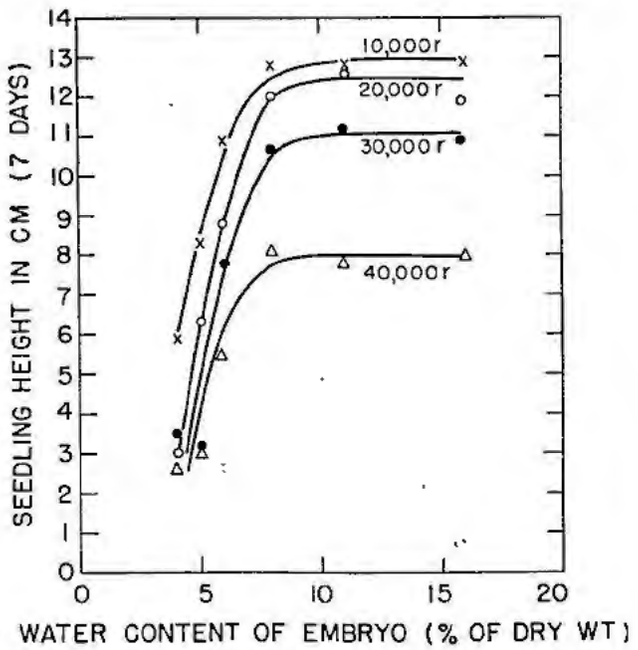

Figure 3. Relation between water content of embryos of dormant barley seeds and their sensitivity to different doses of X-rays

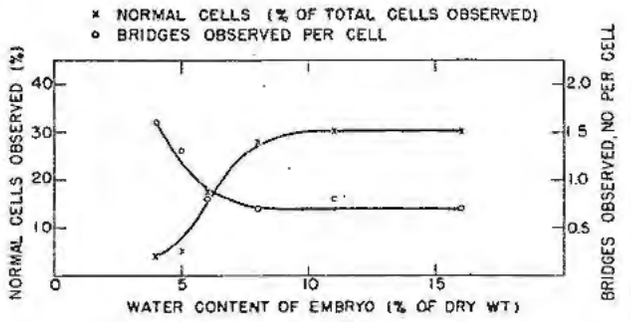

Figure 4. Relation between water content of embryos of dormant barley seeds and their sensitivity to 20,000 r of X-rays as determined by cytogenetic analyses

Table II. The Relation of the Water Content of Embryos and Endosperms of Barley Seeds to the Water Content of the Atmosphere in Which They Are Stored

Treatment Stored in desiccator at 20°C over	Water content of air mg/l	Approx. no. seeds	Water content of embryos and endosperms at equilibrium			
			Embryo		Endosperm	
			Wt, gm	H_2O,%	Wt, gm	H_2O,%
Dry P_2O_5	0.02×10^{-3}	440	0.8430	4.0	24.9801	5.0
Dry $CaCl_2$	0.20	440	0.8274	5.0	25.3545	6.0
Sat $CaCl_2$	5.52	440	0.8813	6.0	25.7708	8.0
Sat $NaHSO_4$	8.89	440	0.9347	8.0	26.7485	11.0
Sat $NaClO_3$	12.82	440	0.9102	11.0	27.7838	14.0
Sat $NH_4H_2PO_4$	15.92	440	1.0146	16.0	30.1678	19.0

Figs. 3 and 4 indicates that there is a good correlation between the degree of seedling injury as measured by plant height and the frequency of chromosomal aberrations in shoot tip cells.

These data mean to the plant breeder that he can increase the X-ray sensitivity of the genetic material of dormant seeds simply by desiccating them over P_2O_5 prior to irradiation. This would be most valuable where X-ray facilities were limited or expensive.

Effect of Soaking

Two of the factors known to limit the germination and growth of seeds are water and temperature. Water provides the working medium for the biochemical reactions incident to growth. The temperature at which growth takes place determines the rate of these reactions. If both the water content and the growth of seeds modify their radiosensitivity, it should be possible to increase or decrease the efficiency, per unit energy absorbed, of any given radiation in producing physiological and genetic changes.

In this connection, seeds originally desiccated over P_2O_5 were soaked at 22°C and 0°C, for various periods of time prior to X-radiation. Following irradiation the seeds were germinated and grown in petri dishes for 7 days at which time seedling height measurements were made. These studies showed that after one hour steeping at 22°C the seeds were much less sensitive to X-rays than controls that had been stored over P_2O_5 and not steeped. However, steeping the seeds at this temperature in excess of 2 hours resulted in a striking increase in their X-ray sensitivity so that after 6 hours they were more sensitive than dry controls. On the other hand, when soaked at 0°C the seeds just reached maximum resistance after about 4 hours steeping and they remained at this level of tolerance for at least 8 hours before they underwent a gradual increase in sensitivity. After 24 hours soaking at 0°C seeds were about as sensitive to X-rays as dry controls (Fig. 5).

Further studies on the nature of the sensitivity changes that accompany soaking at 22°C have demonstrated that seeds soaked for about 8 hours are about 10 times as sensitive to X-rays as dry controls that have been stored over P_2O_5 prior to irradiation. This increased sensitivity resulting from soaking for 8 hours is reflected by both a reduction in seedling growth rate and an increase in the frequency of chromosomal aberrations (unpublished). These studies indicate that soaking seeds at about 20°C for 8 hours or more gives the maximum increase in sensitivity that can be obtained with seeds under the conditions we have tested.

Studies on the effects of increasing the water content of seeds by steeping prior to subjecting them to thermal neutrons have shown that the treatment reduces their sensitivity for about 16 hours at 22°C and for at least 24 hours at 0°C. Other cytogenetic studies on seeds soaked at 22°C suggest that the period at which the increased sensitivity occurs with thermal neutrons is associated with the time at which chromosome doubling takes place in the cells of the seed (unpublished).

The reduction in sensitivity to thermal neutrons that accompanies the initial stages of soaking seems to be best explained by the fact that in the unsoaked seed over 90 per cent of the ionization results from protons and alpha particles from capture of thermal neutrons by nitrogen and boron respectively. Thus, when the water content is increased, the ratio of nitrogen and boron to the total elemental constituents of the seeds is decreased. From this it follows that relatively fewer thermal neutrons would be captured

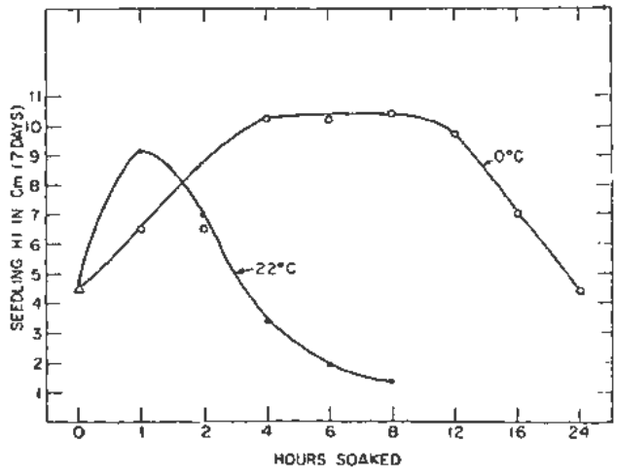

Figure 5. Effect of soaking barley seeds at 22°C and 0°C on their sensitivity to 15,000 r of X-rays

by nitrogen and boron and thus the biological efficiency per neutron would decrease.

In contrast to the observations with thermal neutrons, when seeds are soaked at either 0°C or 22°C and then subjected to fast neutrons their sensitivity increases with increased time of steeping. The rate of increase is greater at the higher temperature (unpublished). Undoubtedly, some of the sensitivity increase is associated with cellular activity but it seems likely that a considerable fraction results from the greater abundance of hydrogen for reaction with fast neutrons.

Influence of Atmosphere

It has been well established that a number of biological materials X-rayed in the presence of free oxygen are more sensitive than when X-rayed under anaerobic conditions.[3] There is some indication that the magnitude of this "oxygen effect" is dependent on specific ionization. Therefore, to help clarify the role of ion density in the oxygen effect, dormant barley seeds with about 10% water in the embryo were placed in atmospheres of air, oxygen, carbon dioxide, and nitrogen for 24 hours and then irradiated with either X-rays, fast neutrons or thermal neutrons. Following treatment the seeds were germinated and height measurements were taken after 7 days (Table III).

The atmospheres had little or no effect on susceptibility of the seeds to either neutron source of radiation. However, the seeds treated with X-rays were distinctly affected by the atmosphere in which the irradiation was given. X-radiation either in the presence of oxygen or air reduced seedling height by about one-half and one-eight respectively over that of the seeds treated in nitrogen and carbon dioxide.

Preliminary cytogenetic studies suggested that the oxygen effect produced when seeds were subjected to X-rays was also manifested in an increase in the frequency of chromosomal aberrations (cf. Hayden and Smith,[8] and Nilan[6]).

These observations are further evidence of the manner in which radiosensitivity can be modified by environment. However, it is still too soon to say conclusively whether or not it will be possible to alter the kinds and frequencies of genetic events obtained from irradiations by modifying the conditions under which materials are exposed.

From the plant breeder's standpoint the most significant feature of this study appears to be the fact that it presents him with a means of increasing the efficiency of the X-ray facility at his disposal.

SUMMARY

Optimum usefulness of ionizing radiations in plant breeding programs can be obtained only if plant breeders have adequate basic information which can be utilized in designing their experiments. In an effort to collate such information we have irradiated barley seeds, subjected to different degrees of hydration and oxygenation, with some of the following radiations: 250 kvp X-rays, cobalt-60 gamma rays, 2-Mev electrons, thermal neutrons and fast neutrons. The studies have shown that the neutron sources of radiation, which in seeds have a high specific ionization, produce from two to four times as many genetic events per unit survival as the more sparsely ionizing radiations.

In other studies it has been shown that the efficiency of X-rays (per r unit) in producing genetic changes and lethality can be increased by about a factor of 10 when seeds are pre-soaked for 8 hours at about 22°C. Such a treatment reduces the effectiveness of the thermal neutron by about 25% and increases the effectiveness of the fast neutron by a comparable amount. In the same way, pre-treatment with oxygen markedly increases the sensitivity of seeds to X-rays but has considerably less influence on their sensitivity to either neutron source of radiation.

It should be emphasized, that while the effectiveness of X-rays per r unit can be strikingly modified by pre-treatments, it has not yet been demonstrated that the degree of genetic damage per unit survival can be altered.

Detailed cytogenetic analyses and seedling mutatinn studies have failed to indicate any appreciable difference in the kinds of genetic changes induced by X-rays, gamma rays, fast neutrons, and thermal neutrons. As mentioned above, because neutron sources of radiation produce more genetic events per

Table III. The Influence of Atmosphere during Irradiation on the Sensitivity of Barley Seeds to X-rays, Thermal Neutrons, and Fast Neutrons as Measured by Seedling Height at 7 Days *

Treatment	X-rays		Thermal neutrons		Fast neutrons	
	No. seeds	Av. ht., cm	No. seeds	Av. ht., cm	No. seeds	Av. ht., cm
Control (no treatment)	80	12.1	147	11.6	147	11.4
Air	40	6.7	142	3.8	147	6.5
Oxygen	40	4.0	147	3.9	147	6.5
Carbon dioxide	40	7.3	149	4.0	—	—
Nitrogen	40	7.9	146	3.9	147	6.9

* Doses of radiation used: X-rays, 20,000 r; thermal neutrons, $1.7 \times 10^{13}/cm^2$; fast neutrons, $6.3 \times 10^{11}/cm^2$.

unit survival than more sparsely ionizing radiations, it appears that for plant breeding purposes densely ionizing radiations should be more useful than sparsely ionizing radiations.

These studies will have particular significance if pile radiations can be made readily available to agricultural scientists.

ACKNOWLEDGEMENT

The writer is indebted to Dr. H. J. Curtis, Chairman of the Biology Department, Brookhaven National Laboratory, for making available facilities used in most of the research reported.

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Genetic Effects Induced in Plants

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In Japan, very few studies of genetics have been carried out with the aid of atomic energy. Several Japanese geneticists, however, have been pursuing studies on the genetic effects of the nuclear detonations at Hiroshima and Nagasaki in 1945. As materials for such work, *Oryza sativa*[1,2] (rice, the most important plant for Japanese as the source of their staple food), *Tradescantia*,[3] *Cassia*[4] and *Veronica*[5] were chosen, and all of them except *Veronica* were collected around the hypocenter and grown in greenhouses for genetical and cytological studies.

Among those works, experiments on rice have been carried out most accurately by Y. Nishimura, and a set of reciprocal translocation strains selected from progeny of the irradiated plants is going to be utilised as a sort of linkage tester. The following is an outline of Nishimura's work: Rice seeds were gathered from paddy fields located as far as 600–2000 m from the hypocenter of the nuclear detonation at Nagasaki, in autumn, 1945. Among F_1 plants grown from these seeds, abnormal plants with morphological changes or of low fertility were found at the ripening stage in 1946. Most of them were dwarf, with a culm length of less than 40 cm instead of the normal length of 66 cm. Other abnormal morphological characteristics were extra palea; dense ears; triangular, narrow, or short grains, etc.

By cytological examination of ten F_1 plants, one haploid plant and one asynaptic plant which would give rise to tetraploid descendants were discovered. The haploid number of chromosomes of normal rice is 12.

To study the sterility of descendants, 1000 seeds were picked up at random from a lot taken at a field about 600 m from the hypocenter. Out of those which were cultivated, 61 specimens were found to be partially sterile. The descendants of these plants were investigated with respect to seed fertility. The result obtained seems to lead to the general conclusion that most of the partially sterile plants segregate fertile plants as well as partially sterile ones in the progeny. When inbred, the fertile progeny derived from the partially sterile parent give rise to very few sterile plants, but partial sterility recurs, although the degree of sterility varies individually.

The fact that most of the partially sterile plants segregate partially sterile and fertile plants in every generation suggested the occurrence of a reciprocal translocation as the cause of their partial sterility. This was investigated by cytological studies of meiosis in the partially sterile strain. Among 52 ever-segregating partially sterile strains, 29 strains were found to have a ring or chain of four chromosomes at prophase and metaphase, and 23 strains had 12 bivalents. When partial sterility was caused by a reciprocal translocation, the fertile offspring were found to be homozygous either with respect to normal chromosomes or interchanged chromosomes. In more than twenty strains the interchanged chromosomes have been detected cytologically, after crossing with normal plants.

Hybrid plants between those interchanged homozygotes and the normal plants segregate two kinds in relation to fertility with a ratio of 1:1 in F_2 plants. When such hybrids segregate by another character in addition to the character of fertility, whether the gene concerned is located on the interchanged chromosomes or not will be judged from the way of segregation of the two characters in F_2 plants. On these bases, a set of reciprocal translocation strains is going to be utilised as the tester.

From the asynaptic plants found in the second generation, two seeds were obtained. Both of them germinated and grew normally. They were considered to be natural hybrids. One of them was fertile and segregated fertile and sterile plants in the ratio of 3:1. So, this asynaptic character is considered to be determined by a single recessive gene, induced by the detonation.

In the progenies of the other atomized plants studied by Japanese geneticists independently, many abnormal traits or partial sterility were often found. These abnormalities seem to have been mainly caused by reciprocal translocations or by a gene mutation resulting in an asynapsis, either of which must have been induced by the detonation.

In addition to the genetical studies of the effects of radiation resulting from the nuclear detonation, a cytological work was recently reported on the radioactivity of Bikini dust by N. Tanaka,[6] A. Suginra[7] and H. Kakehi.[8]

Tests were made using the radiation from the hairs of the patients on the Daigo Fukuryu Maru contaminated with the fission products of the nuclear

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detonation at Bikini atoll on March 1, 1954, as to whether the radiation would induce breakage and reunion of chromosomes in the root-tip cells of *Tradescantia paludosa*. The results were quite positive. For example, in an experiment carried. out even after 90 days, the chromosomal abnormalities were definitely found.

The results mentioned above show that we can select some useful strains for the studies of genetics among the progeny of irradiated plants, but also indicate that the greatest care must be taken about the undesirable effects which may have occurred simultaneously.

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The Comparative Effects of Radiation and Hybridization in Plant Breeding

By W. C. Gregory,* USA

The value of irradiation in the field of plant breeding depends upon the extent to which induced genetic variation either supplements or replaces the natural resources in genetic variability.

Gustafsson,[5] Shebeski and Lawrence,[13] MacKey[11] and Humphrey[9] have demonstrated that certain useful mutations of special character may be of value to the breeder. Freisleben and Lein,[2] Konzak,[10] Frey[3] and Cooper and Gregory (unpublished) have demonstrated that disease resistant mutant plants can be produced by irriadiation. Gregory *et al.* (unpublished) have shown that a continuous spectrum of mutation expressed in numerous ways exists in advanced generation populations of plants following irradiation with X-rays. Gregory[4] has shown that samples of irradiated populations which included only the normal types of intra-varietal variation of a self-pollinating species showed highly significantly greater polygenic variation in yield of fruits than untreated controls. In this study progress from selection resulted in yields much exceeding the mean of the population. When sampling was restricted to plants of only "normal" character, progress from selection did not exceed the non-irradiated controls in yield comparisons. The genetic variance estimates were not made upon samples of irradiated plants which included the full range of phenotypic variation since many of the mutants did not lend themselves to such experiments. When mutant plants of extraordinary vegetative vigor were tested, lines significantly superior in yield to the non-irradiated controls were discovered.

The justification for "mutation breeding" has been made chiefly upon (*a*) the production of new mutations of economic value, such as for example, stiffness of straw in barley and (*b*) the creation of variation in highly adapted but uniform agricultural varieties. In the high latitudes and specialized conditions of Sweden where highly adapted varieties had already been produced the necessary conditions for the successful use of "mutation breeding" were recognized by Swedish plant breeders. It has been under these specialized environments that the most successful and voluminous mutation work has been done.

The question arises as to whether the general problems of breeding under the much more general conditions of the warm temperate and tropical zones of the world will lend themselves readily to solution by mutation breeding.

Most of the world's agriculture and most of the world's crops do not meet highly specialized conditions. This is especially true for tropical and subtropical cross-fertilizing species. The value of ionizing radiations as a consistent part of established breeding procedure under these conditions or under any conditions where natural resources in genetic variability are great and therefore relatively unexploited depends almost wholly upon the following conditions: (1) if new characteristics are desired but do not exist in natural populations; (2) if variation in established characters will be increased by orders of magnitude sufficient to much increase the efficacy of selection beyond the levels expected in the naturally variable populations.

The first case always has a chance of success in that new changes do occur and in that breeders can visualize desirable characteristics which do not exist in their populations. The probability of success however is lowered by the fact that the types of changes desired cannot as yet be specified by the treatment and the chance of a random change being desirable is distinctly low.

In the latter case, that is, increased variation in established characters (and this includes all polygenically controlled variation), success will depend almost wholly upon the magnitude and upon the distribution of the variation induced. The relative magnitudes of quantitative genetic variability expected from hybridization among varieties and from mutations following radiation are unknown.

In order to evaluate the usefulness of atomic energy or of mutagenic agents generally, in the field of plant breeding the following elementary questions must be answered:

1. Can new as well as useful mutations be produced with sufficient frequency to justify adoption of "mutation breeding" generally among crops?
2. Do the relative magnitudes and distributions of quantitative variation induced by radiation and hybridization justify adding the radiation induced variability to the populations under selection?

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3. Will the additional variability induced by mutation add simply to that recovered by hybridization or will irradiation of the hybrids themselves produce greater or less variability than the sum of the variance of the irradiated parents and of the non-irradiated hybrids?

4. Will the hybrids among induced non-useful mutations produce populations of complex variability and manifold usefulness to the breeder, or will simple monogenic distribution of the mutant forms only be recovered?

NEW USEFUL MUTATIONS

New and useful mutations have been produced by radiations in self-pollinating species of higher plants (Gustafsson[5], MacKey[11] and in rapidly reproducing micro-organisms (Hollaender[7], Hollaender *et al.*[8]). An instance of a useful induced mutation has been described in cross fertilizing white mustard by Andersson and Olsson.[1] In a discussion of the potential uses of mutagens in the "rationally directed" evolution of organisms Muller[12] suggests that where inbreeding may be practiced in cross-pollinating species of plants the effects of radiation might be equaled and possibly surpassed by exploiting the accumulated natural mutations which already have some chance of possessing adaptive value. He points out however that, if an organism possesses a complex of desired characteristics which might be lost by outcrossing, radiation might be usefully employed. This is especially true of certain inbred lines of corn which have failed to respond to outcrossing as a means of improving the line itself while retaining the combining properties with other lines for the production of known adapted hybrids which have been established at great cost. Disease-resistant mutants, already established in normally self-pollinating species [Freisleben and Lein,[3] Konzak,[10] and Gregory and Cooper (unpublished)] should just as readily be established in cross-pollinating species where selection is carried out under epidemic conditions.

Where populations may be large and the radiation problem simple as in the case of cross-pollinating species the effectiveness of radiation in the development of improved varieties should almost equal that to be expected in normally self-pollinated organisms. Certainly experience has deviated sufficiently from the expected in the past in the field of mutation breeding to justify a more extended effort in the case of normally cross-pollinated species of economic plants.

HYBRIDIZATION vs RADIATION

The true role of radiation in the field of plant breeding will not be felt by its specialized uses until its more general applicability in plant breeding procedure has been investigated. Success in selection for change in any character in any sexually reproducing organism is intimately associated with the genetic variance of the character and the intensity of selection. In general genetic variance, latent in individual members of a self-pollinating species, may be made most completely labile by hybridization. Once this is done the genetic variance may be estimated in F_2 and subsequent generations during the passage of which the variance rapidly becomes latent again by the laws of progression. Selection opportunity exists at all levels of this process but diminishes at the same rate as the variance is lost. Therefore any procedure which will significantly increase the genetic variance, or retard its return to latency will be of value to the breeder.

Comparable estimates of total genetic variance of polygenically controlled characters such as yield of fruits have been made in peanuts, (*Arachis hypogaea* L.).[4] When the same species, the same fields, similar means, similar experimental designs, and similar population sizes are used, comparable error variances in a quantitative measurement should be obtained.

Under such conditions estimates of genetic variance should also be comparable. By using such data it has been possible to make comparisons of the quantitative variation induced in peanuts by X-rays with that released from latency by hybridization. The error and treatment (variety) variances in yield of fruits (Table I) for four different classes of peanuts: (*a*) F_3 generation progenies of four different hybrids involving eight different parents: (*b*) "normal" X_2 plant progenies (X_3) of X-irradiated plants of the uniform variety; (*c*) mutant X_3 plant progenies (X_4) from the same population as (*b*) (these mutants were of essentially normal appearance exeept for their excessive size and increased vegetative vigor); (*d*) individual plant progenies of a pure-breeding uniform variety (peanuts are naturally self-pollinated) have been developed.

Table I. The Comparison of Quantitative Genetic Variation of Hybrids with That Produced by X-rays in Peanuts (*Arachis hypogaea* L.)

Code Letter	*Source of plants*	*Date*	*Plot size, ft*	*Plot means, gm*	*Treatment variances*	*Error variances*	*Variance ratios*
(*a*)	F_3 hybrids A	1947	15′4″	850	112914	21561	5.24
(*a*)	F_3 hybrids B	1947	15′4″	931	159256	25901	6.15
(*a*)	F_3 hybrids C	1947	15′4″	790	113853	21041	5.41
(*a*)	F_3 hybrids D	1947	15′4″	852	143076	19284	7.42
(*b*)	X_3 of uniform variety	1951	18′0″	747	57717	18909	3.05
(*c*)	X_4 of uniform variety	1952	18′0″	1478	266265	43279	6.15
(*d*)	Uniform variety	1951	18′0″	834	28493	20090	1.42

Except for class (*c*), the mean of which very much exceeded all other means, the error variances were essentially the same in all classes, and the treatment variances of the hybrids were twice as great as the treatment variance of the X_3 "normals".

There is little evidence in Table I to demonstrate that the treatment variances given in the two classes of X-ray progenies arose from numerous small mutations of polygenic character since the variance measured was estimated from differences between individual X_2 and X_3 plants each of which traced back to a separate X_1 seed. That this variation was polygenic is suggested however by the very large assortment of visible mutant sibs of the tested normals in the X_2 and X_3 generations. In addition Gregory *et al.* (unpublished) have shown that the observable variation in each mutant of the population from which the normal and vigorous samples were drawn formed a continuous spectrum of expression from the extreme mutant type to normal.

RADIATION OF THE HYBRIDS THEMSELVES

In order to compare the relative magnitude of and the character of the genetic variation in irradiated organisms with hybrid populations hybridizations among irradiated mutants themselves are required. In 1953 an experiment was designed to make these estimates and at the same time to compare the sum of the variances of non-irradiated hybrids and their irradiated parents with the sum of the variances of the irradiated hybrids and their non-irradiated parents.

The expected relation of the above variables in advanced generations following hybridization and irradiation would be:

$$\sigma^2 g\ F_2X_2 = \sigma^2 g\ \frac{P1X_2 + \sigma^2 g\ P2X_2}{2} + \sigma^2 g\ F_2$$

where $\sigma^2 g$ is the genetic variance of the character measured; F_2X_2 is a hybrid population in the F_2 generation treated with radiation in the F_1 generation; $P1X_2$ and $P2X_2$ are the parents of the hybrid in the second generation following irradiation. However there is no experimental evidence to contradict the hypothesis that the radiation treatments might destroy some of the latent variance in the F_1 generation thereby rendering

$$\sigma^2 g\ F_2X_2 < \sigma^2 g\ \frac{P1X_2 + \sigma^2 g\ P2X_2}{2} + \sigma^2 g\ F_2$$

Gustafsson[6] presented evidence that hybrids are naturally more mutable than pure breeding lines. Assuming this to be so then the greater mutability of hybrids should be accentuated by the radiation of the F_1 generation and

$$\sigma^2 g\ F_2X_2 > \sigma^2 g\ \frac{P1X_2 + \sigma^2 g\ P2X_2}{2} + \sigma^2 g\ F_2$$

It is likewise probable that somatic recombinations and translocations produced in irradiated F_1 hybrid plants would give rise to a wider range of genetic recombinations in subsequent generations. Such additional variance as occurred from this source would be added to that produced by new gene mutations and would therefore be expected to swell the variances of the irradiated hybrids beyond that resulting from mutation alone.

AN EXPERIMENT IN RADIATION BREEDING

Three X-ray induced mutants in the X_5 generation selected because of their superior vigor and yield of fruits were chosen to measure the total genetic variance (yield of fruits) of their hybrids. Each of these mutants arose from a different X_1 plant and each differed distinctly from the other. One produced a remarkably tall, vegetatively vigorous plant; one was a bright golden-green color and the third was intermediate in vegetative vigor. Three superior varieties of widely different and of non-irradiated origins were likewise chosen for estimating the genetic variances arising among their hybrids.

These six lines were hybridized in all possible ways in 1953 to produce fifteen F_1 hybrids. A minimum of one hundred seeds of each cross were obtained. Fifty seeds of each cross and each parent were treated with 15,000 r of hard X-rays. The remaining fifty seeds of each cross and parent were not irradiated. Each parent and hybrid, irradiated and non-irradiated was planted in single row plots in 1954. Represented then were the following classes of hybrids, both treated with X-rays and not treated: (1) X-ray mutant by X-ray mutant; (2) X-ray mutant by non-irradiated variety; and (3) non-irradiated variety by non-irradiated variety.

Because of primary X-irradiation injury appropriate comparisons of the above three kinds of hybrids could not be made in the treated samples in the F_1X_1 generation. The untreated samples however could be used to estimate differences in heterotic yield response of hybrids in the different classes in the F_1 generation.

In these comparisons of the yields, evidence of heterosis would suggest genic differences in the parents even though the absence of heterosis would

Table II. The Pedigrees and Origins of Three X-ray Mutants and Three Non-irradiated Peanut Varieties for the Purpose of Visualizing Their Degree of Genetic Relationship

Symbol	*Pedigree*	*1953 generation*
YT24	Single plant selection NC4*	X_5
X_4YT13	Single plant selection NC4	X_5
X_4YT32	Single plant selection NC4	X_5
A18	NC4 × Sp. 2B ("Spanish" Selection from S.C.)	F_9
B35	(Ga. variety × African) × (N. C. Whites Runner)	F_9
C12	A. *hypogaea macrocarpa* (Argentina) × N. C. Bunch	F_9

* Irradiated with 18,500 r in 1949.

not suggest the lack of genic difference. Furthermore the more distantly related any two lines were the greater the heterotic expression should be. In order to see more clearly the order of relationship among the parent lines their pedigrees and origins are presented in Table II.

From Table II it can be seen that the three X-ray mutants should be the most closely related among themselves and to A18. The next most closely related line to this group of lines is B35 while the most distantly related line to all others is the subspecies cross C12. The average yield in grams of each parent and hybrid in the three classes of material is given in Table III.

Table III. Differences in Yields in Grams of Seeds among F_1 Hybrids and Their Parents of 3 Different Classes of Hybrids Arranged in order of Presumed Relationship

Class of hybrid	*Order of relationship*	*Lower parent*	*Higher parent*	*F_1 hybrids*	*% increase*
X-ray mutant by X-ray mutant	1	53.9	54.5	58.5	7.9
		54.5	56.1	62.4	12.8
		53.9	56.1	69.3	26.0
X-ray mutant by non-irradiated variety	2	54.5	58.3	69.6	23.4
		53.9	58.3	72.3	28.9
		56.1	58.3	76.3	33.4
	3	54.5	55.0	83.1	51.9
		53.9	55.0	68.5	26.4
		55.0	56.1	72.4	30.4
	4	54.5	55.9	85.8	55.4
		53.9	55.9	89.5	63.0
		55.9	56.1	82.5	47.3
Non-irradiated variety by non-irradiated variety	—	55.0	58.3	85.0	50.2
		55.9	58.3	83.4	46.1
		55.0	55.9	90.0	62.5

ESTIMATING THE TOTAL GENETIC VARIANCE

In order to determine with certainty the relative magnitudes of quantitative variation in the various classes of hybrids discussed above, it will be necessary to study the results of plant progeny tests in the F_2X_2 and the F_3X_3 generations. When these tests are made in conjunction with similar tests of the F_2 and F_3, appropriate analyses of variance will disclose: (*a*) the comparative contribution of irradiation and hybridization to the total variance; (*b*) the latent variances of ordinary varieties and of X-ray mutants can be compared; and (*c*) the relative magnitude of the effects of radiation of the same dose can be shown for a hybrid and its two parents.

SUMMARY

The amounts of latent variation in hybrids of X-ray mutants, hybrids of varieties, and hybrids of X-ray mutants and varieties have been discussed and the expression of the F_1 generations compared. An experiment in re-irradiation of the X-ray mutants and their F_1 hybrids, and of the different varieties and their hybrids, and of hybrids between these has been presented. It has been shown how appropriate analyses of the variances of these parents and hybrids and their re-irradiated counterparts will provide information leading to a more rational interpretation of the part to be played by atomic energy in the future of plants through breeding.

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The Effects on Plants of Chronic Exposure to Gamma Radiation from Radiocobalt

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Although the use of kilocurie radiation sources in experimental botany is a relatively new development, results already obtained show that such a tool can be useful in the study of both normal and abnormal plant growth. During the past six years the authors have investigated the effects of chronic exposure to gamma radiation in over 100 species of plants representing 35 different families. Several papers dealing with various aspects of this work have already been published as well as a number of preliminary reports. However, since the technique of chronic exposure of plants to ionizing radiation has previously been investigated by only a few workers using small radiation sources, a great deal of new information is still available concerning the cytogenetic and morphogenetic responses of plants to long continued exposures to ionizing radiation. Since a general review of the morphogenetic abnormalities induced by ionizing radiation has already been published,[11] this report will deal with a few new specific examples.

METHODS

Radioactive cobalt in the form of metallic cylinders or hollow tubes has been used consistently as the source of gamma radiation. For comparative purposes, plants have been exposed frequently to acute radiation with X-rays and in a few experiments beta radiation from P^{32} and fast electrons (800 kvp) from a resonant transformer electron-beam generator were also utilized. The cobalt-60 sources were used in both outdoor and indoor installations. A special greenhouse utilized 1.5- to 14-curie sources, while two different outdoor gamma-radiation fields varying in size from about 2.6 to 9 acres used 16–1800-curie sources (Fig. 2). In all cases the radioactive cobalt could be remotely controlled in such a fashion that when personnel desired to enter the field or greenhouse the source could be lowered into a shield which reduced the level of radiation well below the permissible dose of 50 mr per 8-hour day. Except where special experiments required some deviation, the standard procedure was to expose plants for 20 hours out of every 24. This allows four hours each day for planting, cultivating, examination and collection of the material exposed. Although somewhat inconvenient at times this schedule seems to be a practical one.

The daily dose rate received by any given plant depends on the size of the radiation source (i.e., number of curies) and the distance from the source to the plant. Except for a minor correction due to air absorption, the dose rate falls off according to the inverse square law. Thus, all plants growing equidistant from the source receive the same daily dose rate. However, plants at any given position receive slightly less radiation per day at the end of the season than at the beginning due to the decay of the radiocobalt. Since the decay is only about 1 per cent a month, the change produced during the summer is of minor consequence for most of our work. In this report the daily dose rates given are those determined by measurement and calibration of the source at the beginning of the growing season each year, usually in April or May. More detailed techniques for operation of a gamma-radiation field have been published elsewhere.[34]

TOLERANCE OF CERTAIN PLANT SPECIES TO CHRONIC GAMMA RADIATION

Prior to the establishment of the Brookhaven gamma-radiation field, there was essentially no data on the tolerance of plants to chronic gamma irradiation. During the six years since the first gamma field was put into operation the tolerance of over a hundred species of plants has been determined. Some of this information has been reported elsewhere,[30,34] but since the available information on tolerance may be of value to other institutions which wish to carry on similar work, it was decided to present a brief summary of tolerance data so far available (Table I).

The dose rates indicated in Table I indicate to the best of our knowledge the range in which severe radiation damage (usually severe growth inhibition or dwarfing) would occur. The dose rate which causes severe growth inhibition varies greatly in the different species studied. Some species, e.g., *Tradescantia paludosa* and *Lilium longiflorum*, show severe morphogenetic effects with dose rates as low as 30 to 40 r per day while others such as *Gladiolus* show little or no effect at dose rates of 5000 to 6000 r per day. Very few plants so far studied have shown gross morphological changes at dose rates below 15 r per

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day. However, chromosome aberrations and mutations have been noted in significant numbers at dose rates below 1 r per day.[34,27]

There are undoubtedly a considerable number of factors which determine the radiosensitivity of a given species of plant. The results of our investigations seem to indicate clearly (1) that plants with very large chromosomes have a high radiosensitivity, (2) that plants with smaller chromosomes tend to be less sensitive than those with large chromosomes, and (3) that polyploid species within a genus tend to be less sensitive than diploid species.

GROWTH INHIBITION AND STIMULATION

Previous work, both with acute and chronic exposures of ionizing radiation, has shown clearly that growth inhibition or stunting is a very common effect. Growth stimulation, however, is much more controversial, and the question has been much discussed in the literature for many years.[25] The interest in this phenomenon is not surprising since any treatment that can stimulate plant growth is not only of considerable scientific interest but is also of potential economic value. We report, therefore, the following observations even though the cause for the enhanced growth is not clear.

Plants of *Antirrhinum majus* have been grown in the gamma field over a wide dose range for four years. The first two years it was noted that toward the end of the summer certain plants growing at the higher dose rates grew much taller than average. The following year measurements of plant height were taken at dose rates varying from 14 to 600 r per day. The data indicate that at dose rates above about 125 r per day there is a gradual increase in plant height, reaching a maximum at 230 r per day. At 285 r per day the plants are still much taller than average, but at 330 r the average plant height has decreased appreciably to less than that of normal plants. Associated with this increase in plant height is an increase in average stem diameter. Increased leaf thickness has also been observed (see below).

A number of pure species of *Nicotiana*, as well as certain interspecific hybrids, have been grown in the gamma field and the gamma greenhouse for several years. On several occasions it has been found that plants growing at a certain dose rate grew taller than plants at higher or lower dose rates and also that they bloom earlier. The exact dose rate at which this apparent stimulation occurs varies from experiment to experiment and is apparently influenced by the external or internal environment of the plant, both of which could be expected to vary considerably from experiment to experiment and from year to year. A typical experiment with the hybrid *Nicotiana glauca* x *Langsdorffii* is illustrated in Fig. 1 which shows a plant of average size from each dose rate row as well as a control plant. The photograph clearly indicates that the plant which grew at 15 r per day was much taller and bloomed well in advance of either the control or any of the plants grown at the higher dose rates.

Table I. Daily Dose Rates Required to Produce Severe Radiation Effects on 79 Species of Plants *

Dose rate, r/day	Specie	Specie
30–50	*Lilium longiflorum*	*Tradescantia paludosa*
	Taxus media	*Tradescantia ohiensis*
51–100	*Cornus florida*	*Setcreasia* sp. (4n)
	Impatiens Sultanii	*Vicia Faba*
101–200	*Acer rubrum* (6n or 8n)	*Ilex* (4n)
	A. spicatum (?)	*Magnolia* sp. (?)
	Commelina coelestis (?)	*Pyrus Malus*
	Cosmos	*Rhododendron*(hybrid)
201–400	*Antirrhinum majus*	*Mirabilis jalapa*
	Canna generalis	*Nicotiana bigelovii*(4n)
	Capsicum frutescens (2n 4n)	*N. glauca*
	Chrysanthemum nipponicum	*N. glauca* x *langsdorffii* (6n)
	Coleus blumei (4n)	*N. langsdorffii*
	Dahlia (hybrid) (8n)	*N. rustica* (4n)
	Datura stramonium	*Phytolacca decandra* (4n)
	Gossypium hirsutum (4n)	*Pisum sativum*
	Kalmia latifolia	*Prunus persica*
	Liriodendron tulipifera	*Vicia angustifolia*
	Luzula purpurea	*Vicia tenuifolia* (4n)
	Melilotus officinalis	*Zinnia elegans* (?)
401–800	*Allium cepa*	*Lycopersicon esculentum*
	Althea rosea (6n or 8n)	*Petunia hybrida*
	Celosia cristata (4n)	*Pieris japonica*
	Chenopodium album (4n)	*Ricinus communis*
	Chrysanthemum ircutianum (4n)	*Rosa* (Hybrid Tea Rose)
	Helianthus annuus	*St. Paulia*
	Ipomoea noctiflora	*Sedum aizoon* (?)
	Kalanchoë daigremontiana	*Stachyurus* sp. (?)
	Lactuca sativa	*Xanthium* sp. (4n)
801-1600	*Chrysanthemum arcticum* (8n)	*Lenophyllum pusillum* (?)
	C. lacustre (22n)	*Linum usitatissimum*
	C. yezoense (10n)	*Mollugo verticillata* (8n)
	Cucurbita (pumpkin) (4n)	*Phaseolus vulgaris* (?)
	Iris (hybrid) (4n)	*Sedum acre* (12n)
	Kalanchoë blossfeldiana	*S. album* (16n)
1601–6000	*Digitaria sanguinalis* (4n)	*Kalanchoë tubifolia* (4n)
	Gladiolus (hybrid) (6n)	*Lenophyllum texanum* (?)
	Graptopetalum bartramii (2n)	*Luzula acuminata* (8n)
	Graptopetalum McDougallii (22n)	*L. multiflora* (4n)
		L. pallescens (4n)

* Chromosome number is diploid unless otherwise stated. Question mark (?) indicates degree of polyploidy uncertain.

The above two examples of apparent stimulation are of considerable interest, but they are not typical responses for plants in general. More commonly, the growth of plants exposed to chronic gamma irradiation is inversely proportional to the dose rate (Fig. 4). This relationship between dosage and degree of growth inhibition is the basis for the inhibition of sprouting reported for potatoes following

treatment with X- and gamma rays[29,31,24] and with fast electron beams, (Sparrow, Schairer and Lawton, in press), and for onions following X-radiation.[7]

MORPHOLOGICAL EFFECTS

As indicated above, one of the most common effects of irradiation upon plant growth is growth inhibition and, more rarely, growth stimulation. In addition, the roots, stems, leaves, buds and flowers of most plants may have their normal expression altered by exposure to radiation.[11,23] Most of the early work, particularly X-ray effects, was reviewed by Johnson[15] and later work, particularly with gamma radiation, was reviewed by Gunckel and Sparrow.[11] The present paper will be confined to a few unpublished examples of leaf, bud, stem and flower development following chronic exposure to gamma radiation. In all cases, the response will vary, depending upon the level and duration of the dosage and upon the species, age, and physiological condition of the plants irradiated.

Leaves

In most plants, the leaves already present on the plant at the beginning of the exposure are changed relatively little. Rarely, these older leaves may have a restricted blade or show some growth of the blade between the veins; more commonly the leaf texture may be changed, becoming dry, stiff and coarse or thickened and leathery.

Young leaves formed during irradiation show a wide range of responses. In general, the leaf abnormalities include dwarfing, asymmetrical development of the leaf blade, distorted venation, a change in texture and thickening of the blade. This latter is most interesting and has been studied in some detail in *Antirrhinum majus* at 17 dosages ranging from 0.5 to 600 r per day. At the lowest dosages, the leaf was not significantly thicker than that of the controls. However, with increasing dosage there was a progressive thickening of the leaf up to at least three times that of the controls at 600 r per day. The leaves at dose rates above 240 r per day had a thick, leathery aspect, quite different from the controls. Sections were made from representative leaves and from these measurements showed that two-thirds of the increase in thickness was due to repeated cell divisions in the palisade layer. Enlargement of spongy mesophyll cells contributed about one-third to the leaf thickening.

An additional leaf response may be reported from the 1954 gamma-field studies. Plants of *Graptopetalum MacDougallii* were placed at various dosages and observed for morphological changes. In Fig. 3 we see that leaves which received 590 r per day during their development became not only slightly thickened but underwent considerable enlargement in length and width by comparison with controls.

Root Development

We have previously reported that chronic gamma irradiation of *Kalanchoë* at 1280 r per day seems to have a stimulating effect on the production of roots from stems.[11] It has been shown that acute X-radiation of localized stem areas will cause the formation of roots above the irradiated zone (Christensen;[5] Sparrow, unpublished). Observations on plants grown at fairly high dose rates also indicate that the root system frequently suffers severe damage (Fig. 4). Inhibition of the formation of new roots has also been observed in irradiated sectors of the stem cuttings of *Impatiens*. In one experiment with this plant, cuttings placed in vermiculite showed good root development from the basal node of irradiated stem sectors at 2000 r, whereas sectors irradiated at 4000, 8000, 16,000 and 32,000 r showed no root development at all in the irradiated zone. Roots did develop, however, above the irradiated zone at all dosages and were at least as numerous, if not more numerous, at 16,000 and 32,000 r than in the lower dosages or in the controls (Fig. 5).

Flowers

With chronic gamma irradiation, flowering is generally retarded at the higher dosages and approaches that of the controls with decreasing dosages. However, in some plants, as the snapdragons and tobaccos already cited, flowering may be stimulated in a critical dosage range which varies with the species. Since most of this has been reviewed previously,[11,12,13,15] it will suffice here to mention just one further observation on *Tradescantia paludosa*. This species is of considerable interest because it has been used so frequently for experimentation and is one of the most sensitive plants so far studied. In a critical range between 13 r and 37 r per day the plants showed many abnormal axillary buds and inflorescences due principally to the induction of multiple growth centers. Inflorescences receiving 20–24 r per day show, after 8 weeks, proliferation by leaf-like structures and modified flowers so that by 3 weeks a globose flower head 3–5 cm in diameter results. Removed from the gamma field and brought into the greenhouse, these globose flower heads commonly generate a large number of vegetative shoots

Key to photos on facing page

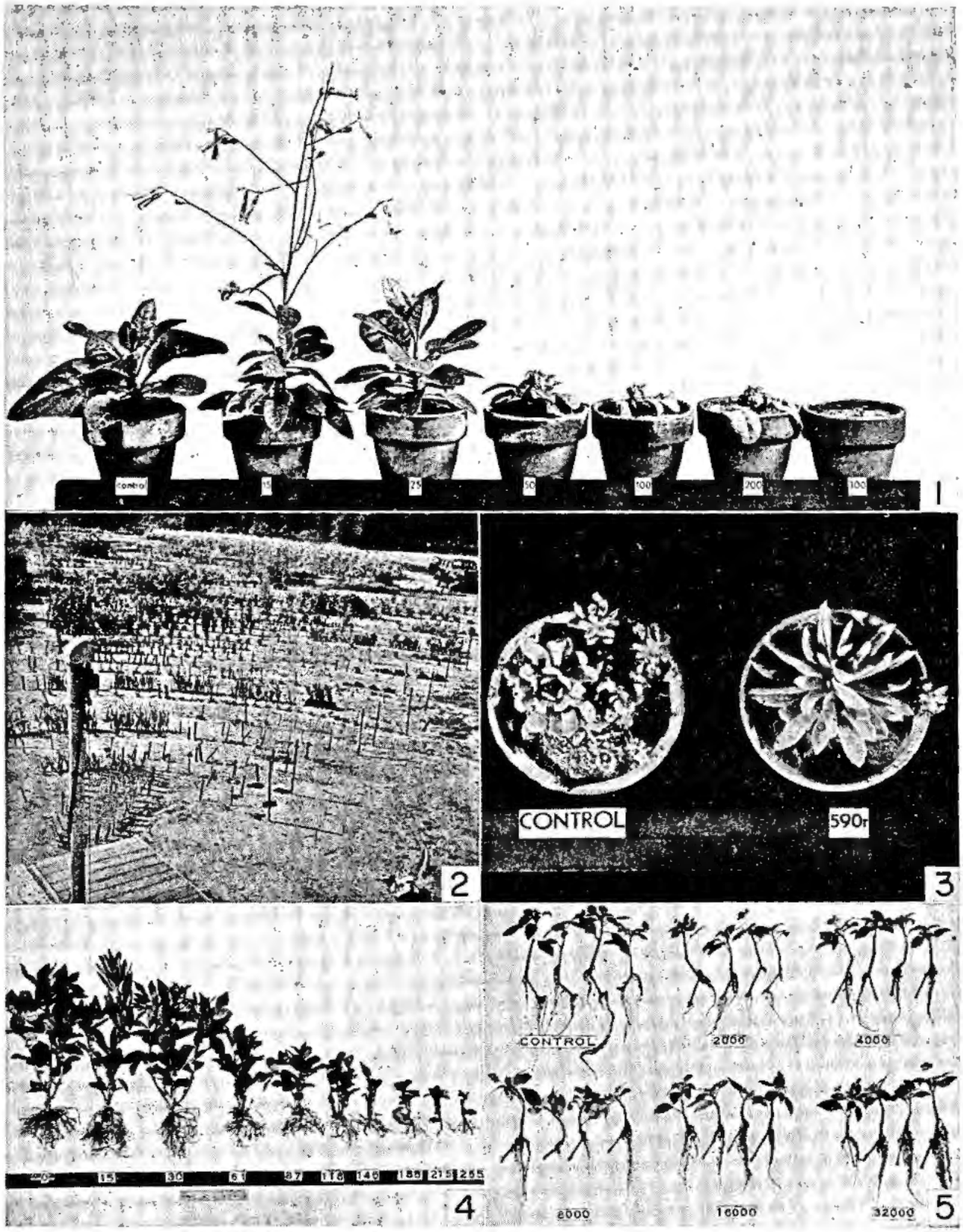
Figure 1. Tobacco plants of the cross *N. glauca x Longsdorffii* after 47 days of exposure in the gamma greenhouse. Irradiation was begun in the first true leaf stage. Stem and root growth are inversely proportional to the dosage level. Note enhanced flowering in the plant which received 15 r per day

Figure 2. General view of a segment of the gamma field at the Brookhaven National Laboratory. The source was approximately 1800 curies of Co^{60}

Figure 3. Control plant of *Graptopetalum MacDougallii* for comparison with a plant which received 590 r per day for 19 weeks. In addition to the usual thickening of irradiated leaves, note the marked increase in length and breadth of the treated leaves

Figure 4. Plants of *Vicia Faba* after 39 days of exposure to a wide range (15-255 r per day) of chronic gamma rays. Both top and root growth are inversely proportional to the dose rate

Figure 5. Cuttings of *Impatiens Sultani* grown in vermiculite after X-ray treatment. Plants which receive 2000 r show root development from the basal stem node, but not those which received 4000, 8000, 16,000 and 32,000 r. Roots developed above the irradiated stem sector at all dosages, especially at 16,000 and 32,000 r



Figures 1-5. Morphological effects on plants exposed to chronic gamma irradiation for varying periods of time

(Fig. 6). At the higher dosages in this range (e.g., 34 r per day) radiation favors vegetative rather than floral production. Accordingly, multiple, leafy shoots are borne in each leaf axil and in internodal positions (Fig. 7) and modified leaves occur in floral positions (Fig. 8). These observations are of interest, particularly in suggesting that one might induce proliferations where the normal stem, leaf, or flower character might be lost. Such is indeed the case and this topic is of sufficient importance to discuss independently of the morphological effects already described.

TUMOR INDUCTION

Plants of the amphidiploid hybrid *Nicotiana glauca x Langsdorffi* have long been known to develop spontaneous tumors on leaves, stems and roots.[16] Plants of this hybrid were exposed to 20 hours per day of chronic gamma radiation at dose rates of 385, 320, 210, 105, 50, and 26 r per day. The percentage of plants showing one or more tumors of significant size was recorded after 37, 44 and 64 days. The results show that dose rates above 210 r promote more tumor development than lower rates, and that longer exposures at any given rate also promote tumor development.[32] For example, after 37 days exposure 47 per cent of the plants at 385 r showed tumors whereas none showed in these at 210 r or less. In the 210 r per day plants, the percentage of plants with tumors increased from none at 37 days to 33 per cent at 44 days and up to 80 per cent at 64 days. This increase in number of plants with tumors was paralleled by an increase in the average amount of tumor per plant. The tumor weight is greater at the higher dose rates, reaching a high of 47 and 23 per cent of the wet weight respectively for tops and roots at 385 r per day, compared with 0.1 and 1.0 per cent wet weight for controls. Similar, but less extensive, work with acute X-irradiation and internal beta irradiation from P^{32} indicates a similar response.

Morphologically, at least 6 types of induced tumor growth patterns may be described. Histologically, at least two growth patterns are evident. First, a tumor may be a composite of a large number of induced growth centers, each consisting of an apical meristem, derived from the surface and subsurface layers, and a number of associated leaves (Fig. 10). Such leafy tumors are characteristically found in axillary positions (Fig. 9). Secondly, tumors may increase by sub-epidermal cell divisions and by cell enlargement, the surface layers remaining fairly discrete and nearly glabrous (Fig. 11). After growing for some months in sterile culture, each of the tumor types may still be recognized, indicating that the induced morphological changes are not due merely to temporary physiological disturbances induced by the radiation. The specific cause of tumor induction is not known, but work is under way to determine the chemical constituents and the nutrient requirements of each of these morphological types of tumors.

PEACEFUL APPLICATIONS OF ATOMIC ENERGY IN PLANT SCIENCE AND AGRICULTURE

The preceding sections of this paper have been devoted to brief descriptions of some of the results that we have obtained from irradiating plants with chronic gamma rays from a cobalt-60 source, with fast electron beams, or with acute X-rays. It might be well to mention some of the problems raised by these investigations and to note briefly some of the prospective practical applications of ionizing radiation in plant science and agriculture.

Applications in Agriculture

By using the techniques described for growing plants in the gamma field one may obtain a significant increase in mutation rates, in some cases (e.g., *Antirrhinum*) at dose rates below 1 r per day. The potentialities of the application of ionizing radiation in agriculture and plant breeding programs have recently been evaluated[25,26,36] and are considered elsewhere in this conference.[27]

Another potential use of ionizing radiation which may prove of practical value is the inhibition of sprouting of certain vegetables. Sparrow and Christensen,[31] and Sawyer and Dallyn,[24] have reported that exposures of potato tubers to gamma-ray dosages in the 10,000- to 20,000-r range is effective in the inhibition of sprouting. Certain other undesirable changes which normally accompany sprouting are also reduced in the irradiated material. It has also been shown[7] that sprouting in sweet Spanish onions can be effectively controlled by 4000 r of gamma rays. There is every reason to believe that the same technique will prove useful in the inhibition of sprouting of other vegetables. With the advent of megacurie radiation sources it seems not improbable that certain

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Figure 6. Proliferated inflorescence of leaves and modified flowers after 24 r per day for 32 weeks. Removed from the gamma field and held in the gamma greenhouse, the inflorescence was producing a large number of vegetative shoots

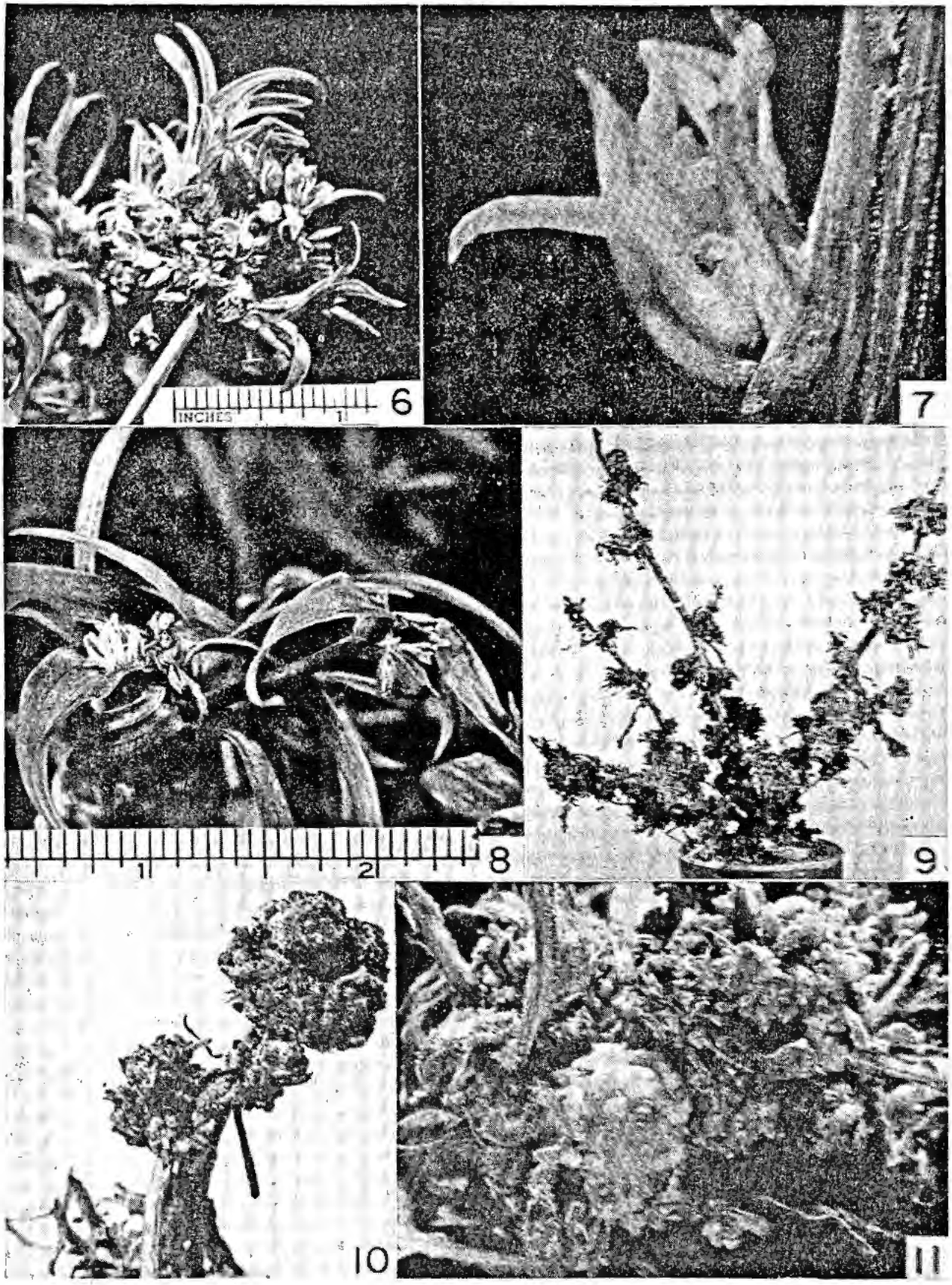
Figure 7. Plant which received 34 r per day for about three months. Multiple leafy shoots were developing from one locus in the internodal region. Such shoots are found usually in axillary positions and are frequently chlorotic

Figure 8. Terminal inflorescence on plant in gamma greenhouse which received 34 r per day for 32 weeks. Note the formation of modified leaves in a floral position

Figure 9. Plant which received 300 r chronic gamma irradiation for about three months. The leaves were removed to show the large axillary stem tumors which developed

Figure 10. Plant which received 9 weeks of chronic gamma irradiation at 325 r per day. This is a close-up view of terminal and axillary tumors essentially as seen in Fig. 9. The tumor mass consists of a large number of induced growth centers consisting of numerous meristematic areas and a series of modified leaves. The axillary bud tumors undergo some elongation while the buds of the terminal tumors are very restricted in growth

Figure 11. Axillary plant tumor induced by 2100 r of X-rays. In addition to some leafy outgrowths there was a yellow, glabrous, central mass. This tumor differed from the leafy in that the surface remained fairly discrete and the mass increased by subepidermal divisions and overall cell enlargement



Figures 6-8. Irradiated flower stalk of *Tradescantia paludosa*
Figures 9-11. Plants of the tobacco amphidiploid *N. glauca* x *Langsdorffii* following X- or gamma irradiation

vegetable crops may be irradiated on a commercial scale within the next few years.[3,17] If the process is commercially adopted, the economic value of the method would be considerable. In addition to the improved storage quality, recent work from this laboratory indicates that an X-ray dose of approximately 20,000 r may be sufficient to prevent reproduction of the Golden Nematode of potato (Fassuliotis and Sparrow, unpublished). If this work is confirmed, and perhaps extended to other plant diseases, the application of ionizing radiation in plant disease control might be considerable.

The difficulty in rooting cuttings of certain plants, even after chemical pre-treatment, makes it desirable to investigate further the commercial possibilities of using radiation for the induction of adventitious roots. The method might prove to be a useful and practical one.

Applications to Basic Problems in the Plant Sciences

On theoretical and practical grounds the work reported on *Tradescantia* has intriguing possibilities. The results suggest that in critical dosage ranges one may selectively discriminate between a dosage level which favors a vegetative response and another which favors a floral response.[12] The mechanisms of vegetative and floral development are little understood and might be effectively studied by such methods.

During the life of the plant various internal systems or cells are more easily modified by radiation than others.[14] Further, it has been shown that radiosensitive systems may be modified by changes in the internal or external plant environment.[4,6,19] A study of these possible mechanisms of effect may help to solve some of the physiological problems of growth and differentiation.

The development of irradiated leaves is interesting in that it shows that the rather characteristic thickening may be due to cell division or to cell enlargement. It is suggested that irradiated leaves might be good material for studying the relative roles and the mechanics of cell division and cell enlargement in the growth process, and for trying to elucidate the manner in which radiation initiates abnormal proliferation.

The work on tumors deserves special comment.[32] We feel that our studies may throw some light on the regenerative capacity of cells. More information on the growth of normal cells is necessary before too much can be said about abnormal cell growth. However, the studies under way on the chemical constituents of these tumor cells may enable us not only to grow these tumor cells more effectively in sterile culture but also to alter experimentally the growth pattern of these tumors. By additives to the nutrient medium we hope to determine the effectiveness of various compounds against radiation damage or, conversely, to induce, by additives, radiation effects in non-irradiated plants.

A recent review of the literature on plant growth following irradiation reports many tentative conclusions which may contribute toward a solution of some of the problems raised here.[11] For example, the relationship between chromosome breakage, mitotic inhibition, and growth inhibition has been considered by several workers.[9,33] A number of physiological disturbances are induced in plants by ionizing radiation. Some of these disturbances, e.g., auxin metabolism, have specific morphogenetic effects. Any combination of the following factors may contribute to the morphogenetic effects described in this paper: (1) growth substance production and distribution,[28,8] (2) the nutritional level of the plant (e.g., Gunckel *et al.*[10]), (3) an effect upon the mechanisms of assimilation,[22] (4) local mobilization of nutrient materials (e.g., Mitchell[20]), (5) organic phosphates,[18] (6) accumulation of free amino acids,[35] (7) changes in enzyme activity,[1,2] and (8) inhibition of synthesis.[21] With the exception of mutations, most of the irradiation effects described are probably secondary effects of physiological disturbances only quantitatively different from those which occur in unirradiated plants. Radiation may thus be a useful tool for the analysis of many problems in plant growth.

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Resistance to Rust Induced by Ionizing Radiations in Wheat and Oats

By W. M. Myers,* E. R. Ausemus,† F. K. S. Koo* and K. J. Hsu,* USA

Breeding for resistance to stem rust in wheat and oats caused by *Puccinia graminis tritici* Eriks. & Henn. and *P. graminis avenae* Eriks. & Henn. and for resistance to crown rust in oats caused by *Puccinia coronata* Eriks. have occupied important places in the program of varietal improvement of these two major fields crops. Resistant varieties developed in these programs have contributed in a large way to increased yield and stability of production.

Extensive variability in pathogenicity within the disease organisms and great variation in prevalence of the different pathogenic races in different years have made necessary continuing programs of searching for new sources of resistance and of incorporating the new genes into improved varieties.

The problem is of such magnitude and complexity that the plant breeders and plant pathologists must use every technic available to them in seeking its solution. The work of Gustafsson and his associates in Sweden suggested the possibility of inducing favorable mutations by irradiation. Konzak and Frey, working independently in the United States, demonstrated that resistance to stem rust in oats could be induced by ionizing irradiation (cf. Singleton[1]). To investigate the potentialities of irradiation induced mutations in the program of breeding for disease resistance, studies were initiated at the University of Minnesota in 1953. This paper reports progress to date in these investigations.

MATERIALS AND METHODS

Lee, an outstanding variety of spring bread wheat, and Ajax and Clintafe, two widely adapted spring oat varieties, were used in the studies. Lee has resistance, derived from Hope and Timstein, to most physiologic races of stem rust. It has been resistant in the field to all prevalent races prior to the outbreak of race 15B, to which it is susceptible.

Ajax has the "Richland" gene which provides resistance to race 7 and several other races of stem rust. It is susceptible to race 8. Clintafe has the "White Russian" gene and is resistant to several stem rust races including 8 but is susceptible to race 7. The "Richland" and "White Russian" genes are allelic or pseudo-allelic.[2] Ajax is susceptible to several prevalent races of crown rust.

Dry seed lots of each variety were irradiated at the Brookhaven National Laboratory in 1953. Different seed lots of Lee were treated with X-ray dosages of 12,000 r and 16,000 r and with thermal neutrons at a dose of approximately $9.94 \times 10^{12}/cm^2$. Seed of Ajax and Clintafe was exposed to thermal neutrons at the same dose used for Lee.

Plants from the treated seeds (X_1 and N_1 generation) were grown in the field at St. Paul, Minnesota. Plants from untreated seed of each variety were grown as controls. Individual panicles were harvested from each treated plant and from the controls.

The X_2 and N_2 generations and controls of Lee were grown and tested (1) in Mexico under a natural rust epidemic in which race 15B was an important component, (2) in the rust nursery at St. Paul with an artificially induced epidemic in which all available prevalent races of stem rust including 15B were used, and (3) in the greenhouse at 78°F, using a mixture of isolates of race 15B. The N_3, X_3 and X_4 progenies of resistant N_2 and X_2 plants were grown in the field under an artificially induced epidemic and in the greenhouse where they were inoculated with race 15B in the seedling and adult plant stages.

Panicle progenies of the N_2 and controls of Ajax and Clintafe were grown and tested for rust reaction in the greenhouse. Progenies of Ajax were tested in the seedling stage with stem rust race 8 and resistant plants were subsequently inoculated with race 7. Progenies of Clintafe were first inoculated with race 7 and resistant plants were then tested to race 8. Resistant plants were grown to maturity and N_3 progenies of these plants were grown in the field where a natural epidemic of stem rust, caused by races 7 and 8, occurred.

Additional N_2 and control progenies of Ajax and Clintafe were grown in the field nursery where an artificial epidemic was created using races 7 and 8 of stem rust and several races of crown rust including 202, 205 and 213. Seed was collected from the resistant N_2, N_3 and control plants for greenhouse progeny tests that are still in progress.

EXPERIMENTAL RESULTS

The results in X_2 and N_2 generations of Lee are shown in Table I. Results from field and greenhouse tests are combined. Since Lee is resistant in the field to prevalent stem-rust races except 15B and since

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15B was an important component in the field epidemics and was used in the greenhouse tests, this combining of results appears to be justified. Two panicle progenies from X-ray treatment at 16,000 r and one progeny each from X-ray, 12,000-r and thermal neutron treatments contained segregates resistant to stem rust. All other progenies from treated seed and all progenies from the control were susceptible.

Table I. Frequency of X_2 and N_2 Progenies of Lee Bread Wheat Containing Segregates Resistant to Stem Rust

Treatment	Number of panicle progenies: Tested	Number of panicle progenies: Containing resistant plants	Per cent progenies with resistant plants
X-ray, 12,000 r	1446	1	0.1
X-ray, 16,000 r	530	2	0.4
Thermal neutrons	244	1	0.4
Control	250	0	

Because of the relatively small number of control plants used and low frequency of progenies with resistant segregates, it cannot be concluded that the resistant types were the result of changes induced by the irradiation. Nevertheless, several things are strongly suggestive of that conclusion. Not only was no resistant plant found in any of the progenies from the control plants, but neither have plants resistant to race 15B been found in large populations of Lee used in breeding and genetic studies at the Minnesota station. In each case in which resistant plants were found, sister progenies from heads of the same X_1 or N_1 plant were uniformly susceptible, eliminating the possibility of heterozygosity of the treated seeds. In the segregating progenies, there were one to five resistant seedlings out of an average of about 15, a larger number than could readily be accounted for by natural cross pollination. Finally, the resistant plants and their progenies appear identical to the controls in head type, plant height, and other agronomic characters except maturity. They have been slightly later in maturity than normal Lee under rust-epidemic conditions, a difference that may be attributable to effects of rust on the susceptible plants.

In most cases, N_3, X_3 and X_4 progenies of the resistant N_2 plants have been resistant to rust, indicating that they were homozygous for resistance. The segregates obtained from the thermal neutron treatment have been highly resistant both in the seedling and at the adult plant stage. Those obtained from X-ray treated material have been moderately resistant. It seems unlikely, on the basis of results reported by Caldecott *et al.*,[3] that this difference in degree of resistance has any significance in relation to kind of irradiation used.

The results in the N_2 generation of Ajax and Clintafe are presented in Tables II and III. In Ajax, 6 of the 988 N_2 progenies (0.6%) contained plants resistant to both races 7 and 8 of stem rust, while none was found in the controls.

In Clintafe, 2.3% of the N_2 progenies had plants resistant to both races, while 1.4% of the untreated progenies likewise had resistant plants. It is apparent that changes similar to those found in treated material are also occurring in untreated Clintafe. Clinton, the recurrent parent of Clintafe, is known to be inherently variable in disease resistance and in morphological characters, due in part at least to heritable changes that occur in the tiller primordia.[4]

Table II. Frequency of N_2 and Control Progenies of Ajax and Clintafe Oats Containing Plants Resistant to Stem Rust Races 8 and 7, Respectively

Variety	Treatment	Number of panicle progenies: Tested	Number of panicle progenies: Containing resistant plants	Per cent progenies with resistant plants
Ajax	Thermal neutrons	988	6	0.6
	Control	410	0	0
Clintafe	Thermal neutrons	434	10	2.3
	Control	204	3	1.4

Table III. Frequency of N_2 Progenies of Ajax Oats Containing Plants Resistant to Crown Rust in the Field

Treatment	Number of panicle progenies: Tested	Number of panicle progenies: Containing resistant plants	Per cent progenies with resistant plants
Thermal neutrons	558	4	0.7
Control	149	0	0

Field observations of Clintafe indicate that similar somatic mutations are common in it. It is noteworthy that the frequency of progenies with resistant plants was higher in N_2 than in untreated Clintafe and that the difference is of about the same magnitude as the frequency of such progenies in N_2 Ajax. Among the 558 N_2 progenies of Ajax grown in the field, 0.7% contained plants resistant to crown rust.

Resistant N_2 plants obtained in greenhouse studies were grown to maturity and N_3 progenies from them studied in the field in 1954. The number of seeds from each plant and hence the number of plants in each N_3 progeny was too small to provide critical evidence. Some progenies appeared to breed true for resistance to both races of stem rust, others bred true for resistance to one race and segregated for resistance to the other. Final conclusions regarding the behavior of this material must await N_4 progeny tests now in progress.

As was the case in studies of Lee wheat, the number of control progenies and the frequency of progenies with resistant segregates are too small to warrant the positive conclusion that the resistant segregates resulted from irradiation induced mutation. Nevertheless, the results are suggestive.

In the case of each panicle progeny containing resistant plants, sister progenies from panicles of the same N_1 plant were uniformly susceptible, indicating that the treated seeds were not heterozygous. The possibility of natural cross pollination cannot be eliminated with available data but some of these data suggest that it is not a likely explanation. The control and N_1 populations of each variety was isolated by

at least 26 feet from any other oats, thus reducing the likelihood of natural crossing. Crosses between varieties with the "Richland" and "White Russian" genes are resistant to both races 7 and 8 of stem rust, as were the resistant segregates obtained in this investigation. However, the progenies of F_1 plants from such a cross segregate for resistance to both races, whereas the data available indicate that some plants obtained in this study produced offspring all of which were resistant to one race while being resistant or segregating to the other race.

Among the N_2 progenies of Ajax and Clintafe, a number of chlorophyll deficient mutant types were found, while none was observed in the progenies of the controls. In contrast no chlorophyll deficient plant was found in any of the N or X progenies of Lee. These results suggest a difference in response of oats and wheat to irradiation insofar as changes in the chlorophyll formation mechanism is concerned.

DISCUSSION

The results obtained with respect to induction of resistance to specific races of stem and crown rust in normally susceptible varieties, although not conclusive, are suggestive and encouraging. Resistance to the races used in this study is already available in existing varieties of wheat and oats and can, therefore, be incorporated into improved varieties by conventional plant breeding methods. Whether the same or different *loci* are responsible for resistance in the treated material and in existing varieties cannot be determined with present information. If the same *loci* are involved, the mutations obtained in the treated material in this study, even though induced by the irradiation as suggested, may not represent a major advance from the standpoint of plant breeding. If, on the other hand, new *loci* are involved and new sources of resistance can be induced by irradiation, the importance to plant breeding is obvious.

Even if new *loci* are not involved the potentiality of induced mutation for resistance to specific races may be an important tool in some cases. For example, Lee wheat is an outstanding variety in all respects except for its susceptibility to race 15B. If the resistant segregates derived from the irradiated material have not been altered in any other character than resistance to stem rust, they will be a real improvement over Lee, accomplished with less work and in fewer years than would have been possible with usual plant breeding methods and with less hazard of losing some of the desirable characteristics of Lee.

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Record of Proceedings of Session 13C.2

MONDAY MORNING, 15 AUGUST 1955

Chairman: Mr. A. Gustafsson (Sweden)

Scientific Secretaries: Messrs. E. O. Hughes, L. F. Lamerton and C. Polvani

PROGRAMME

P/110 The contribution of radiation genetics to crop improvement . . . . . W. R. Singleton *et al.*

P/793 The production of beneficial new hereditary traits by means of ionizing radiation . . . . . L. Ehrenberg *et al.*

P/890 Studies on genetic effects of chronic gamma radiation in plants . . . . . K. Mikaelsen

P/101 Ionizing radiations as a tool for plant breeders . . . . . R. S. Caldecott

DISCUSSION

The CHAIRMAN: Outstanding in any biological discussion on ionizing radiations is the fact that the nucleus, with its chromosomes and its genes, is highly sensitive to irradiation, more so than the cytoplasmic part of the cell, judging from appropriate experiments in plants and animals. Moreover, most changes of the genes, in experiments as well as in nature, are negative, in the sense that they decrease viability, deform or kill the organism when homozygous, in the double dose, and often, though far from always, do so when heterozygous, that is, in the single dose. In addition, ionizing radiations readily induce profound losses, duplications and translocations of the chromosome material, with all their consequences on sterility, zygote lethality and segregation of defect chromosome types. For man, this latter fact implies a severe hazard. Mutations, beneficial in character, do occur, but in number they cannot compare at all with the detrimental ones.

In crop plants where a breeder easily wipes out, by a sort of mass-killing, 99 per cent or more of the individuals in an experimental field, without any feeling of remorse, the beneficial mutants are the ones to catch his eye. Plant-breeders simply ignore that defects are in the majority, or in some cases they even intentionally include them and the corresponding lethal genes into a heterozygous cross-fertilizing population. The laws behind spontaneous and induced mutation are remarkably similar in all organisms studied. We, the plant-breeders, find the King's picture on the front side. You, the animal and human geneticists, just detect disaster. We want the reconstruction of the plant organism by means of ionizing radiation; you fear the destruction of the human race. However, we all share in Muller's fundamental discovery of the genetic implications of irradiation. The positive side of the irradiation effects on genetic material also rests on his findings. We regret that he can not take part in our discussion today.

Mr. W. R. SINGLETON (USA) presented paper P/110, as follows: Radiation genetics has made sizable contributions to crop improvement and promises many more. These improvements are in all stages of development. In Sweden, farmers are now growing superior strains of cereals induced by radiation. In North Carolina, in the United States, extensive field trials are being conducted with strains of radiation-induced peanuts, *Arachis hypogea L,* some of which are resistant to leaf-spot disease, and some which previous trials have indicated are higher in yield than the parent strain.

Other radiation breeding programs in the United States are promising but not as far along as the ones mentioned. At the Brookhaven National Laboratory we have a few strains of oats, *Avena sativa,* with radiation-induced resistance to stem rust (*Puccinia graminis avenae*) and Victoria blight caused by *Helminthosporium sativum var. victoriae.* These samples of oats are in amounts of only a few grams each, but the resistance is inherited and it is only a question of time until some of these strains will find their application in commercial varieties.

We also have a radiation-induced pure white carnation, produced by radiating the White Sim variety, which has small blotches of red on a white background. Some twenty different lines are in a field trial this summer.

In the case of maize *Zea Mays L,* we are still in the "blueprint" stage as far as improved types are concerned. We have learned much in the past seven years regarding the fundamental aspects of radiating the corn plant, and are now embarking on a program to induce disease resistance in maize. There is every reason to believe the program will be successful.

Radiation genetics had its origin in the fundamental researches of H. J. Muller, who used X-rays on *Drosophila* males; and the late L. J. Stadler, who X-rayed seeds of barley and maize. These researches were conducted about thirty years ago, and both men found that X-rays gave rise to many more mutations than found in the controls.

The pioneering work in plant breeding was done in Sweden by the late H. Nilsson-Ehle, and A. Gustafsson, our Chairman of today's session. They began their work shortly after the researches of Muller and Stadler were announced and have had signal success.

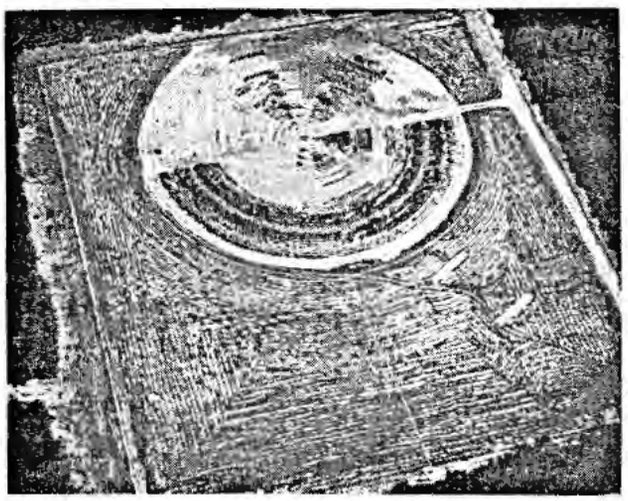
You will hear more of this in the following paper given by Mr. Ehrenberg.

Before the advent of the Atomic Age, research workers were limited mainly to the use of X-rays in their experiments. Radium was available but in extremely limited quantities and at prohibitively high prices. X-rays have proven useful for seed irradiations and many worth while results have been achieved. However, it is not practical to take an X-ray machine into a field for extensive radiation, although Stadler proposed a portable machine for field use. The difficulties of supplying power to a field and protecting the machine while in use made it impractical. While much information could have been gained by radiating plants while growing, this phase of research had to await the development of radioactive sources that could be placed in a field and plants grown near them.

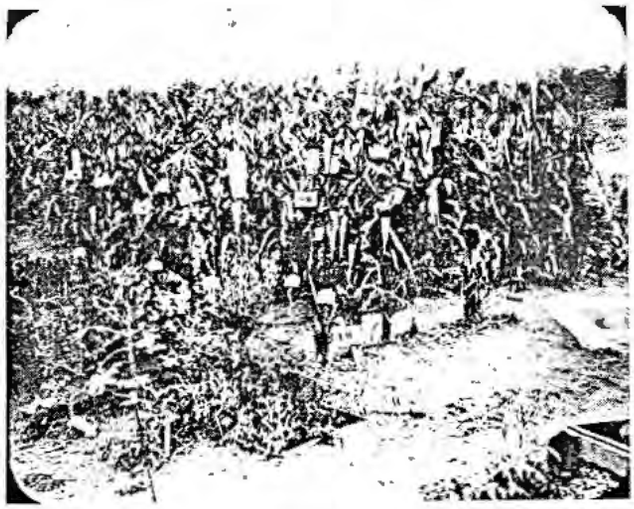
This development was not long in coming. On 2 December, 1942, in a squash court, under the West Stands of Stagg Field, the University of Chicago stadium, the first chain reaction took place. This was the culmination of many years of brilliant research of the late Enrico Fermi, who is perhaps the one man most responsible for the immediate coming of the Atomic Age. Following the war there was an "avalanche of isotopes," as Paul Aebersold told you this last week. One of the most useful of the isotopes for sustained irradiation of plant material is Co^{60} with a half-life of 5.3 years.

This was the one selected by the Brookhaven group when we set up a radioactive Co^{60} source in 1949. The original source was 16 curies, which was in operation in 1949 and 1950.

Slide 1 will give you an idea of how the field was laid out, with the Co^{60} source in the center of the field, and the rows of maize and other crops in concentric circles around the source. Within each circle or arc, all plants receive the same amount of radiation, which decreases rapidly from the center to the perimeter of the field, due to the inverse square law.



Slide 1



Slide 2

Slide 2 shows the adverse effect of the radiation on all plants growing near the source. Plants in the 3-meter row (670 r/day) were killed in the seedling stage, which is much more susceptible to injury by radiation than later stages. Plants nearer maturity will withstand radiation of more than 100 r/day for several days without apparent injury.

Plants in the 4-meter row (370 r/day) were so severely injured they produced no tassels or ears. Plants receiving 230 r/day (5 meter row) produced abundant pollen with a fairly high mutation rate of endosperm characters.

Endosperms (kernel) characters were used for the study of the effects of chronic gamma radiation on mutation rate because of the ease and certainty in scoring, and the large populations readily obtainable. I have one diagram here in which we use mainly four characters: the *sh* character on chromosome (4), the *pr g* on chromosome (5), the shrunken on chromosome (9) and the *r* gene on chromosome (10).

Slide 3 shows the genetic stocks used. The ear on the left is completely blue except for one colorless kernel, a radiation induced mutant. This stock was grown in the radiation field and pollen placed on a recessive type, the third ear from the left. The resulting kernels were all blue, also dominant for all other characters studied, except for mutations. Such an ear is shown between the two parents. It also has

Slide 3

a mutation for colorless, the *r* gene on chromosome (maize has 10 pairs of chromosomes numbered from the longest to the shortest). We will ignore the two ears on the right of the slide.

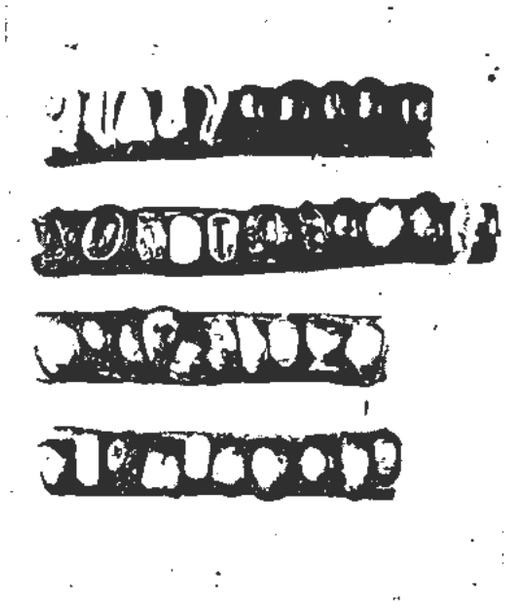
Slide 4 shows the different endosperm characters studied in determining the effect of radiation on mutation rate. At the top left is the recessive type, top right the dominant which was grown in the radiation field. The second line from the top shows from right to left the recessive parent, the dominant one and the different mutants obtained from right to left, *r* (chromosome 10) *pr* (5) *su* (4), 2 *sh* (9), *r sh, r su, pr sh* and *pr su*. We will ignore the last two rows of kernels.

Slide 5 (Fig. 2 of P/110) shows the mutation rate of endosperm characters when plotted against dose rate. These are for 1950 and 1952. We cannot tell how many of the events scored as mutations were induced chromosomally and how many were point mutations. The non-linear effect could be due to a large percentage of chromosomal events (two-hit effects) or to a marked increase in sensitivity during particular stages in the meiotic cycle. Later experiments have shown a marked difference in sensitivity at different stages in the meiotic cycle. This was determined by growing plants in pails and exposing them to a semi-acute dose of one day only.

Slide 6 (Fig. 1 of P/110) shows plants being irradiated in such a manner. Note that the rows are arcs of a circle so that all plants within a row receive the same dose. Plants nearer the source (pipe in the center) received 2000 r/d while those in second row received only 1300.

Slide 7 (Fig. 3 of P/110) shows results of exposing corn plants at different stages in the meiotic cycle.

Letters of the alphabet were used to represent exposure dates with *A* representing the earliest date on July 21. Letter *D* represents meiosis and pollen



Slide 4

was shed about 5 days after letter *Q*, after the plants were removed from the field. Note a 7.5 fold increase from letter *E*, immediately after meiosis to letter *O*, shortly before pollen shedding. At meiosis (*D*) the seed set (open bar) was so low as to make it impossible to determine mutation rate. The ears averaged only 10 seeds per ear. No mutations were found.

The variation in sensitivity of different stages in the meiotic cycle are extreme. Similar differences in sensitivity have been found in *Drosophila* by Mary Lou Alexander and the Texas group.

The finding that there are extreme differences in sensitivity to radiation during the meiotic cycle suggests a somewhat different approach to the use of the radiation field for the production of mutations. Instead of radiating chronically it seems better to give a short, semi-acute dose at the sensitive period.

To be successful in this operation, it is necessary to determine the sensitive period for each species of plants worked with. The sensitive period is known for maize, and there is reason to believe that comparable stages in other plants might show similar sensitivities.

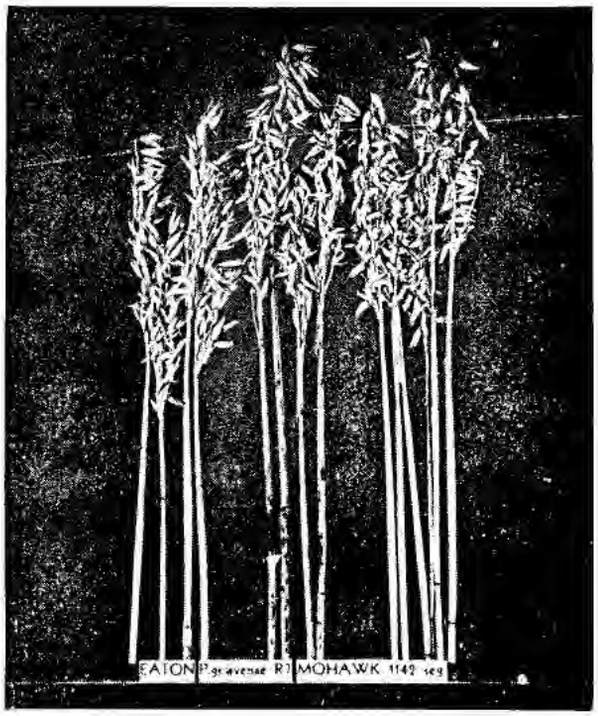
The Co^{60} radiation field at the Brookhaven Laboratory has been in use for seven summers. There are now similar radiation fields in other parts of the world, two in Sweden, one in Norway, which Mr. Mikaelsen is going to tell us about, and at least one in the USSR. It is expected there will be at least 1 and perhaps 2 more in the United States next year.

A Co^{60} machine has been developed at the Brookhaven National Laboratory (Slide 8; Fig. 4 of P/110). This houses an approximately 200-curie source, and it is estimated that it can be manufactured and sold for approximately $5000. With such a machine it should be feasible to set up additional radiation fields in other parts of the globe.

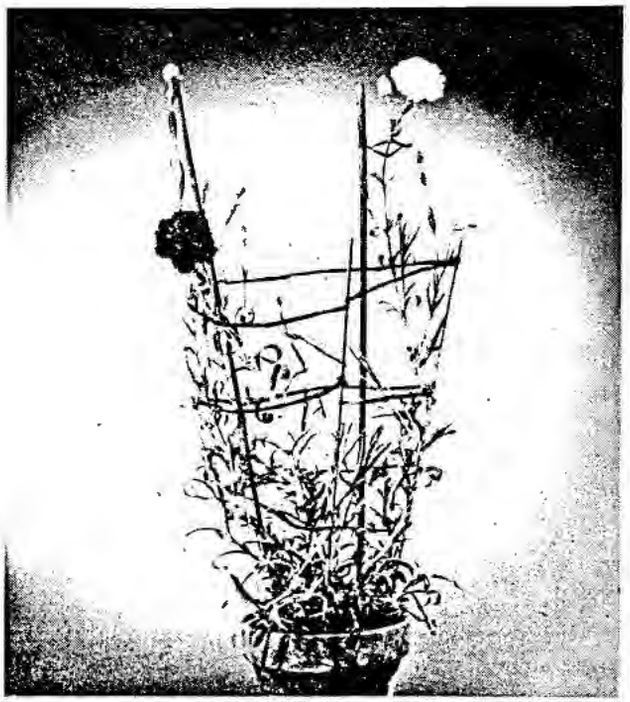
In addition to the Co^{60} radiation field at the Brookhaven Laboratory there are other sources of radiation such as X-ray, ultraviolet light, and thermal neutrons which have proved efficient in producing genetic changes. I think you are going to hear more of that from Mr. Caldecott. The disease resistance in oats, referred to earlier, was induced by exposing seeds in the thermal column of the nuclear reactor.

Slide 9 shows three strains of oats. On the left is Eaton, a variety resistant to stem rust, in the center Mohawk, a susceptible variety, and at the right a resistant type 1142 produced by exposing seeds of Mohawk to thermal neutrons.

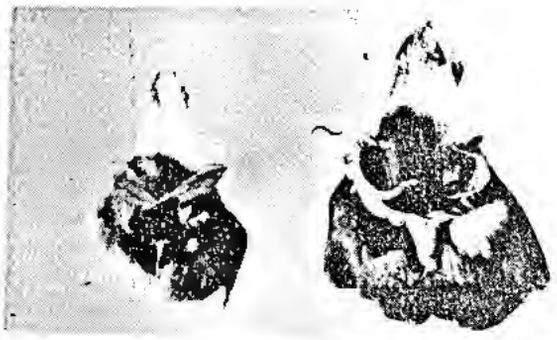
In addition to treating pollen of developing maize, considerable work has been done in producing mutations in somatic tissue. Seed irradiation is one way of producing somatic mutations. In addition considerable work has been devoted to producing somatic mutations in fruit trees, ornamental shrubs and other plant material where the mutations induced can be propagated vegetatively and a new variety established immediately.



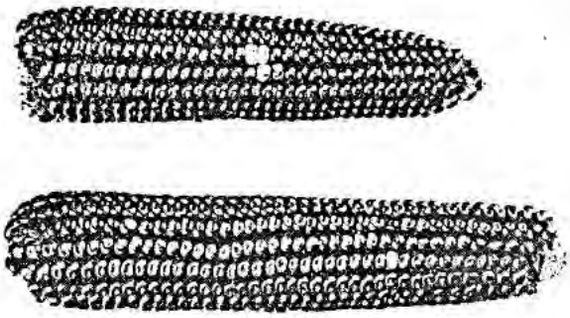
Slide 9



Slide 11



Slide 10



Slide 12

Although it is too soon to expect results from most of the slower growing woody plants, positive results have been obtained with some herbaceous plants such as *Antirrhinum majus* L., in which tetraploid sectors were induced, Slide 10.

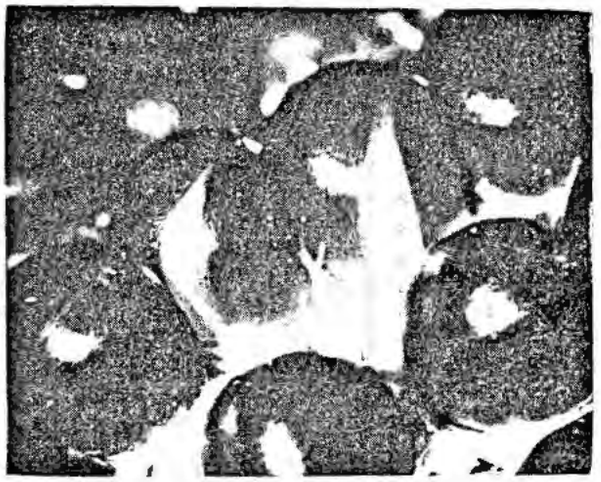
In carnations (*Dianthus caryophyllus* L.) several propagatable somatic mutations have been produced. Eleven of 87 cuttings from plants of White Sim receiving the highest doses of radiation up to 340 r/day produced branches that had all red flowers Slide 11.

Extensive somatic mutation data were obtained with the P^{RR}/p^{r} gene in maize (Slides 12 and 13). More than a million kernels were observed. Most of the somatic changes were rather small, producing in many instances a very fine streak. The mutations are plotted against dose rate (Slide 14; Fig. 14 of P/110). At higher doses the relationship seems linear but at the lower doses of radiation there is not a linear relationship between dose of radiation and mutations. Below 5 r/day there was no apparent effect of radiation.

It was also observed that radiation injured the developing ear. From less than one to approximately 100 r/day, there was an inverse linear relationship between radiation received and number of seeds per ear.

Regarding co-operative research it was mentioned earlier that the gamma field is used co-operatively by research workers in eight different experiment stations and universities, who grow their material in the field.

In addition to this co-operative project other facilities unique to the Brookhaven Laboratory are made available to other investigators. One such facility is the thermal column of the nuclear reactor for treating seeds and scions. Also material is X-rayed for those not having access to an X-ray machine. To date radiations have been made on more



Slide 13

than 60 crop plants for well over 100 investigators located at many places throughout the United States and Canada.

Radiation as a tool in plant breeding has made a modest beginning. We feel that each succeeding year will show progress. It is almost impossible to envisage the eventual good that may arise from the use of radiation in plant breeding—and we thoroughly agree with the late Enrico Fermi who said "I believe truthfully, that the conquest of atomic energy may be widely used to produce not destruction, but an age of plenty for the human race".

Mr. L. EHRENBERG (Sweden) presented paper P/793, as follows: It is well established that ionizing radiations, irrespective of mode of production and of properties, readily induce hereditary changes, so-called mutations, which are stable and lead to new traits manifested also in following generations. Although time does not permit me to go into many details, I want to demonstrate some data which prove the value of the application of mutation in plant breeding work. Since I thus have to speak about some mutations which involve improvements of practical value, it seems necessary to stress that the vast majority of induced as well as spontaneous mutations are detrimental, or inferior to the unmutated type.

According to an older opinion prevalent for several years, all mutations should lead to a breakdown of the hereditary material, thus being exclusively harmful. Recent studies have shown, however, that among several hundred harmful mutations one or two may be obtained which are distinctly positive. Since we deal here with agricultural plants a positive mutation was to be defined as a hereditary change beneficial to man's interests.

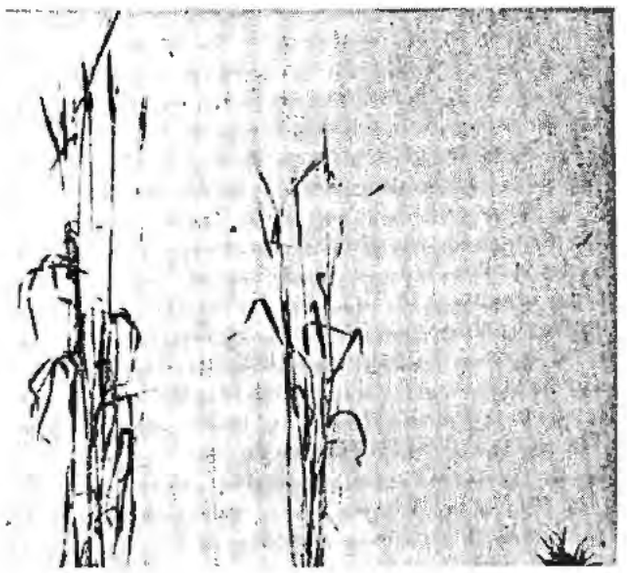
The principles for the induction of positive mutations have now been worked out for a large number of agricultural species. The methods involve irradiation of seeds or growing plants under different conditions. Much work has been concentrated on barley and wheat. Both these organisms are self-fertilizing and very suitable for mutation analysis. Comparing the two species, mutations of the diploid barley show, in striking contrast to the hexaploid wheat, a wide range of morphological variation in the mutants, from small, scarcely perceptible changes to most profound alterations, which may even break the morphological frame of the species.

A few slides will demonstrate this.

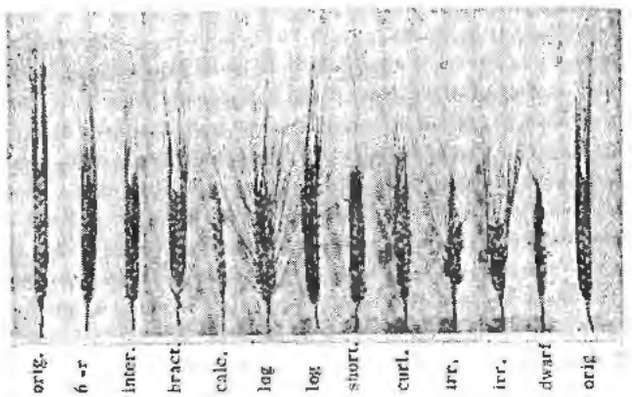
In this picture (Slide 15) you will see in the center the original type used in the experiment—a high-yielding variety of barley. To the right is an example of a dwarf—such dwarfish types are rather common. To the left is the giant mutation, which is sometimes obtained.

Slide 16 shows spikes of different viable but chiefly negative types. There is the spike of a dwarf, several spikes with inherited irregularity and two types which are not known in nature—that is, which represent new properties. A six-rowed barley may also be obtained as a variation from the two-rowed spike.

In spite of their greatly altered appearance many mutations are surprisingly productive. This applies, for instance, to the so-called erectoid mutants in

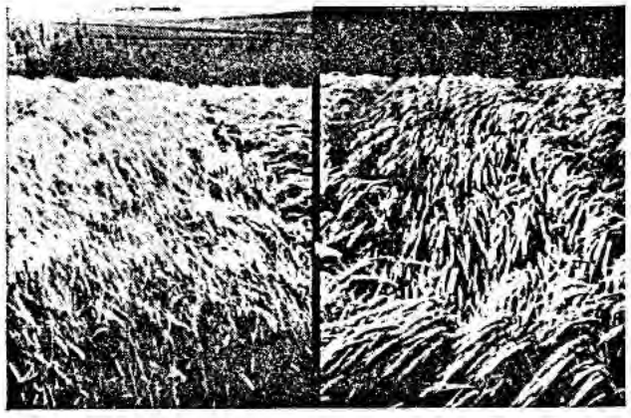


Slide 15



Slide 16

Slide 17



Slide 18

barley (Slide 17). To the left we have the original type; the four in the center are examples of this erectoid mutation with a shortening of the internodes in the spikes, so that the whole spike becomes shortened. Many of these erectoid types show properties of value from an agricultural point of view. The grain and straw production approaches or equals that of the mother strain; in some cases the mutant is even superior by a few per cent.

At the same time the erectoid mutants distinctly surpass the mother strain as regards stiffness of the straw (Slide 18). To the right is the mother strain and to the left one erectoid mutant in a field trial. They were taken at the same time, and you can see the great difference as to the erectness of the straw. This property is of great importance, especially when harvest is mechanized.

Increased earliness can also be obtained in combination with erectoid mutation or otherwise. Slide 19 shows a field trial with an early mutant. You can see that the spikes of the mutant have just appeared, whereas those of the mother strain, sown simultaneously, have not yet developed and, in fact, do not appear until appreciably later.

In addition, the erectoids are able to utilize nitrogen dressing more than the mother lines (Table I on this page). The original type is represented by 100, and at two different nitrogen dressings,

Slide 19

Slide 20

the sum of 3 erectoid mutants is compared with the mother type. You can see that at high nitrogen dressing they surpass the mother type.

Table I. Relative Production of Three Erectoids and Two Bright-Greens Obtained from Bonus Barley. Data of 1951 (Bonus = 100)

Manuring: $Ca(NO_3)_2$, kg/hectare	200–300	400–500	*Difference*	*P*
Ert 23, 28, 32	96.41±1.22	101.88±1.48	+5.47±1.92	$0.01>P>0.001$
Bright-green 3,4	98.72±0.92	95.58±0.85	−3.24±1.25	$0.02>P>0.01$

If we separately investigate this number 32, it surpasses the mother type at the low dressing and is much better at the higher dressing. The bright-green mutants show that this is not a common property to all mutants.

They respond in an opposite way to an increased nitrogen dressing. The changed response to nitrogen dressing is one example of an altered reaction norm, involving the sudden registration of new ecological requirements by induced mutation.

A new ecotype or race has arisen. Now and then, but not always, these erectoid types are obtained simultaneously with chromosome rearrangements. We can see here an example where we have a set of 14 chromosomes of barley where two of the chromosomes have two satellites each (Slide 20). In normal

barley, in all races known, there are four chromosomes with one satellite each.

These changes cause the formation of more or less pronounced sterility barriers between mutants and mother strain. In fact, by one stroke and simultaneously, we may induce all the essential characters distinguishing species in nature: First, the origin of a sterility barrier; second, a drastic change in morphology and anatomy; third, an altered ecological response; and fourth, a new karyotype detectable microscopically.

It has been definitely shown, in barley and other experimental plants, that induced mutation can increase the yielding capacity of a variety, or leave this capacity intact and improve upon special characters of importance in agriculture, apart from the straw-stiffness and earliness, or the positive response to an increased nitrogen dressing, also as regards protein or oil content, baking quality, malting properties, fiber strength, grain or fruit size, in cereals as well as peas, lupines, flax, mustard, tomatoes, fruit trees and others.

For instance: in the Swedish breeding material of sweet lupine and white mustard selection and crossing gave no improvement as to earliness and oil content, respectively. But by irradiation of seeds it was possible to increase variation, and subsequent selection was then successful. Now, the only white mustard variety cultivated in Sweden is the result of irradiation.

Summing up these data it can be concluded that the induction of mutations in agricultural plants by means of ionizing radiations *have given* and *can give* hereditary changes of high production capacity. If the mutants, although highly productive, are not suited for a direct marketing, they form a source of variation valuable for a continued breeding along traditional lines. The utilization of mutation will no doubt lead to a considerable gain of time in the breeding work. Even high-bred lines of agricultural species are still rather old-fashioned as to morphology, anatomy, and karyology. They have to be reconstructed to meet the needs of the high mechanization and intense fertilization of modern agriculture. For example, regarding internode length of the straw, barley should be remade to resemble wheat, and, regarding the cross-section area of the straw, wheat should be changed towards barley. Our experiments indicate also that a reorganization of the ancient karyotype may make possible a better coordination of genes and mutations in the future breeding program.

In mutation work there is a more or less random distribution of the changed types obtained. The second large goal of the mutation breeder is therefore to learn how to direct and control this mutation process, i.e., how to influence the distribution of mutation in a wanted direction. Partly as a first step in studies of this question the effects of different radiation types have been extensively compared in the experiments with barley. These studies have

Table II

	Radiation source				
	Cobalt-60 gamma rays	*160 Mv protons*	*180 kv X-rays*	*Pile neutrons*	*Radon alpha rays*
Rare chlorophyll mutants, % ..	6.6	10.3	8.6	12.3	23.1
Sum of mutants	319	639	248	399	65
Ion pairs per μ tissue...	8	16	100	1000–3000	3700

Table III. Relative Frequencies of Erectoid and Other Viable Mutations in the Second Generation (1953)

Type of radiation	*Ion pairs per μ*	*Number of*		*Erectoids, %*
		Erectoids	*Others*	
X-rays	100	8	22	27
Cyclotron-produced neutrons	400–800	8	8	50
Pile neutrons	1000–3000	6	0	100

revealed a clear influence of linear energy transfer and of other factors on the distribution of mutation types.

In Table II, we have compared here the frequency among the number of those types which belong to a group of rare types. We see that they increase from about 6 per cent with cobalt-60 gamma rays to about 20 per cent with alpha rays. There is an increase with ion density.

Table III shows another example of an influence of ion density on the distribution of mutation type from the 1953 experiment. With X-rays about 25 per cent of the erectoid mutations were found among the total number of viable mutants. But at the higher ion density obtained in the center of the pile, about 100 per cent was found. These data have been confirmed in later experiments. The material is rather small here.

It has also been found that higher frequency of lethal mutants are obtained at higher ion density.

We have therefore proved, in principle, that the spectrum of mutation types can be displaced in different directions. Parallel treatments with radiations of different kinds and with mutagenic chemicals, under varied experimental conditions, will therefore give us means to learn how to increase the probability of obtaining certain wanted mutations.

Mr. K. MIKAELSEN (Norway) presented paper P/890.

Mr. R. S. CALDECOTT (USA) presented paper P/101.

DISCUSSION OF P/110, P/793, P/890 AND P/101

Mrs. A. SAVULESCU (Romania): I should like to put three brief questions to Mr. Singleton.

1. Mr. Singleton has shown that, by irradiation, mutations could be obtained in plants which give resistance to black stem rust. In the paper which has been distributed to us, I read about another mutant-type resistant to the Victoria blight disease.

I should like to ask concerning these two important diseases whether any research work has been done concerning the cytological, morphological or biological characteristics obtained which could be made responsible for the induced resistance.

2. In experimental plots, has any estimation been made of the productivity of the non-irradiated plants and of the irradiated resistant mutants, in controlled conditions for both diseases; in other words, as Mr. Ehrenberg had shown for other characteristics in his paper, has the mutation had no effect on productivity?

3. What is the opinion of Mr. Singleton and of the other authors of the paper concerning irradiation of sexual hybrids of cereals and other crop plants? Would they get, through irradiation, a higher mutation rate?

Mr. SINGLETON (USA): The work to which I referred in resistance in stem rust and also in Victoria blight was done by Mr. Contact in our laboratory. Seeds were exposed in the thermal column. He has not conducted any cytological investigations, as I understand it, on these, and is not sure whether they are co-related with the chromosomal disturbance. These have not yet been subjected to yield trials in comparison with the control. The amounts are still limited and it may be that there will be factors that depress the yield along with the rust resistance or blight resistance. But he feels that the resistance is an important thing and even if crossing and backcrossing are needed to get it into the commercial varieties, it can be done fairly readily.

I am not sure that I can furnish any data on the last question. There is an opinion among some of the corn breeders that hybrids do have a higher spontaneous mutation rate. We have been planning to do some work on that, but have not got around to it yet. I think that in corn we have an ideal tool since we have the inbred strains and hybrids between them to determine whether there is an inherent difference between the hybrid and inbred strains. I am sorry that I can only give a guess on it now.

Mr. I. REIFER (Poland): Could Mr. Ehrenberg please comment on the irradiation work done in Sweden on lupines, particularly as regards degeneration of the sweet lupine into the bitter one, containing large quantities of alkaloids.

Mr. EHRENBERG (Sweden): I am not a specialist in the field regarding alkaloid quantities. I am sorry that I cannot reply to the question.

Mr. BONET-MAURY (France): I should like to ask Mr. Ehrenberg whether he has some idea concerning the very interesting mechanism of the action of ion density on mutation. My second question has been answered in large part by Mr. Caldecott, namely whether high ionic density is advantageous for practical purposes.

Mr. EHRENBERG (Sweden): It is a rather difficult question to answer in a few minutes. As regards the mechanism of mutation of ion density, there are certainly what we call indirect effects at hand, since we have the influence of water content and of other factors on the mutation rate. This influence becomes smaller when we go to higher ion densities. Perhaps the localization of energy along the track will lead to what we call direct effects. This would be no more than a kind of definition. Of course, we do not yet know the exact mechanism.

As regards the second question, I mentioned that when we used high ion densities, we get a higher proportion of lethal mutants. It is one of the proofs that there is an influence of ion density on the distribution of mutation types. In our Swedish experiments, when we compared the number of viable mutants obtained at a corresponding survival of the plant material irradiated, we got about the same number with X-rays and with neutrons, because of the prevalence of the lethal ones in the case of neutrons.

Mr. E. LESTER SMITH (UK): If I remember rightly, at the first Oxford Isotope Conference, it was suggested that useful proportions of mutants could be induced by soaking seeds in solutions containing isotopes such as radioactive phosphorus or sulphur. This effect was attributed to atomic disintegration occurring within the nucleus of the cells. I should like either of the authors to comment on this technique as compared with X-rays or neutron irradiation.

Mr. SINGLETON (USA): I would say in reply that I do not know of any work where we can test that with things like seeds. One of the workers in our laboratory this summer, Mr. Bernard Strauss, has been using P^{32} inside *Neurospora conidia* and the same concentration of the same phosphorus outside the *Neurospora conidia*. He finds a much higher mutation rate when the destination is within the *conidia*. That work will be published shortly. I think it is exciting.

Mr. M. ROCHE (Venezuela): Does Mr. Caldecott have any data regarding the electrolytic content of his hydrated embryos? Does he think that this rather than the water content itself influences their sensitivity to X-ray?

Mr. CALDECOTT (USA): We have no data in that connection. I should like to make one point, however. Ehrenberg and Nyhon have suggested that a long period of respiratory activity which accompanies hydration at different relative humidities, may result in the development of protective substances within the seed material. For this reason we have actually soaked seeds in water at a very low temperature, the object being to allow them to hydrate when there would be a very limited respiratory activity. Under these conditions, we get the same decreased sensitivity with increased hydration. This is about the only comment that I could make.

Mr. SINGLETON (USA): Mr. Caldecott suggests that plant breeders should, if possible, use highly ionizing radiations to produce mutants for agri-

cultural purposes. Since abundant mutations can be produced by several ionizing radiations and since the plant breeders' time is a limiting factor, does it make too much difference how the mutations are produced? With either gamma, X-ray or chronic gamma, many more mutations can be produced than studied. Do we need to stress too much the number of mutations that can be produced, because we cannot study all that we can possibly produce?

Mr. CALDECOTT (USA): I want to emphasize that, in X-raying seed material, one frequently encounters difficulty in obtaining good surviving populations to study. This is emphasized time and again in our work and in the Swedish work. One does not encounter such difficulties when one uses neutrons. Remember that the time during which the seeds are irradiated is just a small portion of the time over which the material is studied. For my part, it would pay to send the seed material to a place where it can be subjected to neutron irradiation. This is a matter of only a few days, or a few weeks, at most, whereas the analysis one will be doing on the material will encompass several years.

The CHAIRMAN: I have two questions from Mr. Bonferroni, of Italy. They are directed to Mr. Ehrenberg.

1. Are the induced mutations stable, or do they show a tendency to fall again into the previous state? If the answer is yes, what is the percentage?

2. What is the ratio between recessive and dominant (if any) induced new characters?

Mr. EHRENBERG (Sweden): The answer to the first question is yes. As regards the percentage of induced mutations, it depends upon dose, but some 5 or 10 per cent can be obtained. Five to 10 per cent of the irradiated seeds could give rise to mutations and 500 times less of these could perhaps be positive.

In reply to the second question, very few dominant mutants are observed in barley but, upon closer inspection of the mutants, one can observe a difference between the heterozygote and the unmutated normal type—a sort of semi-dominance in most cases, although often very weak.

The CHAIRMAN: Ladies and gentlemen, you have now heard four scientists of high rank deliver facts and ideas about ionizing radiations and the production by their means of beneficial mutations. Personally I consider that we are now entering a new phase of plant-breeding. We definitely know that beneficial mutations can be produced in any crop-plant sufficiently worked upon. In the next few years we have to elaborate new methods for the induction of *specific mutations*, changes in just those *loci* where mutations cause an increase of yield or improve upon other characters of significance. Most crop-plants are rather old-fashioned in their morphology and anatomy. We simply have to modernize them. Induced and controlled mutation is a means of doing this. For this sake ionizing radiations ought to be supplemented with chemical mutagens. When this is effected, but only then, we shall have a very wide arsenal of methods. Induced mutation is now incorporated into the regular scheme of plant breeding. Like artificial polyploidy it does not replace or exclude, but rather adds to and forms a part of the traditional methods: selection and hybridization.

Finally, since Dr. Muller is here I think we really should give him our applause and our thanks for starting and inducing this state of affairs.

Session 14C

RADIOACTIVE ISOTOPES AND IONIZING RADIATIONS IN AGRICULTURE (*concluded*) - TRACER STUDIES IN AGRICULTURE

LIST OF PAPERS

(Continued overleaf)

LIST OF PAPERS

(Continued)

Studies of Special Problems in Agriculture and Silviculture by the Use of Radioisotopes

By J. W. T. Spinks,* Canada

It is now over thirty years since Hevesy introduced the application of radioactive isotopes as indicators in plant studies.[1] The discovery of artificial radioactivity by Curie and Joliot in 1934 greatly enlarged the scope of the method, but it is probably fair to say that it was not until the advent of the atomic pile that the use of radioisotopes in agricultural research became at all widespread. Today isotopes such as H^3, C^{14}, Na^{22}, P^{32} and Co^{60} are available in quantity and have been used to investigate a number of problems of interest to agriculture—problems of analysis, animal metabolism, the relation of the plant to its soil food, trace elements, fertilizers, photosynthesis, and weed and pest control.

FERTILIZER UTILIZATION

General Principles

Within the last few years radioisotopes have provided an invaluable tool for investigating the availability of plant nutrients under field conditions. While a number of different isotopes have been used, the economic importance of phosphate fertilizer and the relative ease of handling P^{32} have resulted in particular attention being paid to phosphorus. Qualitative soil and plant studies using P^{32} began in 1936 and were followed ten years later by quantitative field studies of phosphate fertilizer uptake by wheat plants.[2,3,4,5] By combining the radioactive measurement of fertilizer uptake with the measurement by ordinary chemical methods of total phosphorus uptake, the uptake of soil phosphorus could be determined by difference. In the past, the recovery of phosphate fertilizer by a crop was determined by a comparison of the uptake of phosphorus by crops grown with and without fertilizer. The extra phosphorus in the fertilized crop was taken as the quantity coming from the fertilizer. This method assumed that fertilized and unfertilized crops take up the same amount of soil phosphorus, but tracer experiments indicate that this is often far from being the case.

Numerous experiments have been reported during the last decade covering such diverse topics as utilization of various phosphatic fertilizers by different types of crops at various stages of growth grown on different types of soil, using different methods of placement and different rates of application. In the first field experiment, 1 mc P^{32} was used. Four years later, 30 curies was used in the United States alone, and now the labelled phosphate fertilizers are made by the ton!

Preparation of Labelled Material

It is important that the P^{32} be in the same valence state and chemical form as the phosphorus in the phosphate fertilizer being studied. For example, radioactive ammonium phosphate ($NH_4H_2PO_4$) may be prepared by adding phosphoric acid of high specific activity to a solution of inactive ammonium phosphate and subsequently evaporating this solution to dryness with stirring.[6] Standard methods have been worked out for preparing various labelled phosphate fertilizers, and the procedures have in some cases been stepped up to pilot plant scale.[7] It should be pointed out that the seemingly attractive neutron irradiation of phosphatic materials is likely to lead to difficulties since the P^{32} produced will almost certainly be present in several different chemical forms and give rise to ambiguity in interpretation of results.[8,9,10,11]

Assay—Radioactive and Chemical

In the early work, the phosphorus in the plant was converted to magnesium pyrophosphate and counted under an end-window counter.[4] Since then the solution counting technique has been used, utilizing either dip counters[12] or immersion counters.[13] However, most workers,[13,14] now measure the activity of briquettes of dried plant material, using an end-window counter,[14,15] or the activity of hollow cylinders of dried plant material placed around a thin wall counter of the thyrode tube type.[15,16] By using as standard a cylinder made from an aliquot of the original labelled fertilizer mixed with inactive plant material, the uptake of the fertilizer is very simply and directly measured. The hollow cylinder method has increased both the sensitivity of the measurements and the ease of making them, so that it is now possible to work with much lower specific activities and still do many hundreds of analyses in a relatively short time.

Phosphorus is determined chemically using the phosphomolybdate blue reaction with hydrazine as a reducing agent.[16]

* Dean of Graduate Studies, University of Saskatchewan, Saskatoon, Saskatchewan.

Field Experiments

In a typical field experiment each treatment plot consists of five rows, the two outside rows being guard rows, with no fertilizer. The three inner rows receive the designated fertilizer treatment, but only the centre row is treated with radioactive fertilizer. The level of radioactivity in most field experiments is 100 μc P^{32} per gram of inactive P^{31}. The rows are 5 metres long, the centre 3.7 metres being harvested. Six similar plots of land are used in a random arrangement, or a balanced lattice design. Except for placement experiments, fertilizer and seed are at the same level, the fertilizer and grain usually being sown together.

Typical Results of Tracer Fertilizer Experiments

The following results were obtained in experiments with the soils of Saskatchewan, Canada, and may not apply in all details to other types of soil.

Stage of Growth

There is little uptake of fertilizer or soil phosphorus in the earliest stages of growth of the wheat plant. The uptake of fertilizer phosphorus reaches a maximum at 2–6 weeks and begins to fall off at about 8 weeks as the plant reaches the heading stage. In the earlier stages of growth, the wheat plant obtains a larger portion of its phosphorus from the fertilizer than from the soil, but later on the situation is reversed.[17,18]

Type and Variety of Crop

In a typical tracer experiment, plots were set out at two locations, one on a less responsive, brown heavy clay (Regina Heavy Clay),[19] and the other on a highly responsive, moderately heavy black soil (Melfort Silty Clay). Four varieties of wheat (Thatcher, Apex, Redman, and Rescue), three varieties of barley (Titan, Montcalm, and Vantage) and three varieties of oats (Ajax, Exeter, and Fortune) were included in the test. The tests were conducted on fallow land, and the fertilizer used was mono-ammonium phosphate at 24 lb P_2O_5 per acre. The order of yield increase in the two trials was wheat (least), barley, oats, and it is to be noted that barley utilized a greater portion of the fertilizer phosphorus and showed a greater uptake of total phosphorus on the fertilized plot. This was also the case for the uptake of phosphorus on the unfertilized plots. While, as might be expected, there were a number of significant differences in yield between varieties of the grain attributable to inherent varietal characteristics, it was only in barley that differential response to the fertilizer treatment was found. The varieties, Montcalm and Vantage, gave a greater response to the phosphate fertilizer than Titan at Birch Hills, but not at Rosetown on the less responsive soil. Titan is an earlier maturing variety, and this factor may be related to the result obtained. It is of interest that such differences between varieties can exist, and conceivably such differences could be of considerable interest to the plant breeder.[20,21,22]

Type of Soil

In a typical experiment, using wheat, barley, and oats with ammonium phosphate fertilizer, two types of soil were compared. One was a less responsive heavy clay (Rosetown, a chestnut soil on a black lake-bed base material), and the other a more responsive silty clay at Birch Hills (a thick black chernozem-like soil on lake-bed material). All three crops showed a greater utilization of fertilizer phosphorus on the more responsive soil at Birch Hills (33% compared with 24% at Rosetown).[21]

Placement

In a typical placement experiment with Thatcher wheat and 11-48-0 fertilizer (mono-ammonium phosphate), seed was placed at depths of 7.5 and 11 cm, with fertilizer at depths of 5, 7.5 and 11 cm. The experiment was done at three locations using fertilizer in the powdered and granular forms. The results indicated a maximum yield and maximum utilization of fertilizer with seed and fertilizer (granular form) both at depth of three inches.[21,22]

Type of Fertilizer

Numerous tracer experiments have been done with wheat, oats, and barley, and various phosphatic fertilizers such as ammonium phosphate standard super-phosphate, monocalcium phosphate, etc. The experiments all indicate that for the Saskatchewan prairie soils, ammonium phosphate provides the most readily available source of phosphate.[6,22,23] In other experiments, the effect of nitrogen addition on fertilizer phosphate availability has been studied in some detail.[24] Field tests were carried out in ten plots located in the Brown, Dark Brown, Black and Grey soil zones of Saskatchewan. Fertilizers were mono-ammonium phosphate and monocalcium phosphate applied at 24 lb P_2O_5 per acre (lb /acre ~ kg /hectare), varying amounts of NH_4NO_3 being added to give ratios of N to P varying from 1 : 4 to 1 : 0.8. A marked increase in the uptake of fertilizer phosphate from both carriers occurred as the amount of nitrogen was increased. Stubble trash reduced fertilizer availability considerably. Later experiments in which the $N : P_2O_5$ ratio was varied from 1 : 4 to 4 : 1 have confirmed the above findings.[25]

Rate of Application

The effect of rate of application has been intensively investigated. For example, in 1950 experiments were done at Melfort and Watson using Thatcher wheat and 11-48-0 applied at 6, 12, 24, 28 and 96 lb P_2O_5 per acre (or kg /hectare).[22] With increasing rate of application, the fertilizer uptake increases while the soil phosphorus uptake steadily decreases (Fig. 1). Before the advent of tracers, fertilizer uptake was estimated by comparing the phosphorus uptake from a fertilized plot with that from a control plot, the assumption being made that an equal quantity of soil phosphorus is taken up from fertilizer and control plots. The values obtained in this way are shown by the broken line in Fig. 2. They can obviously be greatly in error.

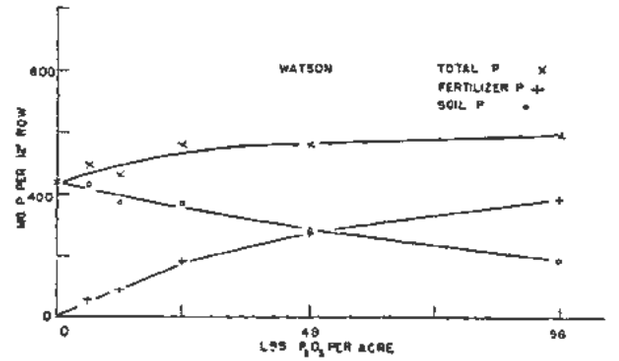


Figure 1. Relationship between total phosphorus in plant and phosphorus uptake from soil and fertilizer, as influenced by rate of application at Watson, Saskatchewan, 1950

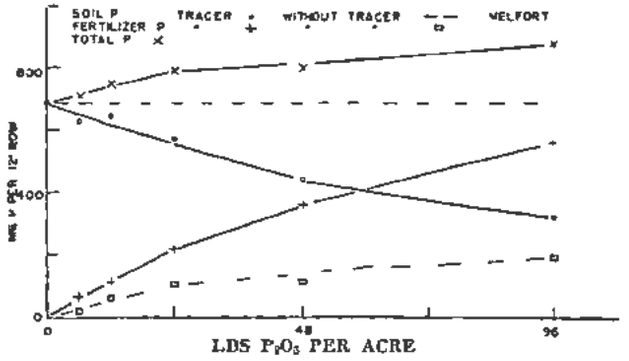


Figure 2. Relationship of uptake of phosphorus from soil and fertilizer with increasing rate at Melfort, Saskatchewan, 1950. Broken lines indicate results as might be calculated without the information provided through the use of tracer phosphorus

Available Soil Phosphorus; "A" Value[26]

In Fig. 3 the rate of application of fertilizer has been plotted against the ratio, (P from the fertilizer)/(P from the soil). It is of considerable interest that the points lie on a reasonably straight line, the slope of the line being different for different types of soil. This behaviour has been found by a number of workers and appears to be quite general.[26] Having established that this behaviour is quite general, the slope of the line, usually called A, can be determined by doing experiments for just one or two rates of application of fertilizer. In fact,

$$A = \text{(rate of application of fertilizer)} \times \frac{\%\ \text{P from soil}}{\%\ \text{P from fertilizer}}$$

From the figure for Melfort soil it appears that when 11-48-0 is applied at 60 lb P_2O_5 per acre, soil and fertilizer contribute equal amounts of phosphorus to the plant, or in other words, the soil phosphorus made use of is equivalent to that supplied by 60 lb P_2O_5 as 11-48-0 per acre. The 60 lb per acre is just the slope of the line, and thus A gives a measure of the available soil phosphorus in terms of 11-48-0. The theoretical basis for this expression has been discussed by Fried and Dean[26] and by Larsen.[27] Actually, this expression is just what we would expect if we were doing a quantitative analysis by means of the isotope dilution technique. In order to determine, for example, the amount of a given substance in a liquid sample by isotope dilution, a known amount of tracer of known specific activity and in the same valence form is added to the sample. After thorough mixing, a portion is pipetted out and the specific activity redetermined. In the soil-plant case, the sample is the phosphorus in the soil available to a given crop (plant or plants), the tracer is the tagged fertilizer, and the crop represents the pipette. It has sometimes been claimed that the occurrence of surface exchange between the phosphate fertilizer and the phosphate in the soil invalidates the method, but actually, as was explained in one of the earliest papers,[4] surface exchange will occur to the same extent for labelled and unlabelled fertilizer molecules and the mathematical result is that the conclusions drawn are not affected.

The available soil phosphorus, as indicated by the A value, has recently been used to help evaluate chemical methods used in determining soil phosphorus availability.[28]

Radiation Damage

It is well known that the radiation from radioisotopes will inhibit the growth of plants. It is therefore important to use the tracer at a specific activity which is sufficiently low that radiation effects do not invalidate the method. Just what the safe level is has not yet been settled with certainty. There is an added difficulty that the safe level will depend on the particular design of the experiment, and it is unfortunate that different workers have used different techniques in trying to establish the critical level. Russell and Martin claim that in nutrient solution, 10 μc P^{32} per litre may interfere with the growth of young barley plants.[29] The same authors have claimed that P^{32} produces variations in the ratio, soil phosphorus/fertilizer phosphorus, although the variations seemed quite irregular.[30] Bould *et al.*[31] reported effects on plant weight with as little as 5 μc P^{32} per pot, but failed to find any effect on the soil phosphorus/fertilizer phosphorus ratio with 300 μc P^{32} per pot! Blume[32] and Bould *et al.*[31] both concluded that in experiments in which activity levels were much greater than those normally used, the smallness of the radiation effects was the most startling feature of the experiment. Numerous field experi-

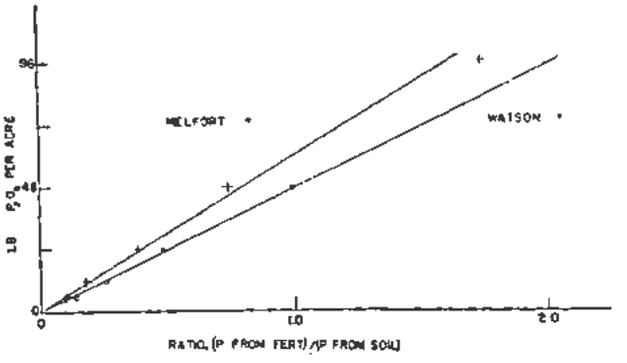


Figure 3. Ratio of fertilizer phosphorus to soil phosphorus in the plant as related to rate of application of phosphate fertilizer. Melfort and Watson, 1950

ments have failed to show any radiation effect on yields of fertilized crops grown with and without P^{32} up to quite high levels of P^{32} activity.[33,34,35]

In an elaborate series of greenhouse experiments conducted by Penner,[36] no deviations were found when radioactive $NH_4H_2PO_4$ was applied in granular form with specific activities from 0 to 3600 μc P^{32} per gram P^{31}. In particular, no alterations in the soil P/fertilizer P ratio occurred in any of the experiments even when the fertilizer was applied in solution. Small radiation effects on plant weight were thought to occur when the P^{32} was applied in solution at 12 μc per gram P^{31}, but an examination of Table 1 of reference[36] makes it questionable whether this effect was really due to radiation since 120 and 1200 μc P^{32} per gram P^{31} showed no effect, and even the 12 μc showed no effect in the ratio, soil P/fertilizer P, the per cent fertilizer absorbed, the total fertilizer absorbed, etc. Field experiments using 0 to 3600 μc P^{32} per gram P^{31} produced erratic results: at one location no effects were found for three harvest dates, whereas at another location (where there was, incidentally, a considerably smaller uptake of fertilizer) radiation effects were claimed at the second harvest, and even here the criteria showing an effect varied from time to time![37] A further field experiment at two locations and two harvest dates showed no effect in three of the trials, and the effect in the fourth was such as to be quite suspect—a slight decrease in fertilizer P uptake at 120 μc as compared to 12, 60, 240, and 720 μc per gram P^{31} (Table 7 in reference 37). While a good deal more work on radiation damage is desirable, it seems that activity levels in the usual field experiments, with less than 200 μc P^{32} per gram P^{31}, are low enough to avoid appreciable radiation damage.[38]

Diffusion of Ions in the Soil

Another factor which has to be considered in carrying out field tests is the amount of phosphorus which might be obtained by the plant from adjacent rows. In our experiments the row spacing is 15 cm, three rows being fertilized, only the fertilizer in the centre row being tagged. For ammonium phosphate, the maximum "piracy" occurring between rows was less than 1% of the applied phosphorus, indicating that this effect can be neglected in our experiments.

The actual movement of ions in soil is, of course, of considerable interest and can be studied using radioactive tracers (see, for example, reference 39).

Comments

Considering that the whole development in this field has taken place in the last decade, it is, perhaps, not surprising that there is still some discussion, at times acrimonious, over the exact interpretation of some of the results.[40,41] However, it cannot be denied that tagging provides the only means of determining the path of the fertilizer phosphorus as distinct from that of the soil phosphorus. It is also quite clear that the active research of the last nine or ten years has led to a very considerable increase in understanding of the complex relationship existing between plant and soil, and that this increase in understanding has not been entirely due to the effect described in Aesop's fable of the husbandman who, when he died, told his sons that he had left gold to them, buried in the ground.

NEUTRON MEASUREMENT OF SOIL MOISTURE

The amount of moisture in the soil is of obvious importance to plant growth as is also the problem of moisture movement in soils. Measuring changes in soil moisture in a given sample of soil involves using a non-destructive method of sampling, and heretofore no completely satisfactory method has been available. The neutron method seems to offer certain advantages over those previously suggested in that it is non-destructive, is adaptable to all types of soils, is independent of the state of the moisture (whether vapour, liquid or solid), is able to indicate rapid changes in moisture content, and is relatively independent of salt content of the soil. In the neutron method, fast neutrons emitted by a neutron source are scattered by the atoms in the soil, thereby losing their energy and becoming slow neutrons (apart from loss by capture). The slow neutrons are then measured by means of a slow-neutron detector such as an indium or rhodium foil or a BF_3 chamber. The fractional loss in energy when a neutron collides with an atom is at a maximum for hydrogen,[42] and thus soils with a high moisture content are particularly effective in slowing down neutrons. The activity induced in a slow-neutron detector is correspondingly high and is, in fact, proportional to the moisture content of the soil when standard conditions of neutron irradiation are adopted.[43,44,45,46] It can be shown theoretically that the distribution of slow neutrons about a point-source of fast neutrons is a function of the ratio of the slowing-down length to the diffusion length.[47] The slowing-down length is defined as the average distance a neutron must travel in a medium before it is slowed down to thermal velocity. It is made relatively small by hydrogen in comparison with other common elements in soil. The diffusion length is one-sixth the average distance from point of origin to point of capture of the neutron. It does not vary, with changing moisture content, as strongly as does the slowing-down length. The net effect is that if a slow-neutron detector is placed near a source of fast neutrons in soil, the activity will be largely a function of the hydrogen (or moisture) content of the soil.

Neutron Moisture Meter

The underlying principle and general experimental arrangement of the moisture meter is shown in Fig. 4, while mechanical details are given in Fig. 5. The apparatus consists of a probe head, *D*, Fig. 5, which is lowered into a 5-cm diameter vertical hole in the soil by means of an aluminum probe cylinder *A*. A 50-mc Ra-Be neutron source is then lowered into its seat using an electromagnet. A foil holder, *C*, containing indium foil is lowered into its seat by

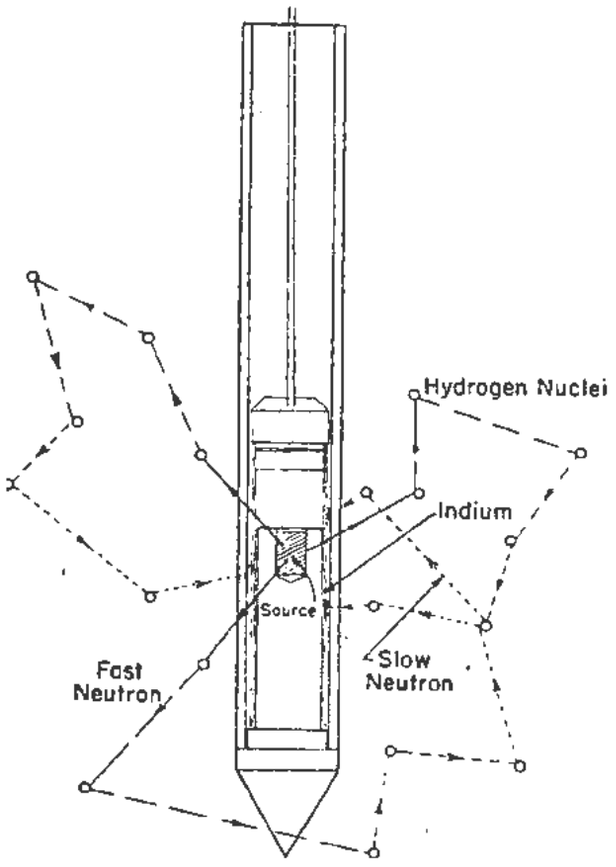


Figure 4. Diagrammatic sketch illustrating principle of the neutron moisture meter

means of a cord attached to the foil holder cap, *B*, thus placing the indium foil around the neutron source. After exposing the foil for a definite time (measured using a stopwatch), the foil in its holder is withdrawn and placed around the Geiger tube of a portable beta-gamma rate meter, which is read at a definite time after removing the foil from the neighbourhood of the source. After some experimentation, a 10-minute exposure followed by a 1-minute delay before measuring the activity was chosen as standard procedure.

Field Experiments

In a typical field experiment, a series of holes, six feet deep and two inches in diameter, were drilled at various levels on the banks of the South Saskatchewan River to give a variety of soil types, densities, and moisture contents. The soil types encountered in these holes ranged from sandy silts to medium plastic alluvial clays. The holes were drilled with a 5-cm auger and lined with 5-cm diameter aluminum pipe 0.125 cm thick. The depth of the source could be varied by adding lengths of aluminum tubing to the source holder and lowering it down to the desired depth in the hole. Samples of the soil were taken every 15 cm during excavation of the holes for determination of moisture content by the standard oven-drying method. At each depth, three readings for moisture content, using the rate meter, were averaged to give a mean value. This was plotted in Fig. 6 against the average measured moisture content in the 15 cm above and below the source, since the zone of influence of the source is primarily a sphere of about 15-cm radius. Figure 6 shows a reasonably linear relation between count rate and mass of water per unit volume. This curve was used as a standard calibration curve for subsequent tests. Figure 7 shows a comparison of moisture content determinations in a vertical hole on the campus of the University of Saskatchewan for the neutron meter and the usual oven-drying method. All determinations are within 3 per cent moisture, and most are within 2 per cent moisture or less.

Improved Neutron Moisture Meter

It has already been noted that the zone of influence of the neutrons is a sphere of about 15-cm radius, and that as a consequence the neutron moisture meter readings represent an average value for a sphere of about 15-cm radius, the average being weighted somewhat in favour of points close to the neutron source. For some agricultural purposes it is desirable to have a somewhat more detailed knowledge of the variation of moisture content with distance. After varying a number of the parameters involved, the arrangement illustrated in Fig. 8 was adopted.

The neutron detector now consists of a thin strip of rhodium foil, 2 mm wide and 66 mm long, bent

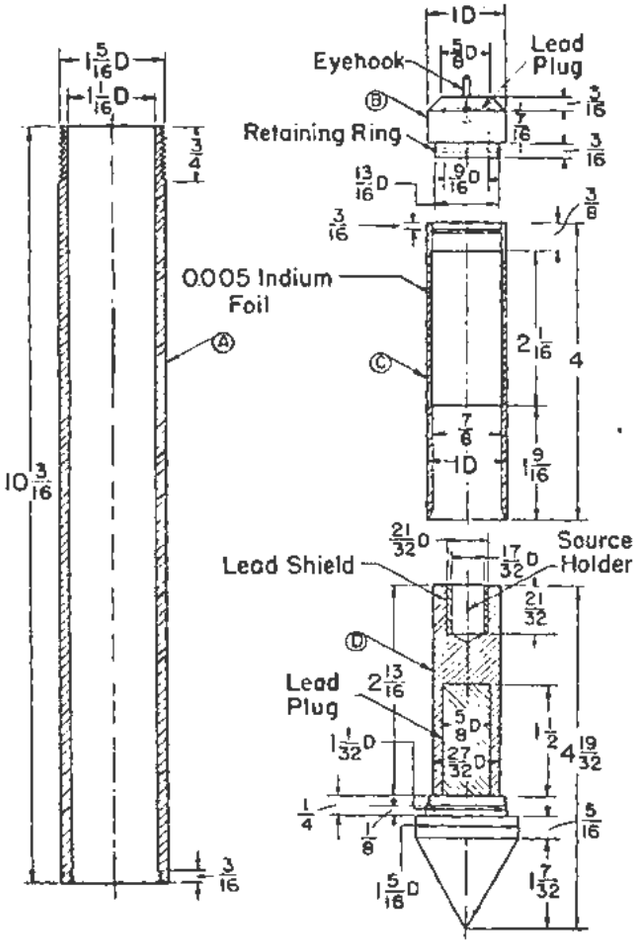


Figure 5. Moisture meter details

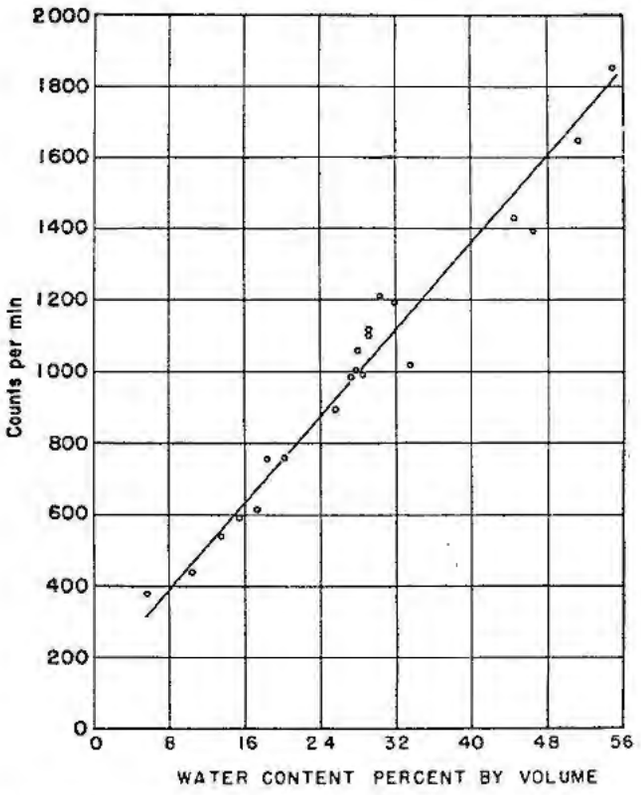


Figure 6. Calibration curve for neutron moisture meter using a 50 mc radium-beryllium source

into a ring and mounted in a lucite holder. During irradiation, the lucite holder is slipped over the neutron source so that the centre line of the rhodium foil is at the same elevation as the centre line of the neutron source. After 4 minutes' irradiation time, followed by a lapse of 30 seconds, the activity of the rhodium strip is measured by slipping it over a "thyrode" thin-walled Geiger counter tube (connected to a scaler) and counting it for 4 minutes. The neutron beam is collimated to some extent by using two shields, each consisting of a hollow Cd cylinder, 5 cm long and 4.75 cm in diameter, with a central hole 2.5 cm in diameter, the hollow cylinders being filled with paraffin. The Cd shields fit snugly onto the probe cylinder. It has been found that optimum results are obtained with a shield spacing of about 1 cm. The sensitivity of the device was tested using a simulated soil profile consisting of a 23-cm layer of dry sand topped by a 12.5-cm layer of paraffin wax, the whole being contained in a box of rectangular cross section (25-cm square). A 5-cm diameter vertical aluminum tube down the axis of the box made it possible to take meter readings at various positions with respect to the sand-wax interface. The results are recorded graphically in Fig. 9. They indicate that the modification does effect an improvement, but that the device could probably be improved still further, possibly by using a somewhat larger diameter cadmium shield. It has been suggested by Gueron[48] that a further improvement can be obtained by measuring the epithermal neutrons, using indium foil sandwiched between two pieces of cadmium foil. By measuring the neutrons in process of being slowed down, the effects of capture of thermal neutrons by various elements in the soil can be avoided.

TRACING AGRICULTURAL INSECTS AND PESTS

Radioisotopes have also been used extensively to study agricultural pests with respect to insect dispersal and behaviour, food relations, disease transmission, mode of action of insecticides, including systemics, dispersal of insecticides and deterioration of insecticides in the soil. (For a general reference, see reference 49.)

In tracer studies of insect dispersal and behaviour, insects or groups of insects are tagged with a suitable radioactive tracer and then followed after release using a radiation detector. Among the desirable characteristics of the tracer are: (*i*) ease of application, (*ii*) minimal effect on the insect, (*iii*) ease of recognition of the tag, (*iv*) persistence, (*v*) suitable half-life, and (*vi*) availability. These points will be illustrated by reference to tagging experiments with mosquitoes, blackflies, grasshoppers, wireworms and cutworms.

Dispersal Studies of Insects

Mosquitoes

During the last few years, experiments have been reported in which highly radioactive mosquitoes were reared from larvae left in a solution of radioactive

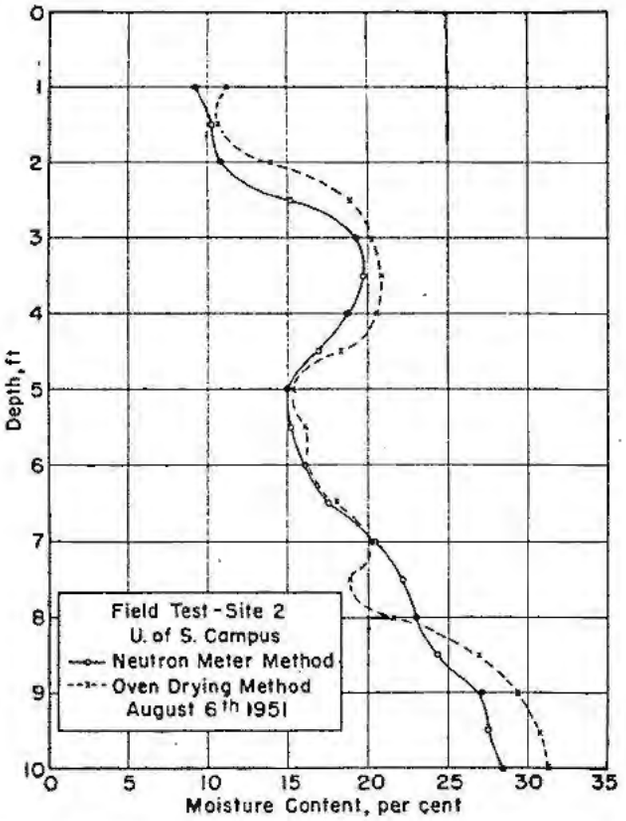


Figure 7. Moisture profile comparison between standard oven-drying method and neutron meter

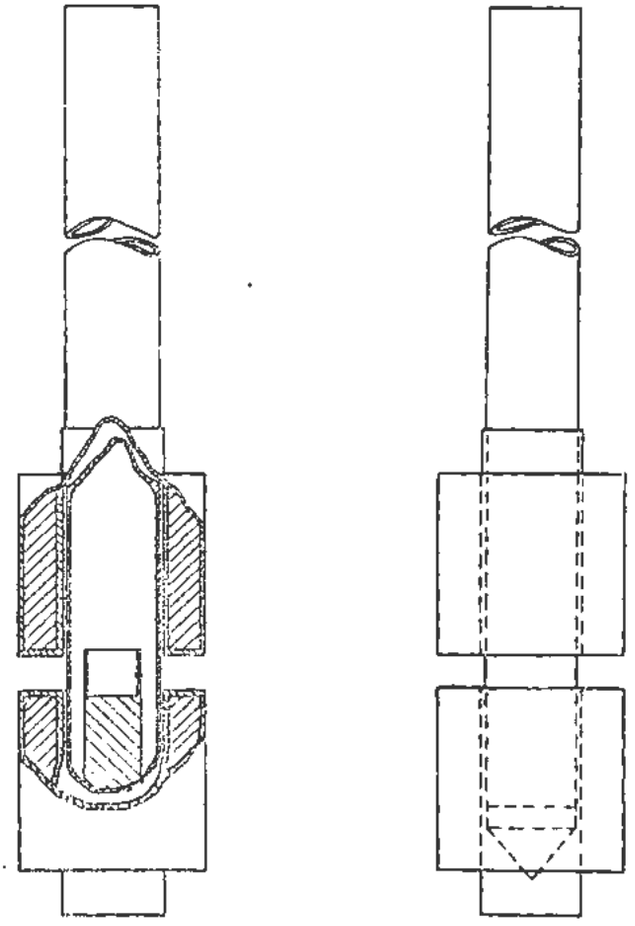


Figure 8. Modified moisture meter with cadmium shields

phosphorus.[50,51] Subsequent field experiments in Saskatchewan have shown that large numbers of adult mosquitoes can be economically tagged by keeping larvae at the fourth instar stage of development in a 0.1 μc per ml solution of P^{32} (in the form of PO_4^{---}) for 24 hours at a density of 1 larva per ml, and then allowing the tagged larvae to complete development to the adult stage in their original or normal habitat. In a typical experiment, about 45,000 larvae were placed in 45 litres of pond water in a tub, together with 4.5 mc P^{32} as H_3PO_4. After 24 hours the larvae were placed in the pond and a further batch of larvae added to the tub together with a sufficient quantity of P^{32} to maintain the level of P^{32} activity. Approximately 450,000 larvae were tagged in this way. In the following month about 500,000 mosquitoes were caught in the neighbourhood of the pond. Radioactive mosquitoes were found as far as 7 miles from the release point, but the majority were within one-eighth of a mile of the point of release. A total of 84 radioactive mosquitoes, 19 flies, 3 parasites and 1 leaf hopper were found at various points. It was found that larvae lost 50% of the absorbed activity within 2 days after removal from the tagging solution, but after that the loss was relatively slow. The average retention of activity through to the adult stage was about 15%.

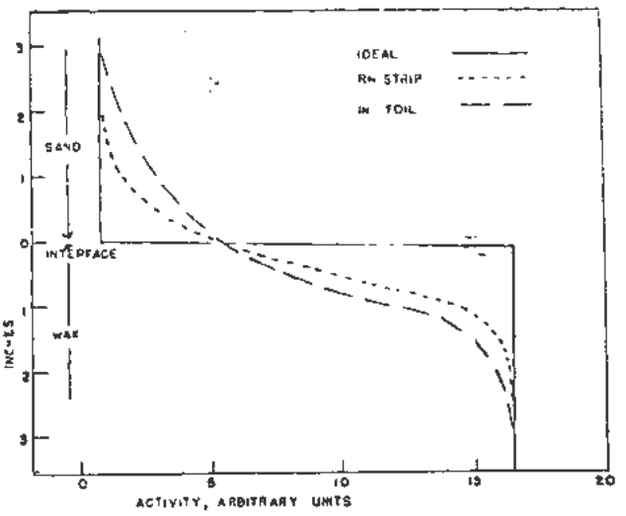


Figure 9. Variation of induced activity in meter with respect to distance in inches relative to wax-sand interface as zero

Control experiments indicated that larval mortality was no greater with 0.1 μc P^{32} per ml than with inactive media. For the first 60 hours, the uptake of radioactivity is proportional to the time of exposure to the P^{32} solution. There is also a very strong dependence of absorption on temperature.[52,53]

Blackflies

The method successfully used to tag mosquitoes had to be modified somewhat for application to the blackfly. In laboratory experiments, blackfly larvae were reared in 5-litre jars containing water circulated and aerated by compressed air. As before, P^{32} was in the form of H_3PO_4. It was found that from 2 to 4 larvae per ml could be kept in a solution at 0.2 μc P^{32} per ml without excessive mortality and that an adequate amount of activity was absorbed in 24 hours. After transferring the larvae to an inactive solution, there was a relatively large loss of radioactivity in the first two days, but the loss thereafter was relatively small.[54] Field tests were made in the Torch and Battle Rivers in Saskatchewan in 1950 and 1951, the larvae being tagged in a tub aerated with a stream-driven paddle wheel. As before, the same solution was used to tag successive batches of larvae, the level of activity being brought up to 0.2 μc per ml by addition of P^{32} as necessary.

On being released into the stream, the tagged larvae attached themselves to rocks and leaves in the stream. They were detected as far as 520 yards down stream from the point of release. The recovery of tagged blackflies was extremely poor, mainly, it is thought, due to inadequacies in collecting methods. In one experiment 300,000 larvae were tagged. Subsequently, 16,000 adults were caught in the neighbourhood, only 1 being tagged. In another similar experiment, 19 tagged predators were recovered. It is believed, however, that the method for mass tagging is basically sound and that it could be used, with minor modifications, in similar studies of stream-inhabiting insects.

Grasshoppers

In a grasshopper dispersal study, 20,000 grasshoppers (nymphs and adults) were allowed to feed on wheat seedlings 6 inches high, in a cage of 4 ft^2 area of cross section for several hours. The wheat seedlings had been sprayed with 0.5 mc P^{32} in 50 ml solution. About 14% of the activity was taken up and retained by the grasshoppers. The loss in activity by the grasshoppers was high for the first few days, but was very small thereafter. Loss of activity through moulting was negligible, and survival was normal.[55] The method was used to measure the dispersal of nymphs from the second instar and adults of *Camnula pellucida* (Scudd) and *Melanoplus mexicanus mexicanus* (Sauss). When released on bare cultivated fields, they showed no ability to orient themselves and move toward a food supply. The average rate of movement at 70°F was about 7 yd per hour. In these particular experiments, the position after 7 days corresponded to random movement plus a response to wind direction.[56]

Behaviour of Wireworms and Cutworms

In making behaviour studies, it is necessary to tag individual specimens. Where the species is of the soil burrowing type, the tag must be a gamma emitter in order for it to be detected through several inches of soil. The feeding of activity to the animal has its limitations since any material excreted will contaminate the soil and make it difficult to follow. In studies of wireworm movement, this difficulty was avoided by sticking a minute piece of radioactive cobalt wire to the wireworm with vinylite plastic. Once tagged, the larva may be followed by using a Geiger probe connected to a rate meter. By moving the Geiger probe over the soil surface and determining the position of maximum counting rate, the wireworm is localized in the horizontal plane. By previously calibrating the instrument for varying soil depths, the position in a vertical plane is also determined. About 20 μc Co^{60} per tag is a suitable amount of activity. No harmful effects are observed over some months, and the long half-life of Co^{60} (5.3 yr) minimizes corrections for radioactive decay. The external tag is, of course, lost at each moult. This difficulty is avoided by tagging the wireworm internally. Successful insertions of cobalt wire have been made in both wireworms and cutworms. No apparent abnormality or loss of movement was evident up to three months after treatment. When using highly active cobalt wire, it was found that the body fluids of the wireworm slowly attacked the wire so that activity was excreted by the insect. This difficulty could probably be avoided by using gold-plated Co wire.

Tagged insects have been used to measure the response of the prairie grain wireworm to moisture, food and temperature, and information on the rate of movement has been obtained. Radioactive larvae of the red-backed cutworm have been used to determine the fate and underground activity of larvae placed in open cages utilized for chemical control studies and observation on habits of the larvae.[57] *Ctenicera destructor* larvae moved very quickly at temperatures above 90°F, a little slower between 80 and 90°F, and were found to prefer temperatures in the range 72 to 80°F.

C. destructor larvae were observed to avoid dry soil when offered a choice of moist or dry. However, larvae sometimes entered the dry soil region when food was placed there. For the types of food tried, the movement of larvae to food was found to be a random process.

A great deal of information on the rate and extent of movement was obtained. The larvae were capable of moving several yards in the space of a few days. A maximum rate of 12.5 cm in 3 minutes was observed, although the usual rate was much slower.

Other observations have been made on cannibalism in wireworms and the eating of eggs of other insect pests (e.g., grasshopper eggs).

Automatic Plotting of Position of Tagged Insect

It has been suggested from time to time that an automatic device for continuously following and recording the position of a suitable tagged insect would be of some value.[58] Tracking or following continuously requires, first of all, a sensitive head and recorder which will follow the insect's movements. The sensitive head must be such that when it is not directly over the insect, a definite type of error signal is produced. The error signal is amplified and the amplified signal applied to a servo mechanism which causes the head to move in such a way that the error signal is minimized by restoring the head to a position directly over the insect. The movable parts must be carried on a suitable movable framework. If the surface over which the follower operates is reasonably smooth and solid, the movable framework can consist of a cart or trolley mounted on wheels. Here again considerable mechanical variation is possible. One of the simplest schemes is to have two main coaxial traction wheels capable of independent rotation in both forward and reverse directions, the third point of support for the framework being merely a caster. The path taken by the insect could be marked directly on the surface traversed or recorded remotely, using a suitable remote recording device.

In the present apparatus, the sensing element is a "thyrode" tube (Victoreen Co. 1B85) which is rotated about a vertical axis at the end of a fixed arm. The axis of the tube lies in a plane parallel to the operating surface (soil). The axis is tangential to the circumference of a circle lying in this plane and about whose centre the arm supporting the tube rotates (Fig. 10).

If a radioactive source lies on the axis of revolution, the distance between the source and the Geiger tube will not vary during a revolution, and the counting rate of the Geiger tube per unit of revolution will be "constant" except for variations arising

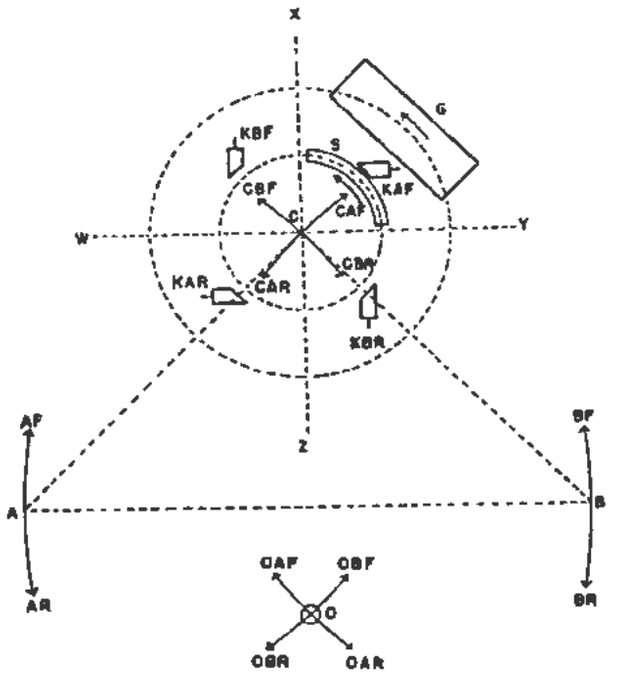


Figure 10. Schematic diagram for interaction of sensing element and carriage unit of automatic following device

from the usual statistical fluctuations associated with radioactive disintegration and background.

If, however, the source does not lie on the axis of revolution, the distance between the tube and source will vary during a revolution. The count rate generated per unit angle of revolution will not then be "constant" during a revolution and will tend to be higher in those areas of revolution where the Geiger tube is nearest the radioactive source. This variation, which is superimposed on the usual background, can be used to cause the carriage to reposition the sensing unit so as to bring the axis of revolution closer to the radioactive source. Shielding may be used to intensify these variations.

In the present model of the following unit, the Geiger pulses for each quadrant of revolution are separated by mechanical commutation. These pulses, in effect, are stored temporarily in order to develop voltages which are a function of the average count rate for each quadrant. The voltages resulting from collections from opposite quadrants are balanced against each other. These two different voltages are used to control the carriage movements (Fig. 11).

The carriage chosen for the present tests involves a "free" unit which is capable of a crab-like motion. This machine is free to move over a horizontal area limited only by the boundaries of a suitable operating surface and the length of the attached electrical cable.

There appear to be at least two possible "crab" carriage mechanisms which can utilize the error information provided by the sensing element for explicit position correction. In the one chosen, the axis of revolution of the Geiger tube holder passes through the vertex of a triangle which is isosceles and approximately right-angled, the axis of revolution being at right angles to the plane of the triangle.

This triangle lies in a plane approximately parallel to the plane of revolution of the Geiger and in or near the operating surface. Tractive effort produced by reversible electric motors is applied at each end of the base of this triangle. The direction of application of this effort is approximately at right angles to the base of this triangle. Most of the weight of the unit is distributed equally on the traction members. The remaining weight is carried by a small polished surface (coaster) which can glide freely to any position.

Small movements by either tractor result in the machine pivoting about the opposite stationary traction point. The vertex of the triangle describes a small arc of a circle for movement of either traction unit. The arcs meet approximately at right angles. The quadrants of collection for pulses from the moving Geiger tube are approximately bisected by these arcs (Fig. 10).

The motors which produce the tractive effort may operate in either direction and may operate singly or simultaneously. If the source position does not lie on or close to the axis of Geiger revolution, error signals develop and the appropriate corrective action by one or both motors is initiated. This method of steering has proved "dodge" free.

There are many general methods of converting the measured error signals into corrective tractive efforts, and for each of these general methods there are many possible variations in the arrangement of electronic and electrical components. The electrical circuits were selected mainly on the basis of availability of components. The model in its present form is not the ultimate in performance or simplicity, but it does demonstrate that a simple, economical and practical unit is possible, and its present performance is equal to expectations.

Control in the model is of the "on-off" type, and traction is supplied by relay-controlled series-wound electric motors. The relays are driven from the error signal voltages by dc amplifiers using hard vacuum tubes. The error signal voltages are obtained by amplifying, lengthening, storing, and balancing the Geiger pulses. Control of hunting was obtained by limiting the speed of correction. One method used was to limit position correction to small "parcels". These "parcels" were approximately half the size of the error which could be detected. The application of these "parcels" was followed by a forced inoperative interval. This interval allowed the storage and balancing circuits to readjust and accumulate reliable error data as a basis for each succeeding parcel of correction.

All translatory movements of the axis of Geiger revolution are recorded on the operating surface by a stylus which is almost coincident with the axis.

A record of the time at which the machine and active source were in a given position is obtained from a clock-driven printing wheel carrying suitable marking symbols, representing the time, on its peri-

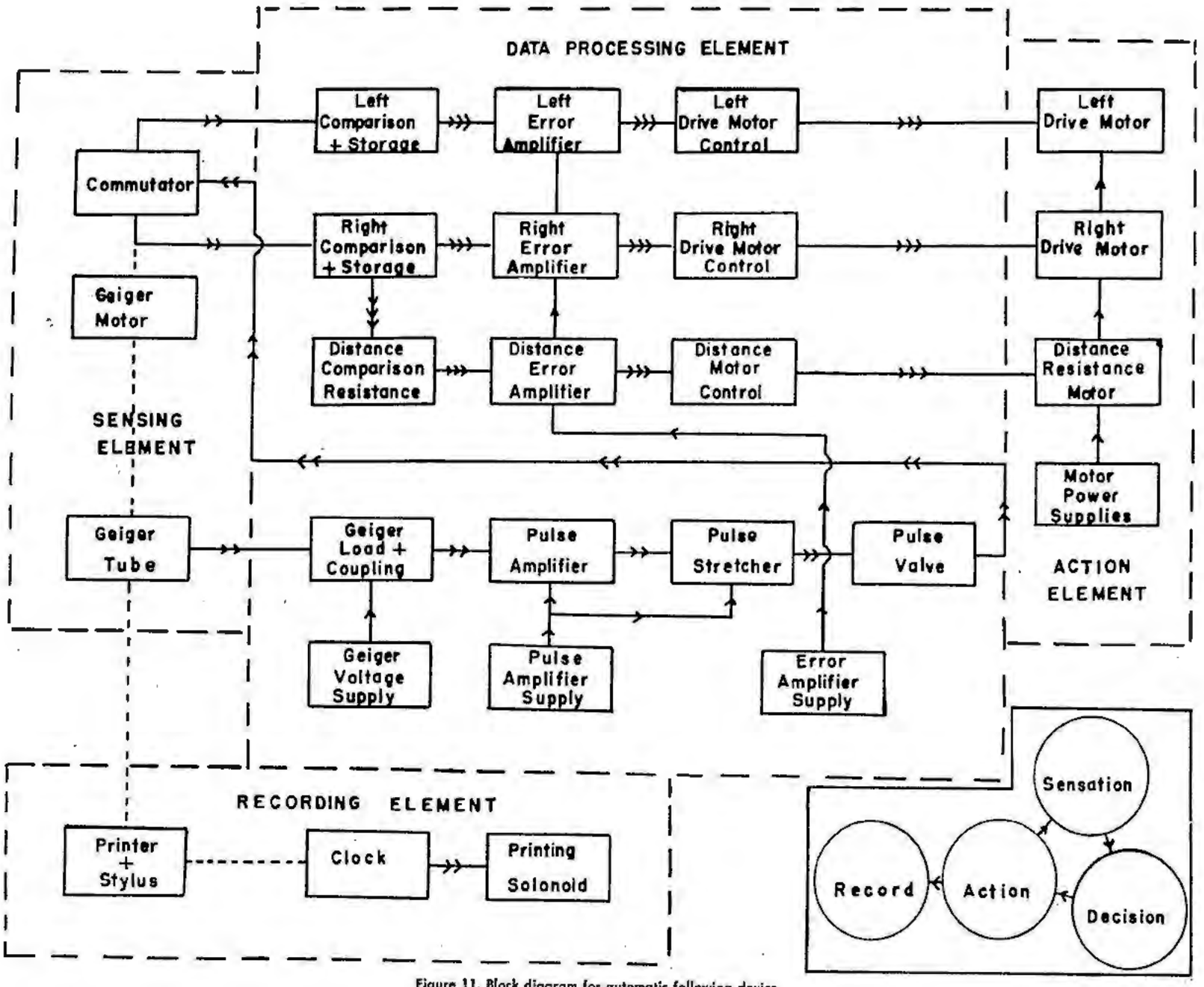


Figure 11. Block diagram for automatic following device

phery. At chosen intervals this wheel is inked and momentarily pressed against the operating surface. The printer is presently activated by a solenoid controlled by the clock through a switch, a sensitive relay, and a slowly charged condenser.

A record of the distance of the radioactive source from the operating surface is also available. This is obtained by measuring the mean of one pair of the voltages collected for position error determination. The magnitude of these mean voltages depends on the distance of the radioactive source from the operating surface. It is measured with a self-balancing bridge. The collected voltage is compared with the voltage across the slider and ground end of a potentiometer by a third dc amplifier. If these voltages are not in close agreement, a relay-controlled meter repositions the potentiometer slider so as to obtain agreement. Rotation of this potentiometer shaft moves a crank which changes the distance between the time printing wheel and a similar and angularly synchronized distance printing wheel. The printing operation, therefore, produces two identical characters whose character indicates time, and whose separation is a measure of the distance of the radioactive source from the operating surface.

The apparatus described has been built and is able to home onto a wireworm tagged with 20 microcuries of Co^{60} from a distance of about 40 cm. It is able to follow such a tagged wireworm through 10 cm of soil, with an average position error of about one-half cm. It is capable of continuous operation at the usual speed for wireworms, a few centimetres per hour. It can, if necessary, travel much faster than this.

The principle of a rotating Geiger would seem to be capable of numerous other applications.

USE OF ISOTOPES IN CANADIAN SILVICULTURAL RESEARCH

Plant Nutrients

Although the translocation and ultimate disposition of nutrients absorbed by plant roots have long been of interest to plant physiologists, there are still differences of opinion as to the mechanism of transfer, the path of movement and the functions of the different nutrients in the metabolic activities of the plant. Radioactive tracers have been used in investigating a variety of such problems related to silviculture. One of the earliest of these studies concerned the movement of phosphate in trees.[59]

A recent Canadian study describes the location of radiophosphorus in various parts of red pine seedlings after being absorbed from the surrounding soil[60] by a part of the root system. The most noticeable feature observed was the uneven distribution of the radioactivity in the active plant. Often the activity would be concentrated in one of the branches, suggesting that certain roots provide nutrients to certain branches. Phosphorus was accumulated in the base of the leaf where the leaf meristem is located. Phosphorus is generally thought to concentrate in meristematic regions.

In other studies, radioisotopes such as Ca^{45} and Rb^{86} were injected into the trunks of yellow birch and white pine.[61] The movement of the radioisotope was followed using a newly developed portable scintillation counter. The maximum rate of upward movement of the Rb^{86} in the xylem of yellow birch approximates 30 cm per minute along a narrow channel spiralling upwards (usually dextrally) from the point where the isotope was first introduced. Movement in decadent yellow birch was very slow with an apparent increase of permeability of the bark tissue as indicated by lateral diffusion of the isotope. In October no upward movement was discerned in healthy trees, but rather an active downward translocation in the phloem.

Radioarsenic, As^{76}, has been used in debarking studies of the Douglas Fir.[62] The initial rate of rise of arsenic in the tree was 120 cm per hour. The arsenic is absorbed immediately by the sapwood, rises through the outer layers of the wood and apparently diffuses into the bark.

Other tracer studies of interest to silviculture are the use of C^{14} and P^{32} in uptake studies of stomatal cells and parasitized leaves.[63,64]

Plant Pests

Radioisotopes can obviously be used to study forest insect pests. In one such Canadian study,[65] the white-pine weevil was tagged with Co^{60} as nitrate dissolved in cellulose acetate and acetone to form an adhesive. The Co^{60} was applied to the elytra and the tagged weevils were then released in a white pine plantation. Sixty-four were tagged with 0.2 to 0.5 mc each, and after two months, 21 were still alive and tagged. This is about the usual percentage survival for untagged specimens under similar conditions, but it was thought that a smaller amount of radioactivity might have been better since in the following spring only 19% emerged as compared to 56% in a control group. The tagged insects could be detected from a distance of about 2.75 metres.

Other tracer studies are in progress in Canada on the use of radioactive carbon in metabolic studies of forest insect viruses.[66]

Radioactive carbon has also been used in studies of the action of DDT on houseflies,[67,68,69] while radioarsenic has been used to tag mealworm larvae and tomato hornworms.[70]

CONCLUSION

While this paper has been restricted to a consideration of applications of radioisotopes to studies of plant nutrients and pests in agriculture and silviculture, numerous other biological applications have been made in Canada. These cover such diverse topics as radiation-induced mutations in drosophila and barley, cobalt deficiency in sheep, phosphorus and calcium metabolism in hens, and the mode of action of the anti-blood-clotting agent, dicumarol. All in all, we can say that Canada is pursuing these

studies actively, and that her atomic scientists are trying to live up to the ideals expressed in Swift's "Brobdingnag": "He gave it for his opinion that whoever could make two ears of corn, or two blades of grass to grow upon a spot of land where only one grew before would deserve better of mankind and do more essential service to his country, than the whole race of politicians put together."

ACKNOWLEDGEMENTS

We wish to thank the Engineering Institute of Canada and the Agricultural Institute of Canada for permission to reproduce some of the figures. Much of the work described was supported financially by the Dominion Department of Agriculture and the National Research Council of Canada.

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Studies on Plant Nutrition, Fertilizer and Soil by the Use of Radioisotopes

By S. Mitsui,* Japan

In Japan studies were started about fifteen years ago by Y. Nishina by the use of minor quantities of artificial radioisotopes supplied by cyclotron. The studies reported by G. Hevesy *et al.*, which were among the first to suggest the possible use of radioactive phosphorus in the study of plant nutrition, undoubtedly stimulated this work. Unfortunately, however, the investigations were discontinued during the war and not started again until the importation of radioisotopes mass produced in the USA began in 1949. Though importation was limited, the use of radioisotopes has since been expanded to a considerable extent for fundamental research on plant nutrition, fertilizer and soil. The programs have naturally been directed principally toward the solution of the agricultural problems peculiar to Japan, e.g. those related to lowland rice, mulberry trees and water-logged soil. Large-scale field experiments using radioisotopes will be left to the future.

S. Mitsui *et al.* have studied the movement of P^{32} into, within, and out of the rice plant. By the divided root technique with water culture they could confirm downward movement of P^{32} simultaneous with the upward movement of non-labelled P in the divided root. Moreover, a minor loss of P^{32} from the root indicated almost no possibility of anionic exchange, as generally is the case with cations. Further, S. Mitsui *et al.* suggested the existing form of Ca^{45} in the normally metabolizing wheat and barley roots. Ca^{45} was depleted by K, NH_4, Rb, Mg, Ca, Sr and Ba as well, much more slightly by Na and Li and almost none by H_2O. This was in marked contrast to K^{42} which was reported to be depleted exclusively by K. It was concluded that the status of Ca held in the metabolizing plant root would be a more or less loose bonding as compared with K and still a little stronger than Na, its linkage being almost, if not entirely, independent of normal metabolism.

In a series of studies on the effect of respiration inhibitors such as H_2S, NaCN and NaN_3, S. Mitsui *et al.* confirmed a simultaneous loss and uptake of phosphoric acid by rice root by the use of P^{32}, the net absorption being negative in a comparatively high concentration of NaN_3.

H. Okajima *et al.* studied the uptake and translocation of P^{32} by rice plants as influenced by H_2S. Hydrogen sulfide is considered as one of the principal causes of unproductive soil for lowland rice associated with heavy application of sulfuric fertilizers. With increased concentration of H_2S, the uptake of P^{32} gradually decreased and the isotope even exuded out of the root, confirming the above-mentioned work of S. Mitsui *et al.* Moreover, the P^{32} applied through leaves almost stopped moving downward from those leaves, indicating the possible inhibition of a certain metabolic activity by the hydrogen sulfide moving up the stem from the roots.

Foliar absorption of P^{32} by rice, mulberry tree and other crop plants was studied in connection with application of urea to the leaves. T. Ushioda observed that P^{32} applied through leaves was absorbed and translocated to other parts of mulberry trees much faster than the soil dressing. The intake was faster when the treated leaves were young, and differed according to the accompanying cations in the order $NH4 > K \doteqdot Na > Ca$, corresponding to the lyotropic series of these cations. Radioautograms of wheat and tomato leaves treated with P^{32} were taken by M. Yatazawa. Further, he confirmed that P^{32} absorbed through leaves was rapidly transformed into various organic phosphorus compounds, such as sugar phosphates, nucleic acids and lipoids. The remarkable acceleration by various sugars led him into some discussion of the mechanism involved in the foliage uptake of P^{32}.

S. Mitsui *et al.* proposed a contact solutional uptake of P^{32} in a water-insoluble but 2% citric acid-soluble phosphate fertilizer, fused magnesium phosphate, by a dynamic interaction among acidoidal plant root—base unsaturated soil colloid—fertilizer particle. The availability of phosphoric acid increased remarkably, in some cases seventy per cent, over control, by mixing it with acid soil. But the excess retarded it again. On the other hand, the availability of a water-soluble phosphate fertilizer, superphosphate of lime, gradually decreased as the amount of mixed acid soil increased, without showing any maximum peak of availability. The hypothetical inference was confirmed by a series of model experiments in the laboratory.

K. Ohira *et al.* studied the uptake of Fe^{59} and P^{32} by rice plants in the various nutritional states with respect to iron and manganese. The absorption and translocation of P^{32} was high in the iron-deficient

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plants. The uptake of Fe^{59} was rapid in the iron-deficient plants and the translocation of iron in the plant body appeared to be in antagonism with manganese.

The absorption and translocation of P^{32}, Sr^{89}, S^{35}, Zn^{65} by rice plants were studied by M. Yatazawa. The top dressing of nitrogeneous fertilizers definitely increased the uptake of P^{32}. A. Okuda *et al.* also studied the absorption and translocation of P^{32}, S^{35}, Ca^{45}, Zn^{65} by wheat, straight bean, rape and sesame.

The general trend differed according to time of application and to previous nutritional status. K. Nishida *et al.* detected the absorption and translocation of P^{32} applied in the leaf-pitcher of *Sarracenia*. P^{32} moved very slightly to the upper younger leaf-pitchers. The same author also studied the uptake and translocation of Sr^{90} either by foliage or root application to kidney bean. S. Aso noticed a considerable difference in the translocation and distribution of Ca^{45} and P^{32} applied from the root of soy-bean plant. Ca^{45} was distribnted rather evenly through the whole plant body in contrast to the remarkable accumulation of P^{32} in the younger organs such as buds and root tips.

N. Yamada *et al.* observed a considerable uptake of P^{32} by the young pods of the peanut. The uptake progressed parallel by pods and roots after flowering, indicating that a suitable improvement of fertilizer placement is desirable for peanut cultivation.

A moderate-scale field experiment with P^{32} applied in a water-soluble form for mulberry trees was conducted by T. Ushioda. The movement of P^{32} in the soil was extremely slow, amounting to only about 5 cm in 21 days in a sandy soil. Most of the applied P^{32} was transformed into insoluble phosphate, only seven per cent remaining in water-soluble state after 21 days. The movement was clearly demonstrated by an autoradiograph. In conclusion, he suggested that the phosphate fertilizer, even in a water-soluble form, should be applied as near as possible to the root system.

T. Egawa *et al.* studied leaching of $P^{32}O_4$ through columns of soil. In case of a highly degraded paddy soil the leaching was remarkable as compared with a normal paddy soil, as logically expected on the basis of the extreme deficiency of active iron in the former soil. Further, an isoionic exchange of preliminary fixed $P^{32}O_4$ by soil with inactive phosphate in the external solution was reported by the same authors. The isoionic exchange of phosphate was much more remarkable for an alluvial soil than a volcanic ash soil, the latter having a much greater ability to fix phosphate. These workers suggested that the earlier and rapid part of exchange was possibly due to an ion exchange explicable by Donnan membrane equilibrium, and the later slow part of exchange to a chemical reaction. The elevation of temperature considerably promoted the exchange, but the soil drying prior to exchange remarkably retarded it, indicating that the principal mechanism involved must necessarily have been of a chemical nature.

Possible sources of error in handling radioisotopes were reviewed by S. Mitsui *et al.* In diluting $H_3P^{32}O_4$ samples of extremely high specific activity, an addition of at least 0.5 ppm of inert PO_4 as carrier was necessary to avoid surface absorption of P^{32} on glassware. Further, despite the high maximum energy of β-ray emitted from P^{32}, 3 and 5% self-absorption errors of plant ash could not be avoided with 20 and 30 mg ash per square centimeter respectively.

Y. Yamada compared various methods of eliminating radiation from natural radioactive potassium in the measurement of radioactivity of agricultural substances. Several chemical methods to separate radioactive potassium were always inadequate, giving results 20–30 per cent too low. The best and most logical method was to independently subtract the natural counts of potassium as calculated from the potassium content of the material from the raw original values obtained.

Applications of Radioisotopes to the Study of Soils and Fertilizers: a Review

By L. A. Dean,* USA

The theoretical and applied aspects of soil-plant relationships, soil fertility and fertilizer use have been under consideration for many years. The application of radioisotopes to these fields of study, although relatively new, has done much to advance our knowledge in these areas, and this trend will undoubtedly continue. During the past 15 years about 300 titles have appeared in the scientific literature which relate to the application of radioisotopes in the study of soils and fertilizers. The discussion presented here will review most of these studies in an attempt to evaluate progress resulting from the application of radioisotopes. A number of reviews and general discussions dealing with isotope usage in studies of soils and fertilizers have preceded this consideration of the subject. The reader is referred to the articles by Stout *et al.*,[122] Comar and Neller,[24] Parker,[98] Collier,[23] Low,[77] Hendricks and Dean,[53] Blume,[11] Fried,[38] Deribère,[29] Schuffelen,[104] Herbst[55] and Behrens.[9]

The majority of the investigations which have utilized radioisotopes has dealt with some phase of the phosphorus problem. In addition to the relative importance of this problem the isotope P^{32} has a number of desirable properties from the standpoint of an experimenter. Among these are its relatively short half-life and strong beta particle of 1.71 Mev which tend to minimize disposal and measurement problems. However, as experience and facilities have developed, research projects involving a number of isotopes have become relatively common; these include Ca^{45}, S^{35}, Zn^{65}, K^{42}, C^{14} and others.

The employment of radioisotopes allows the undertaking of experiments and measurements where ordinary chemical and physical measurements are not practicable. Two underlying principles are involved. Populations of ions or molecules can be labeled and readily detected, traced, or a quantitative determination made of dilution by change in specific activity. Secondly, ready identification and measurement at extremely low concentrations is possible. Radioisotopes have been used to advantage in a variety of investigations including ion mobility in soils, distribution and growth of roots, uptake and exchange phenomena, plant nutrition and in certain problems of analytical chemistry involving the soil system.

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ION MOBILITY IN SOILS

Radioisotopes are convenient tools for studying the movement of elements in the soil. Their use for this purpose seems to have been largely neglected. Substances move through soils by diffusion and as the result of mass flow of the soil solution. On the other hand, various processes may restrict mobility. Of primary interest is the movement of the constituents of soil additives such as liming and fertilizer materials.

The earliest report on the use of radioisotopes to study movement in soils was that of Henderson and Jones[52] in 1941. Monocalcium phosphate labeled with P^{32} was placed on the surface of soils and water applied to simulate 2.5 inches of rain. Movement of phosphorus varied from 1.25 inches in a Cecil clay to 4 inches in a Crosby silt loam. Not more than 5 per cent of a potassium application labeled with K^{42} moved over 1.6 inches.

The movement of phosphate ions away from the site where they are introduced into a soil is limited by the phosphate fixation processes. Ordinary chemical methods do not permit a study of this movement in adequate detail because of the relatively large quantity of phosphorus already present in most soils. Through the use of radiophosphorus it has been possible to trace the movement of phosphatic fertilizers under actual field conditions. Ulrich *et al.*[125] added 2300 lb P_2O_5/acre of P^{32} labeled phosphoric acid to a soil in conjunction with 4 inches of irrigation water. The phosphorus penetrated about 12 inches and the water about 20. After 11 and 43 days, 99 and 86 per cent respectively of this phosphorus was in the first 6 inches. Olsen, *et al.*[89] compared the distribution of band placed superphosphates with that of phosphoric acid applied in irrigation water. The movement of phosphorus from the superphosphate was much less than for the liquid phosphoric acid. Very little superphosphate moved beyond 3 inches. The phosphoric acid moved to a depth of 12 inches, but 85 per cent of the total application was found in the top 4 inches. Increasing increments of irrigation water were shown by Jordan *et al.*[66] to increase the downward movement of broadcast applications of superphosphate. In another field study Fiskell *et al.*[35] showed that only 2.5 to 5.2 per cent of the superphosphate top-dressed on permanent pasture moved more than one inch.

The dispersion of salt solutions labeled with P^{32} injected in the soil was studied by Lecrenier *et al.*[75] and graphs were prepared for the distribution in different soils. Rainfall did not appear to affect the distribution of the P^{32}. Egawa *et al.*[33] and Herbst[54] have also studied the leaching of phosphate fertilizers. Procedures have been suggested by Johnston[65] for applying radioautographic techniques to soil sections in the study of fertilizer phosphorus movement.

The foregoing discussion has dealt with the movement of fertilizer phosphorus under the influence of the penetration of rain and irrigation waters. The dissolution and migration in the absence of mass flow have also been studied. Heslep and Black[56] investigated the one-dimensional diffusion of phosphorus from solid phosphatic fertilizer into the adjacent soil by experiments employing fertilizers tagged with P^{32}. The extent of diffusion of different fertilizer materials increased with the fraction of the fertilizer phosphorus that was in water-soluble form. The distance of diffusion increased with time and rate of phosphorus application. In 4 weeks the fertilizer phosphorus diffused only 3 to 4 cm. Under otherwise uniform conditions, diffusion of fertilizer phosphorus differed between soils and was considerably less in calcareous soils than in acid soils. The rapid dissolution of phosphorus from granular superphosphate in contact with moist soil was experimentally verified by Lawton and Vomocil.[72] Even in soils as low as 2 to 4 per cent moisture, 20 to 50 per cent of the phosphorus moved from the granules to the soil in one day. Bouldin and Black[18] tested the validity of activity measurements as estimates of phosphorus diffusion from tagged phosphate sources. Although significant changes in the apparent specific activity of diffusing phosphorus were found, the over-all picture of phosphorus diffusion was not substantially different from that found by total phosphorus analysis. Irregularities in phosphorus distribution by diffusion were found and the possibility of periodic precipitation was postulated.

The leaching of calcium in soils and its relation to problems of soil acidity has long been investigated. Ririe *et al.*[99] and Blume[12] performed laboratory experiments dealing with the movement of calcium from Ca^{45}-labeled compounds under severe leaching conditions. Their findings were essentially the same. Apparently calcium moves downward in the soil by a continuous series of displacements, the calcium appearing in the leachate being that originally present in the lower layers of the soil. Lysimeter studies using Ca^{45}-labeled calcium carbonate and a calcium silicate slag were conducted by Harris *et al.*[50,51] and Davis *et al.*[25] Varying rates were used and the additive calcium compounds were mixed throughout the soil mass. The leachates were examined for Ca^{40} and Ca^{45}. The interpretation of the findings was complicated by the rates of dissolution of the added calcium and by incomplete isotopic exchange with the native soil calcium.

Thoroughness of mixing soil amendments such as lime greatly affects their efficiency. Little information is available as to the soil-mixing characteristics of various tillage implements, although their tilth-creating characteristics may be well known. Radioactive phosphorus was effectively used as a tracer in an experiment reported by Hulburt and Menzel[58] where a series of tillage operations were compared.

ROOT DISTRIBUTION AND ACTIVITY

A thorough understanding of the development and activity of plant roots in their soil environment is needed in the development of scientific crop production. Most of the root studies conducted in the past have entailed some system whereby a direct observation of root systems is made. Hall and co-workers[48,49] developed a tracer technique to measure growth and activity of plant root systems. The method consisted of strategically locating a small quantity of P^{32} at given distances from and below plants growing in the field and measuring its uptake by plants at various time intervals. Corn, cotton, peanuts and tobacco were studied. Cotton was found to be the most shallow rooted of these crops, while tobacco showed the greatest root activity at 16 inches below the surface. A similar technique was employed by Burton *et al.*[20] to investigate root penetration of grasses in relation to drought tolerance. Radiophosphorus was placed in the soil at depths up to 8 feet and various species of grasses planted. Root penetration measured in this way was found to be related to drought tolerance. Lott *et al.*[76] used tracer techniques to study the cross feeding in a muscatine grape vineyard.

A technique involving the use of tracers in studying the penetration into and absorption of nutrient from dry soils was explored by Hunter and Kelley.[59,60]

EXCHANGE PHENOMENA

There are a number of exchange reactions which are of importance to soil chemistry and plant nutrition. The study of these is frequently facilitated through the use of radioisotopes. The general expression for an isotopic exchange reaction is given by the following equation:

$$A^* Y + AX = AY + A^* X$$

where A^* represents the radioactive isotope and A the stable isotope. Starting with $A^* Y$, measurement of the rate of exchange can be accomplished by noting the appearance of $A^* X$ or disappearance of $A^* Y$. The first applications to soils were a series of studies dealing with the "contact" and "soil solution" theories of ion absorption conducted by Jenny and Overstreet.[63,64] Through the use of Na^{24}, K^{42} and Rb^{86} the rates of absorption of cations by barley roots was shown to be greater with soil and clay suspensions containing absorbed sodium, potassium and rubidium than from the corresponding filtrates. Thus it was shown that there is no clear relationship between the uptake of cations in the presence of clay

surfaces and ion absorption from corresponding salt solutions.

The equilibrium of cations between solutions and exchange materials such as soils, clay minerals and resins has been followed with radiocations. The exchangeability of trace concentrations of cations as influenced by degree of saturation and nature of complementary ion was studied by Wiklander and Gieseking.[129] Using K^{42} and Sr^{90}, they found that a certain limit value is approached which depends on the exchange ion, complementary ion and exchanger used. Wiklander[130] has observed that the rate of exchange between the exchangeable and fixed potassium of soils is quite slow. Employing Co^{60}, Banerjee *et al.*[2] and Spencer and Gieseking[112] investigated the chemistry of cobalt absorption and release in the cation exchange system. Easily exchangeable and strongly adsorbed cobalt were distinguished and cobalt was found to be harder to replace than calcium. Krishnamoorthy and Overstreet[70] used K^{42}, Ca^{45}, Rb^{86}, Cs^{137} and La^{140} to test the validity of several expressions for the equilibrium of cation exchange reactions.

Kinetic exchange studies on clays by Borland and Reitemeier[17] demonstrated that Ca^{45}, added to clay suspensions, equilibrated rapidly and completely with the exchangeable calcium adsorbed on the clay minerals kaolinite, halloysite, hydrous mica, beidellite and montmorillonite. Advantage was taken of this principle, and Barbier and Tyszkiewicz,[5] Smith *et al.*[108] and Blume and Smith[14] have proposed isotopic dilution methods for determining exchangeable calcium, base exchange capacity and the rate of reaction of liming materials in soils. Such methods have certain advantages especially in the study of soils containing free calcium carbonate.

Only a fraction of the amount of phosphorus necessary for crop production exists in the soil solution at any given time. If, for the purposes of this discussion, it is assumed that the soil solution is the sole source of phosphate for plants, then it would be necessary for this solution to be renewed many times during the cropping season. McAuliffe *et al.*[78] applied P^{32} to a study of the exchange between solution and solid phase phosphate. A part of the soil phosphate was found to rapidly equilibrate by exchange with the ambient solution. The amount of this rapidly equilibrating surface phosphate was found to parallel other estimates of phosphorus availability. A slower, less reversible exchange of phosphates was also recognized.

The mechanism of phosphate sorption and the kinetics of phosphate exchange in soils was investigated with the aid of P^{32} by Wiklander.[131] It was concluded that the soil phosphorus occurs in essentially two different ways, namely: a fairly reactive fraction and a fraction of low solubility which is difficult to mobilize but tends to be in equilibrium with the first fraction. The series of investigations by Barbier and Tyszkiewicz[3,4,7,8] dealt with the exchange of phosphate ions within the solid phase of soils. They have also demonstrated an exchange between several of the forms of soil phosphorus. Related studies have been reported by Seats[106] and Egawa *et al.*[34]

The amount of phosphate on the surface of soil particles and apatites has been measured in several studies by Olsen and co-workers.[90,91,92,22] The phosphate associated with the solid phase which readily equilibrated with P^{32} in the ionic form was termed surface phosphate. Good correlations were found between the amount of this surface phosphate in soils and the plant-available phosphorus. From 19 to 31 per cent of the phosphorus added in long-time rotation experiments was found to accumulate as surface phosphate. Monolayer absorption of phosphate on calcium carbonate and calcareous soils was found from dilute solutions, but with higher concentrations the percentage of the phosphorus absorbed appearing as surface phosphate decreased. Similar findings, but using an iron-phosphate system, were reported by Fried and Dean.[40]

RELATIVE ABSORPTION OF NUTRIENTS FROM SOIL AND FERTILIZER

Starting in 1947 with experiments by Spinks and Barber,[113] Dean *et al.*[26] and Nelson *et al.*[83] there have been a large number of greenhouse and field experiments conducted utilizing fertilizers labeled with radioisotopes. Fertilizer of known specific activity in respect to phosphorus was incorporated in soil and a crop grown. The ratio of the specific activity of the phosphorus in the crop to that of the original fertilizer has been taken as being indicative of the fraction of the total phosphorus absorbed by the crop that was derived from the fertilizer. This fraction has been shown to increase with increasing rate of fertilizer application and to be lowest for soils high in available phosphorus.

Four groups of workers have proposed that the phosphorus status of soils may be measured by comparing the specific activity of the phosphorus absorbed by plants with that of the fertilizer added to soil; Larsen,[71] Fried and Dean,[37] Gunnarsson and Fredricksson,[45] and Barbier and Lesaint.[6] The methods employed by these investigators were all essentially the same, however, the mathematical treatment and interpretation of results varied. The expression derived for estimating the available phosphorus supply of the soil has several forms, the simplest being:

$$A = B\left(\frac{Sf}{Sp} - 1\right)$$

where A and B are the amount of soil and fertilizer phosphorus and Sf and Sp the specific activities of the fertilizer and plant phosphorus, respectively. This method has been applied to a number of investigations dealing with the phosphorus fertility status of soils and has been one of the tools used in calibrating soil tests; Dean,[27] Olsen *et al.*,[93] Olson *et al.*[95] and Thompson and Pratt[124]. This method for measuring

the phosphate status of the soil which is based on a comparison of the specific activity of the fertilizer phosphorus with that of the phosphorus absorbed by crops has a number of experimental limitations as was pointed out by Fried and Dean.[37] Russell *et al.*,[103] from a consideration of isotopic equilibria between phosphates in soils, consider the method to have serious limitations and suggest that an examination of the total phosphorus content of plants may be a better means. On the other hand, Dean[28] reports that similar information regarding the soil's phosphorus supply is obtained with three methods; namely, the extrapolation of growth curves, the extrapolation of yield-of-phosphorus curves and from radiochemical data. All three methods offer both advantages and limitations.

Phosphorus is applied to soils as a variety of materials ranging from commercial fertilizers to manures. Since the availability to plants of the phosphorus in these materials is known to vary, an accurate evaluation of each material is of considerable economic importance. Radiophosphorus has been utilized in a study of this problem; the principles involved are discussed by Fried.[39] In general, the procedures are a counterpart of the techniques described previously for assessing the phosphorus status of soils. Instead of applying the same fertilizer to a variety of soils, a number of fertilizers are applied to the same soil. Special techniques for preparing a number of different tagged fertilizer materials for this purpose are described by Hill *et al.*[57] Fertilizer evaluation by this method has both advantages and limitations. The possibility that isotopic exchange may interfere has been suggested by McAuliffe *et al.*[80] The method is quite sensitive and differences between fertilizers as measured by the relative amount of soil and fertilizer phosphorus absorbed may not, in turn, always be reflected in crop yields.

A number of factors are known to affect the availability of the different phosphate fertilizer materials. Soil properties, crop growth, time and method of application have all been shown to influence the availability. In all instances, superphosphate has been adopted as a basis of comparison. With but few exceptions, this material has been found to supply as much, if not more, phosphorus to plants than other materials, as indicated by tests utilizing P^{32}.

The chemistry of phosphorus in neutral and alkaline soils, where calcium predominates the system, differs from that of acid soil systems. Thus, the behavior and effectiveness of the various fertilizer materials added to these two systems may not be the same. Olsen and co-workers,[88,89] using P^{32} labeled fertilizers, compared a number of materials on alkaline soil; wheat, barley, sugar beets, and alfalfa were among the test crops. The phosphorus available for plant growth was generally in the order: superphosphate = monoammonium phosphate > calcium metaphosphate > dicalcium phosphate > tricalcium phosphate. Dicalcium was always inferior to superphosphate but the results with calcium metaphosphate varied with time, soil and crop. Superphosphate, monoammonium phosphate and liquid phosphoric acid were compared by Fuller,[44] Olsen *et al.*[89] and Allen *et al.*,[1] and little difference was found in the effectiveness of these sources. On the other hand, studies by Dion *et al.*[31,32] with wheat, suggest ammonium and sodium phosphate to be more effective than monocalcium phosphate. Schmehl and Brenes[105] have investigated the effectiveness of the Rhenania type phosphate on calcareous soils and found it equal to superphosphate except when applied broadcast. A pot experiment comparing the relative efficiency of various phosphatic fertilizers on both an acid and an alkaline soil reported by Speer *et al.*[110] has shown forms of low water solubility to be less effective on the alkaline soil.

A number of fertilizer source comparisons, similar to those discussed above but dealing with acid soils, have been reported. These include studies with cotton and corn by Hall *et al.*,[47] with oats and alfalfa by Stanford and Nelson,[118] with pasture species by Blaser and McAuliffe[10] and with soybeans by Bureau *et al.*[19] The findings were in some respects different from those reported for the alkaline soil region. In general, calcium metaphosphate was a more effective material when applied to acid soils. Dicalcium phosphate, when mixed with acid soils, was also relatively more effective. Studies with neutron irradiated phosphate rock by Fried and MacKenzie[36] have demonstrated the marked influence of soil acidity on the effectiveness of this material as a phosphate fertilizer. Reports by Starostka *et al.*,[116] Borisova[16] and Koritskaya,[68] dealing with the availability of granulated phosphatic fertilizers, show an interaction between particle size, water solubility of the fertilizer and soil acidity. Generally speaking, all of the conclusions drawn from the experiments with P^{32} labeled fertilizer materials parallel those arrived at by a study of crop yield. However, the P^{32} experiments do show a relatively greater difference between the materials compared.

Radiophosphorus has also been used in studies designed to compare the availability of the phosphorus contained in organic farm residues with that of superphosphate. Because of the limited amount of suitably labeled material that it is practical to produce, all comparisons were made in greenhouse pot culture. Fuller and co-workers,[41,42,43] White *et al.*[128] and Nielsen *et al.*[87] studied leguminous and non-leguminous crop residues while McAuliffe *et al.*[79,80] applied similar techniques to a study of sheep manure. The availability of the phosphorus in the materials studied compared favorably with that in superphosphate. In considering these findings, perhaps it should be recognized that more than 50 per cent of the phosphorus in the materials studied was in inorganic forms.

The half-life of P^{32} precludes studies which extend for a period exceeding 120 days. Thus a direct measurement of the residual value to succeeding

crops of phosphatic fertilizers is not possible. However, the effects of previous fertilizer application can be studied through an extension of the technique involving radioactive labeled fertilizer. McAuliffe *et al.*[81] measured the effect of phosphorus applied 8 years previously by comparing per cent phosphorus in the crop derived from the fertilized obtained from P^{32} labeled superphosphate applied to permanent fertility plots. A highly significant decrease in the per cent of phosphorus derived from the fertilizer as a result of past phosphorus applications was observed, thus demonstrating the presence of residual phosphorus in available forms. More quantitative estimates of residual values are possible if 'A' values (see Fried and Dean[37]) are calculated and compared. Using this technique, Webb and Pesek[126] studied the residual effect of a number of fertilizer materials. Superphosphate and calcium metaphosphate applied at equal rates of P_2O_5 over a period of years appeared to have an equal effect upon the residual level of available soil phosphorus. Fused tricalcium phosphate compared favorably with these materials on an acid soil but was less effective on a calcareous soil. Phosphate rock at equal or higher rates was less effective on all 4 of soils studied. Olsen *et al.*,[94] Rubins,[102] Prince,[97] Stelly and Morris[121] and Neller and Lundy[83] have also used P^{32} labeled fertilizers to study the residual effect of previous phosphorus applications.

FERTILIZER UTILIZATION

The mineral nutrition of crops leads to a consideration of the amounts and rates of absorption. The recent studies involving the use of P^{32} have supplied additional information regarding the influence of soil and fertilizer phosphorus on phosphorus absorption. A number of crops growing under a variety of conditions have been studied, including cereals, pasture species, potatoes, sugar beets, sugar cane, tobacco and cotton (Bonnet and Riera,[15] Dion *et al.*,[30] Kapp *et al.*,[67] Krantz *et al.*,[69] Jacob *et al.*,[61,62] Mitchell *et al.*,[82] Stein and Marais,[120] Strzemienski,[123] Spinks *et al.*,[114,115] Welch *et al.*[127] and Woltz *et al.*[133]). With few exceptions, the results of these studies have followed a rather typical pattern. Phosphate fertilizer is usually applied at the time of planting and this application is localized in the proximity of the seed. In the early stages of growth, seedlings absorb phosphorus at a faster rate from the fertilizer than from the soil, but as the plant develops and the root system enlarges, the amount of soil phosphorus accessible increases. Thus the absorption of soil phosphorus increases as compared with fertilizer phosphorus. The net result is that young plants characteristically have a higher per cent phosphorus in the crop derived from the fertilizer than mature plants. This pattern of decline in the per cent phosphorus in the crop derived from the fertilizer with maturity varies with crop species. Relatively little decline has been observed for potatoes as compared with corn. This difference between crops is attributed to differences in root systems and their development.

Crops utilize phosphorus from applications of phosphatic fertilizers with low efficiency. The experiments which compare the phosphorus absorption by crops also supply information relative to the efficiency of fertilizer utilization. The percentage of the phosphorus in a given application which was absorbed and thus utilized by the crop grown during the first season was found to vary between the limits of 2 and 35 per cent. With increasing rates of fertilizer application the total amount of fertilizer phosphorus absorbed usually increases, but the efficiency of utilization usually decreases. The question is frequently asked: Does the application of fertilizer phosphorus influence the amount of soil phosphorus absorbed by plants? With many soils the addition of fertilizer phosphorus has little effect on the amount of soil phosphorus absorbed. Under some conditions of phosphorus deficiency where the phosphate fertilizer increases root growth the addition of fertilizer phosphorus increases the amount of soil phosphorus absorbed. Other experiments have shown that adding fertilizer phosphorus actually decreases the amount of soil phosphorus absorbed. The exact mechanism involved is not understood.

Soil management practices such as fertilizer placement have been designed to enhance the efficiency of fertilizer use by crops. Many of the studies with P^{32} labeled fertilizers included comparisons of methods of fertilizer application. Experiments with corn and cotton by Nelson *et al.*[86] and Stanford and Nelson[117] and with sugar beets by Olsen and Gardner[98] and Lawton *et al.*[73] are cited as examples. The most pronounced effects of placement of superphosphate on the utilization of this fertilizer phosphorus were observed during the early stages of growth. The closer this material was placed to the seed, the greater its utilization. The pronounced differences observed during the early stages of growth largely disappeared as the crop matured. Broadcast applications are the most convenient method of applying fertilizers to pastures and other established stands of crops. Since it is known that phosphates penetrate soils only to a limited degree, this method has not been considered as a very satisfactory means of applying phosphate fertilizers. Stanford *et al.*[119] top-dressed established meadows using P^{32} labeled superphosphate. This method of application was found to be surprisingly effective. Subsequent experiments by Caldwell *et al.*[21] and Lawton *et al.*[74] with established stands of legume hays have confirmed these findings.

Phosphate fertilizer utilization has been shown to be affected by liming, nitrogen fertilization and irrigation practices. Neller[84] and Robertson *et al.*[101] have studied the phosphorus utilization from soils of varying sesquioxide content as influenced by lime applications. Liming above pH 6.5 reduced the fertilizer utilization regardless of the sesquioxide content of the soil. Robertson *et al.*[100] demonstrated that more phosphorus was utilized from a mixture

of phosphate and nitrogen fertilizers applied to corn than when these same materials were applied in separate bands. Rennie and Mitchell[98] and Smith *et al.*[109] have shown nitrogen fertilization to enhance the absorption of fertilizer phosphorus. Haddock[46] has shown that the frequency of irrigation of sugar beets increases the uptake of fertilizer phosphorus.

The foregoing discussion on the use of radioisotopes to study fertilizer utilization has been limited exclusively to P^{32}. However, other isotopes have been used on several occasions. The average mixed fertilizer sold in the United States contains 17 per cent CaO. Under conditions of low soil calcium supply, it seemed possible that crops might obtain substantial quantities of their calcium from the fertilizer applied. To test this premise, a field experiment with tobacco involving the use of Ca^{45} was carried out by Blume and Hall.[13] The results indicated that plants derived only 2 to 6 per cent of their calcium from the fertilizer. In contrast, 25 to 36 per cent of the phosphorus absorbed was derived from this fertilizer. Studies on the plant uptake of Zn^{65} from soils and fertilizers have been confined to greenhouse experiments. Such studies have been reported by Shaw *et al.*[107] Speer *et al.*[111] and Woltz *et al.*[132]. The efficiency of utilization of average applications of zinc fertilizers was lower than that observed for average phosphate applications. Liming was shown to reduce the uptake of zinc applied to soils.

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Uptake and Transport of Mineral Nutrients in Plant Roots

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The passage of solutes between cells and the ambient chemical pool forms one of the principal topics of physiology. The permeability of cells to many types of substances has been studied—to large and small molecules, dyes, and organic and inorganic electrolytes. Autotrophic plants do not have to obtain organic materials from the environment; hence, interest in the flux of solutes between plants and their medium centers around the inorganic electrolytes present in the solution bathing the roots.

Experimental biology advances in proportion to the degree in which phenomena are quantitatively described in terms of physico-chemical reactions and processes. It is natural, therefore, that investigators of permeability in cells and tissues have focused attention on recognized physico-chemical reactions of molecules and ions that might result in transfer of these substances into and out of cells—diffusion, ion exchange, Donnan equilibria, membrane potentials, and others. A measure of success has certainly attended these endeavors, for every one of the phenomena mentioned has been observed in various types of cells and tissues, ranging from unicellular microorganisms through the roots of higher plants to the kidney tubules, to mention but a few examples.

However, it is being increasingly recognized that none of these mechanisms adequately describe ion absorption and secretion in many systems, including plant roots. These mechanisms are all of such a nature as to lead to equilibria, and can fairly readily be duplicated in the laboratory by model systems (see, for example, Sollner[17]). But one of the chief characteristics of ion transport in biological systems is its dependence on active metabolism, and another, the fact that the living cell is part of an open system not at equilibrium with the environment.[18] Yet another feature difficult to account for on the basis of simple model systems is the high degree of selectivity characteristic of biological ion transport.

The dissatisfaction with earlier proposed mechanisms has found expression in a subtle change of terminology, as noted by Steinbach.[18] Whereas papers dealing with this general topic used to bear titles referring to the "permeability" of some cell or tissue to the substance in question, now reference is made more often to the "uptake," "absorption," or "transport" of the substance. This does not, of course, signify a return to vitalistic or other non-mechanistic ideas. It merely reflects an increased awareness of the complexity of the phenomena under investigation.

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TRACER ISOTOPES

Before giving an account of current findings and concepts regarding ion transport by the roots of higher plants, it may be appropriate, in the present context, to review briefly the usefulness of radioactive isotopes for research in this field. In addition to carbon, hydrogen, and oxygen, thirteen mineral elements have been identified as being essential to the growth of higher plants: potassium, calcium, magnesium, nitrogen, phosphorus, sulfur, iron, manganese, zinc, copper, molybdenum, boron, and chlorine. Of these thirteen, only nitrogen and boron have no radioactive isotopes suitable as tracers. Several other elements, though not known to be essential, are of great physiological interest, and suitable radioactive isotopes exist. In this group belong sodium and cobalt. It may be mentioned that the first biological application of radioactive tracers was in the very field discussed here. We are referring to Hevesy's use, in 1923, of thorium B (Pb^{212}) in a study of the uptake of lead by plants.

The usefulness of radioactive isotopes for research in ion transport by plants is twofold. They serve as superior analytical tools, extending the sensitivity and specificity and increasing the ease of analysis beyond what can be achieved by conventional analytical methods. And, secondly, they enable the investigator to measure ion fluxes in a given direction even when there is no net flux of that ion, or when the net flux is in the opposite direction.

Of these two, the first, or "analytical" use of radioisotopes, represents an extension and improvement of existing techniques. But the second, or "exchange" type of application, is a new departure entirely, undreamed of before the advent of tracer isotopes, and has made possible new insights into the dynamics of living things that could be achieved in no other way. We shall have occasion to demonstrate both types of application of radioactive isotopes in the study of ion transport in plant roots.

EXPERIMENTAL METHODS

The method used in our laboratory is essentially a modification of the excised root technique described by Hoagland and Broyer.[9] The modifications consist largely of a reduction in the scale of the experimental

set-ups, and in the amount of root tissue used. These modifications permit quicker and more flexible experimental procedures, and enable us to reduce experimental periods to very short intervals when desired — of the order of a minute. Briefly, barley seeds are germinated and grown for five days under strictly controlled conditions. The nutrient solution is incomplete, consisting of a single calcium salt, usually the sulfate; but when sulfate absorption is to be studied, calcium phosphate is used during this preliminary stage.

Just before the experiment, the roots are excised, suspended in water, and 1.00 gram portions are weighed out and transferred to aeration tubes containing 50 ml of water. (The roots are blotted before weighing, to remove water adhering to the surface.) The water is then replaced by an equal volume of experimental solution containing a radioactively labeled ion. During the absorption period, the solution is constantly aerated, and the temperature maintained at 30°C. The experimental period may vary from one minute to four hours. After the absorption period, the roots may be assayed directly for the radioactively labeled ion. Alternatively, in exchange studies, the roots may subsequently be exposed to water or non-radioactive solutions containing the same or other ions. In this way, data is obtained concerning diffusible, exchangeable, and non-exchangeable fractions.

CONCENTRATION EFFECTS

In many physico-chemical systems, increasing concentrations of a variable result in progressive saturation of the system, until eventually complete saturation may be achieved. Familiar examples are the adsorption of gases on charcoal and other solids and the exchange reactions between solutes and solid exchangers, such as clays or synthetic resins. The usual interpretation in such cases is that the system possesses a finite capacity for holding the substance taken up. A familiar instance is the base exchange capacity of soils and other cation exchangers.[11] When the rate of absorption of an ion by excised roots is measured as a function of the concentration of that ion in the solution, the same type of curve we have been discussing is frequently obtained. Upon plotting the rate as the ordinate, and the concentration as the abscissa, the curve rises steeply and almost linearly at first, but at the higher concentrations, the curve flattens out and asymptotically approaches a limiting value. The conclusion suggests itself that in this case, also, some finite system is being progressively occupied by the ions in question. However, there is this important difference between the latter case and the others we have mentioned, such as cation exchange on clays. The clay, on being immersed in a solution, quite rapidly comes to equilibrium with the solution, and thereafter no net movement of ions between the clay and the solution occurs. We measure *amounts* taken up at equilibrium. In the case of ion absorption by roots, on the other hand, we measure *amounts per unit time*, or *rates*. Once the rate of absorption of a given ion, at a certain concentration of that ion, is established, it may be maintained for many hours.

If we nevertheless wish to maintain the idea that at increasing concentrations, the ions progressively occupy some finite system within the root, additional assumptions are necessary. It is necessary to assume, first, that the ions, having occupied the system, are subsequently released again into a compartment other than the ambient solution (for in that case, no net transport would occur). And, secondly, in order to account for the typical relation between concentration and absorption rate, it must be assumed that the postulated release of the ions from the system is the rate-limiting step in the process, so that the rate is proportional to the extent to which the system is occupied by the ions at the given concentration. The maximal rate approached at high concentrations indicates complete saturation of the system by the ions under those conditions.

The kinetics we have just outlined are precisely those that have been found to apply in many cases of enzymatic catalysis. It is only necessary to substitute "substrate" for "ions", and "enzyme" for what we have vaguely called a "system," and which henceforth we shall speak of as "carriers." The Michaelis-Menten equation applies:[14]

$$v = \frac{V\ (S)}{K_s + (S)}$$

where v = the observed rate of absorption of an ion present at concentration (S), V = the maximal velocity attainable at complete saturation of the carriers, and K_s = the Michaelis constant corresponding to the substrate (ion) concentration at which half the maximal velocity is attained. Lineweaver and Burk[13] showed that when the reciprocal of the above equation is written, straight lines are obtained in a plot of $1/v$ against $1/(S)$.

THE EFFECT OF INTERFERING IONS

In this treatment, interfering ions assume the role of inhibitors or alternate substrates. Interference may be competitive or otherwise, depending on whether or not the interfering ions attach themselves to the same binding sites on the carriers that are, or may be, occupied by the substrate ions. In the double reciprocal, or Lineweaver and Burk plot, the presence of a competitive ion results in an increase in the slope of the line obtained by plotting $1/v$ against $1/(S)$, with no change in the intercept. The increase in the slope is by the factor $[1 + (I)/K_i]$, where (I) is the concentration of the interfering ion and K_i its Michaelis constant. If the interference is not competitive, the intercept is increased.

Figure 1 shows the results of an experiment on sulfate absorption by excised barley roots.[12] The

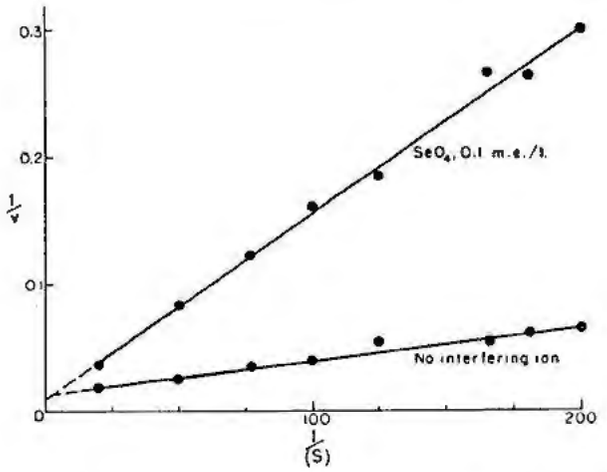

Figure 1. Double-reciprocal plot of sulfate absorption by excised barley roots. Concentration of $K_2S^{35}O_4$, (S), in meq/l. Rate of absorption, v, in mμ e/gram fresh weight/ 3 hours

concentration of potassium sulfate (labelled with S^{35}) was varied over the range 0.005 to 0.05 meq/l. Selenate interfered competitively, i.e., selenate and sulfate are bound by the same binding sites on the carriers effecting the transport. Neither nitrate nor phosphate (as the potassium salts) competed with sulfate. In fact, in the presence of these salts, the rate of sulfate absorption was increased somewhat.

GENERALITY OF THE CARRIER MECHANISM

To recapitulate, the evidence on concentration effects and the effects of interfering ions indicates that the absorption of ions by plant roots is mediated by carriers to which the ions are temporarily attached. The carriers are equipped with different binding sites characterized by differential affinities for different ions. Table I indicates that this general concept applies to a wide variety of ions. The radioisotope used is indicated for each substrate ion.

Table I. Competing and Non-competing Ions*

Substrate ion		*Competing ions*	*Non-competing ions*	*Reference*
Rb^+	(Rb^{86})	K^+, Cs^+	Na^+, Li^+	6
Br^-	(Br^{82})	Cl^-, I^-	NO_3^-	3,4
Sr^{++}	(Sr^{89})	Ca^{++}, Ba^{++}	Mg^{++}	7
SO_4^{--}	(S^{35})	SeO_4^{--}	$H_2PO_4^-$, NO_3^-	12

*At high concentrations of the ions, the indicated specificities break down in some instances, and ions listed as "non-competing" may become competitive under those conditions.

In addition, Hagen and Hopkins,[8] in a study of phosphate absorption by excised barley roots, concluded that the two ionic species, $H_2PO_4^-$ and HPO_4^{--}, are bound by different sites, and that OH^- ions compete with both phosphate species.

It would appear on the basis of these findings that the carrier concept of active ion transport in plant roots is quite generally useful. However, as indicated earlier, it should be recognized that the active mechanism we have outlined is not the only one whereby ions may enter plant roots.

PASSIVE ENTRY OF IONS INTO PLANT ROOTS

Cation Exchange

Figure 2 shows the time course of the uptake of Sr^{89} from a solution of $Sr^{89}Cl_2$ at 1 meq/l. The curve may be resolved into two components. The first is rapid and approaches completion in 30 minutes. The second is linear with time, and no equilibrium is attained in the experiment. The ions taken up by the former mechanism are readily exchangeable with ambient non-radioactive Sr (see the dotted line). Other cations also exchange with this fraction, but the amount lost to water is only a fraction of the amount displaced by salts. The exchange proceeds even under anaerobic conditions. By contrast, the ions taken up by the second mechanism are non-exchangeable, and this uptake is virtually abolished under anaerobic conditions. This second mechanism is active transport as discussed above. The rapid process is straightforward cation exchange, in which the root acts as a solid cation exchanger, like clays or synthetic exchange resins. This mechanism does not result in a net uptake of ions.

The observation that it takes approximately 30 minutes for the cation exchange equilibrium to be reached indicates that the negative exchange sites are not merely superficial, but lie within the tissue so that uptake by this mechanism does indeed represent penetration of the tissue by the ions in question. Figure 2 shows that there may be a loss of ions from this exchangeable fraction while simultaneously there is a net influx of the same ion into the root — a finding that would not be possible without tracer isotopes.

Diffusion

When roots are removed from a solution containing $K_2S^{35}O_4$, blotted to remove the solution adhering to the surface, and transferred to water or to a solution containing non-radioactive sulfate, they lose a con-

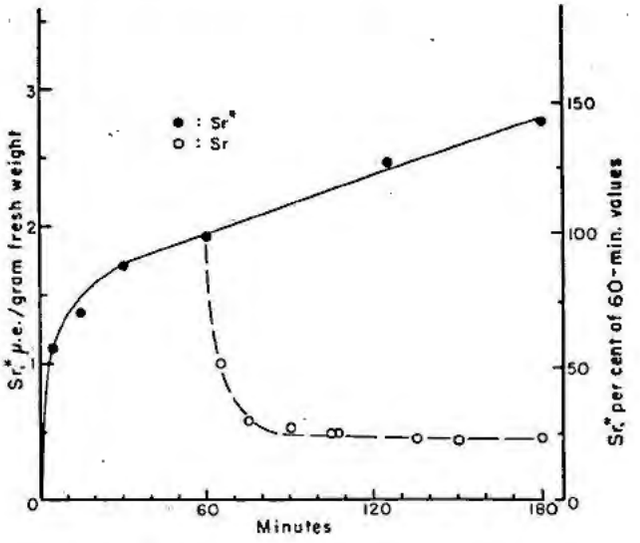

Figure 2. Uptake of Sr^{89} by excised barley roots and its loss to non-radioactive Sr. Concentration of $Sr^{89}Cl_2$, 1 meq /l. Concentration of non-radioactive $SrCl_2$, 1 meq/l

siderable amount of their radiosulfate (Fig. 3). The amount lost to water is identical with the amount lost to non-radioactive sulfate, indicating that there is no need for replacing or exchanging ions to effect this removal. Rather, there seems to be a fraction of the volume of the tissue (the "outer" space) to which the ions have free and reversible access by diffusion.

Equilibration with the ambient solution or water is essentially complete in 60 minutes, and no further loss to water or non-radioactive sulfate takes place thereafter. A fraction of the radiosulfate taken up during the initial period in the radioactive solution is retained by the roots after their transfer to water or non-radioactive sulfate. This represents the amount absorbed by the active transport mechanism, and we shall refer to it as being in the "inner" space, to contrast it with the diffusible "outer" space sulfate.[5]

As pointed out, the loss of radiosulfate to water was identical with the loss to non-radioactive sulfate (Fig. 3). Nevertheless, these two processes differ in a way which could not be demonstrated without the use of tracer isotopes. The exit of radiosulfate into water denotes a net loss of sulfate from the roots. The movement of radiosulfate into the solution of non-radioactive sulfate, on the other hand, represents no net movement of sulfate at all. The concentration of the initial radiosulfate solution was 20 meq/l, and that also was the concentration of the solution of non-radioactive sulfate to which the roots were transferred at the 90-minute point.

We may make the reasonable assumption that at equilibrium, the concentration of sulfate in the outer space of the root is the same as in the ambient solution. In the experiment shown in Fig. 3, the concentration of $K_2S^{35}O_4$ was 20 meq/l. Sulfate in the outer space (i.e., the amount lost by diffusion) was 4.45 μe/gram fresh weight. On the basis of the above assumption, the volume of the outer space of the root tissue is 4.45/20 = 0.22 ml/gram fresh weight. The water content of this tissue is 94 per cent, so that over 25 per cent of the total tissue water is accessible to the ions by diffusion. This magnitude of the space has been verified over a wide range of sulfate concentrations. It is independent of pH over the range 4.0 to 7.7.

It is of some interest to compare the outer space of barley roots, i.e., the fraction of the volume of the tissue accessible to ions by diffusion, with the corresponding values for other types of cells and tissues. Conway and Downey,[2] for a variety of solutes, found a value of approximately 0.1 for the "outer region" of yeast. For *Escherichia coli*, Roberts, *et al.*,[15] using $S^{35}O_4$, found a "water space" essentially identical with the total cell water. In frog nerve, Shanes[16] measured spaces of the order of 0.62 to 0.65, using chloride tagged with Cl^{38}. In Hope's[10] experiments with bean roots, the "apparent free space" varied with the concentration of KCl used in measuring it, indicating a Donnan effect. The true space was of the order of 0.14. Butler[1] found values for wheat roots

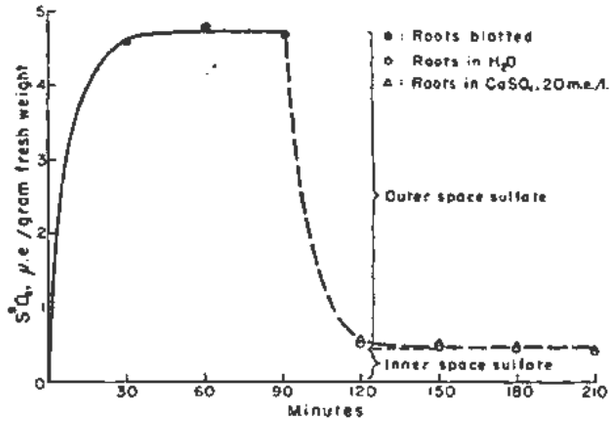

Figure 3. Uptake of $S^{35}O_4$ by excised barley roots, and its loss to non-radioactive sulfate and to water

that varied from about 0.25 to 0.34. He used chloride, phosphate (P^{32}), and mannitol.

CONCLUSION

Radioactive isotopes have vastly accelerated research into the mineral nutrition of plants, and have made possible an understanding of the dynamics of biological ion transport that would be inconceivable without them. Specifically, studies on ion transport in the roots of higher plants have shown that ions freely move into and out of an "outer" space of the roots, by diffusion and exchange, independent of the simultaneous active transport of the same ionic species which results in their transfer into an "inner" space where they are no longer exchangeable with the same or other ionic species. The exchange and diffusion processes are reversible, non-selective, non-metabolic, and come to equilibrium within an hour, approximately, after immersion of the roots in a new solution.

Sustained, selective absorption of ions due to metabolic activity of the cells and rendering the ions non-exchangeable with ambient ions of the same or other species result from the activity of carrier molecules which operate in a turn-over fashion, like enzymes. The carriers possess different binding sites more or less specific in their affinity for various ions. This concept of active ion transport applies to a wide variety of both cations and anions.

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Factors Affecting the Availability to Plants of Soil Phosphates

By R. Scott Russell,* E. W. Russell,* P. G. Marais*† and W. N. M. Foster,*‡ UK

The limited ability of plants to absorb phosphate from many acid soils gives rise to major agricultural problems. Despite numerous investigations the mechanism for the process normally called "phosphate fixation" remains largely obscure. In general the cause of the poor uptake by crops from such soils has been sought in the reaction of the added phosphate in the soil. Recently, however, it has been realized that physiological effects of other ions in plant roots may significantly influence the transference of phosphate to plant shoots.[1,2] Tracer methods provide valuable additional methods for studying both the form of phosphate in the soil and the mechanism of transfer of phosphate from the soil to the root and thence to the shoot.

ESTIMATION OF LABILE SOIL PHOSPHATE BY THE USE OF P^{32}

Isotopically Exchangeable Soil Phosphate (*E*)

If a soil is shaken with a labelled orthophosphate solution, the P^{32}/P^{31} ratio in the solution is reduced in consequence of isotopic exchange with phosphate already in the soil. If the solutions are assayed after different periods, the isotopically exchangeable phosphate (E_t) and the sorbed phosphate (S_t) at time (t) can be calculated by the equations:[3]

$$E_t = \frac{x_t}{y_t} y - x \qquad S_t = x - x_t$$

where x and y are the amounts of P^{31} and P^{32} originally present in solution and x_t and y_t the values determined at time t. This estimate of E_t is valid only if the sorbed phosphate remains in isotopic equilibrium with the external solution. This condition has been satisfied in all soils so far studied.

The Larsen Value (*L*)

If labelled phosphate is intimately mixed into a soil and plants are subsequently grown in it, the amount of soil phosphate, L, with which the labelled phosphate has been apparently diluted on absorption by the plant can be calculated by the equation:

$$L = \frac{Y_f (X_p - D)}{Y_p} - X_f$$

where X_f and Y_f are the quantities of P^{31} and P^{32} added to the soil, and X_p and Y_p are the total plant content and D is the phosphate content of the seed. This type of procedure was first used by Larsen.[4] Subsequent workers described the quantity thus obtained as the A value for the soil, implying that it measures the "plant available" phosphate in the soil[5], but because this interpretation is not always warranted (see below), the term "Larsen" (L) value is considered more appropriate. Identical values of L are obtained when carrier phosphate, X_f, is varied by a factor exceeding 10^3, and it has been found practicable to use values of X_f so low that the phosphate equilibrium of the soil is not affected. The advantage of this procedure is that the phosphate absorbed by the plant, as measured by $X_p - D$, is identical with the values obtained in soil to which no labelled phosphate has been added. L value determinations are open to serious errors when X_p is so small that exchange between seed and soil phosphate significantly affects the P^{32}/P^{31} ratio in the plant.

EXPERIMENTS IN SOIL

Measurements of isotopically exchangeable phosphate E, phosphate sorption S, the Larsen value L, and uptake of phosphate by test plants have been made in four soils (Table I) which ranged from a calcareous alluvium, in which no apparent fixation of phosphate occurred (Soil I), to a basaltic acid soil (Soil IV) from which plants were unable to absorb significant quantities of phosphate in the absence of fertilisers. Brief descriptions of the soil are given in Table I. It is apparent from the table that phosphate extracted by 1% citric acid in 24 hr does not give a satisfactory measure of the amount of phosphorus the plants can extract from the soils.

In the experiments reported here, the soils were used either in their natural state or after the addition of varying quantities of KH_2PO_4. Prior to some experiments the soils were stored moist for periods up to one year after the addition of the phosphate. The values of E here used are those found after 7 days of shaking with labelled phosphate, by which time the rate of exchange had become slow. The values of both E and L are given in the units of mg P/5 gm soil, and are reproducible under a wide variety of conditions. S varies with the phosphate concentration of the equilibrating solution and the

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Table I. Characteristics of Soils Investigated

Soil	*I*	*II*	*III*	*IV*
	Calcareous alluvium	Medium-heavy loam derived from silurian and old red sandstone	Loam basaltic	Loam basaltic
Origin	Thames Valley	Edinburgh	Greenhall, Aberdeen	Northern Ireland
pH	7.6	6.0	5.8	5.5
Phosphate extracted in 1% citric acid (mg P/5 gm soil)	0.56	0.93	0.89	0.64
Relative values for available phosphate relative to soil I determined by plant absorption	1	0.8	0.2	<0.01

amount of phosphate absorbed by test plants depends in addition on all factors influencing plant growth.

The Relationship between the Labile Phosphate in Soil and Absorption by Plants

In *each* soil absorption by plants increased approximately linearly with both E and L when aliquots of soil, to which different quantities of phosphate had been added, were compared (Fig. 1). In no case did absorption amount to more than a very small fraction of the labile phosphate. This simple relationship did not however obtain for the comparison of *different* soils. Figures 2 and 3 show results for two experiments in each of which three soils were compared at different levels of phosphate. The experiment reported in Fig. 2 was conducted in summer with barley as a test plant in soils enriched with phosphate immediately before the experiment, whereas the experiment reported in Fig. 3 was conducted in winter with winter rye in soils enriched with phosphate 118 days prior to the experiment and stored either moist or dry in the intervening period. In both figures the soils have been arranged from left to right in order of decreasing plant uptake. It is apparent that neither E nor L provided any guide to the absorption of the test plants in the different soils.

The Relationship between Sorption of Phosphate by Soil (S) and Absorption by Plants

Figures 2 and 3 show that plant absorption bears a relatively close relationship to the reciprocal of the values for sorption. In considering this relationship it is to be borne in mind that the soils sorbed phosphate to very varying extents, in the order

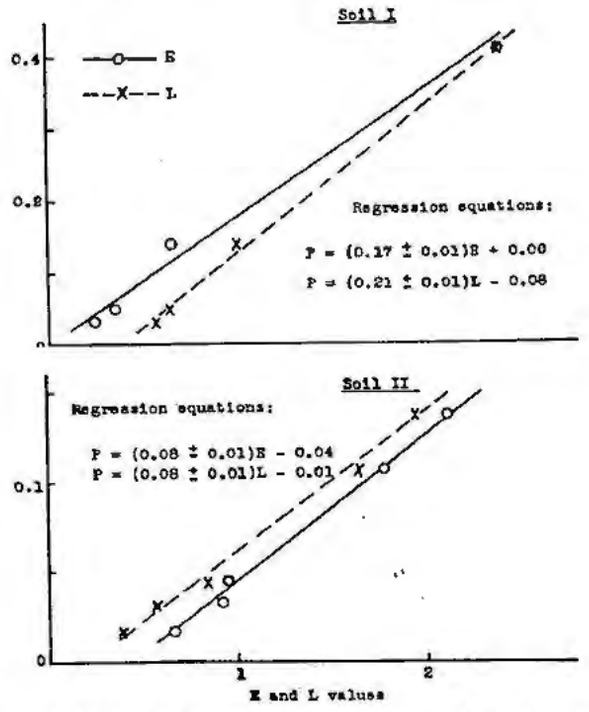

Figure 1. Relationship between plant absorption and E and L values in soils I and II. Results are expressed as mg P/5 gm soil

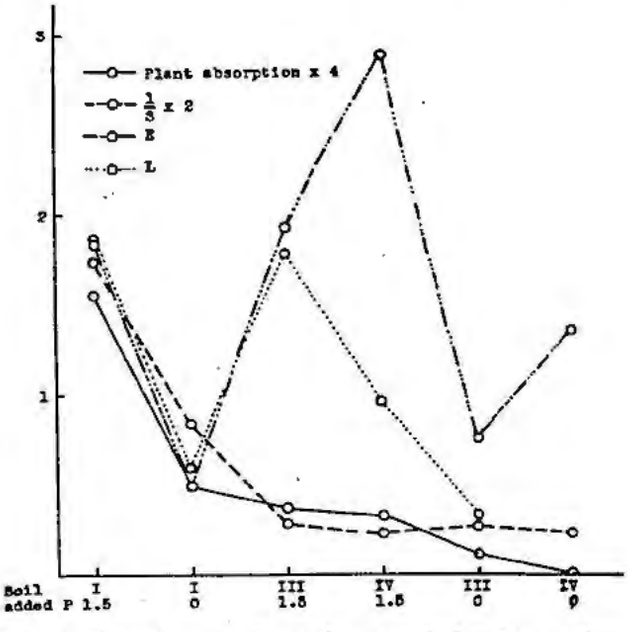

Figure 2. Plant absorption, E, L and reciprocal of S determined in soils enriched with phosphate to varying extents. Test plant, barley. Values are expressed as mg P/5 gm soil

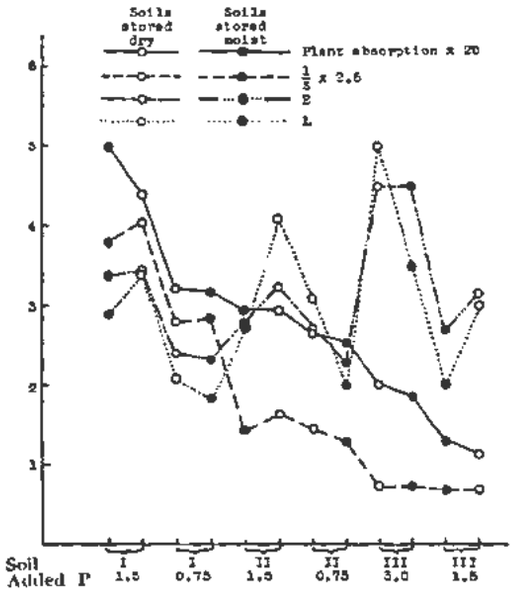

Figure 3. Plant absorption, *E*, *L* and reciprocal of *S* determined in soils enriched with phosphate to varying extents and stored moist and dry for 118 days prior to experiment. Test plant, rye. Values are expressed as mg P/5 gm soil

Soil I < II < III < IV. Thus, when the concentration of phosphate in the solution in which soils were equilibrated was within the range in which sorption by Soil I could be precisely measured, the solutions used with Soils III and IV were virtually depleted. This difficulty could not be overcome by employing solutions of different concentration as this would have affected the extent of sorption. None the less the procedure placed the soils in the right order with respect to sorption. The extent of sorption of a soil may be regarded as bearing an inverse relationship to the activity of phosphate in the soil solution or to Schofield's phosphate potential.[6] Thus the results in Figs. 2 and 3 suggest a dependence of plant absorption on the phosphate potential of the soils though different methods would be necessary for any precise relationship to be determined. It is to be noted that this relationship between plant uptake and the reciprocal of sorption has not been found to hold in all cases when soils are compared which contrast sharply in the phosphate treatments they have received. The influence of factors not yet defined is therefore to be envisaged.

The Effect of the Addition of Phosphate in the Labile Phosphate Fraction of Different Soils

In all soils approximately linear relations were obtained between *E* values and the quantity of phosphate with which the soils had been enriched. When the soils were examined either immediately after the addition of phosphate or following short periods of dry storage the increment in *E* equalled the amount of phosphate added in Soil I but in the other soils the corresponding values were approximately 70% of the added phosphate (Fig. 4). Storage, especially at field capacity, reduced this value significantly. Linear relationships were also apparent between the

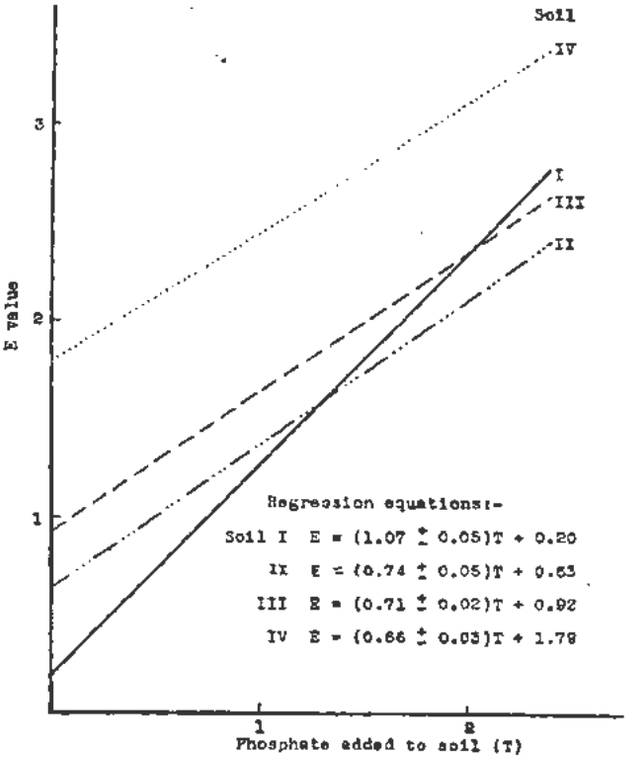

Figure 4. Relationship between *E* values and phosphate added to soil. Results are expressed as mg P/5 gm soil

L value and the quantity of phosphate added. However there were significant differences between the *L* values obtained with different test plants in the more highly sorbing soil treatments.

Effect of Plant Species

The uptake of phosphate by rye was compared with that of barley in some experiments (Table II). Whereas both species showed similar results from the soil which yielded most phosphate, the ratio of the uptake by rye to that by barley increased progressively as the quantity of phosphate taken up by the barley decreased. The *L* value of the soils however was the same whether rye or barley was used as the test plant, except for Soil IV from which the

Table II. Comparison of Absorption and L Values for Rye and Barley

*Soil**	*I*	*II*	*III*	*IV*
Phosphate added in (mg P/5 gm soil)	1.5	0.0	1.5	1.5
Uptake of phosphate in mg P/pot, 28 days after planting				
Barley	8.74	2.00	1.49	0.25
Rye	8.74	2.68	2.76	1.36
Ratio, rye/barley	1.00	1.34	1.85	5.44
L value, in mg P/5 gm soil				
Barley	1.82	0.50	1.63	0.94
Rye	1.80	0.49	1.74	1.80

* Soils III and IV without added phosphate could not be included in this comparison because of the negligible uptake of phosphate by the plants.

Table III. Interaction of Ferric Iron and Phosphate in Absorption by Barley Plants during 24-Hour Periods

External concentration		P or Fe per plant, μg			Ratio, Shoot
P, ppm.	Fe, ppm.	Root	Shoot	Total Plant	Total Plant
Absorption of Phosphate					
Iron and phosphate applied simultaneously					
0.1	0	3.61	3.18	6.79	46.8
0.1	0.2	5.10*	2.11*	7.21	29.3*
0.1	2.0	7.50*	0.99*	8.49*	11.7*
10	0	19.6	35.5	55.1	64.4
10	0.2	20.6	19.0*	39.6*	48.0*
10	2.0	47.7*	9.4*	57.1	16.5*
Iron applied 24 hours prior to the addition of phosphate					
0.1	0	4.23	3.88	8.11	47.8
0.1	0.2	4.48	2.56*	7.04	36.4*
0.1	2.0	4.72	1.40*	6.12*	22.9*
10	0	22.4	38.6	61.0	63.3
10	0.2	22.1	25.6*	47.7*	53.7*
10	2.0	20.5	15.6*	36.1*	43.2*
Absorption of Iron					
Iron and phosphate applied simultaneously					
0.001	0.2	8.50	0.46	8.96	5.11
0.001	2.0	44.9	2.65	47.6	5.57
10	0.2	7.25	0.16*	7.41	2.17*
10	2.0	39.7	1.44*	41.1	3.50*

* Significant differences from control value ($P = 0.05$).

uptake by both plants was very low. On this soil higher values of L and greater absorption of phosphate were found with rye and also with cabbage than with barley. Since this soil had the strongest affinity for added phosphate, this result could be interpreted as showing that rye and cabbage can take up phosphate from very dilute solutions more strongly than barley.

PLANT PHYSIOLOGICAL EXPERIMENTS

The Effect of Iron on the Absorption of Phosphate by Plants

Since there is evidence that phosphate and ferric ions can mutually affect the mobility of each other within the plants, this question was examined in water culture. The use of tracer techniques allowed the accurate determination of the absorption of both in such short periods of time that no appreciable differences in growth occurred between treatments. Thus the direct effects of ionic interactions could be distinguished from indirect effects more readily than in prolonged experiments. Barley was used as the experimental plant, and the iron was added as ferric citrate.

The most striking effect of iron was to increase the phosphate content of the roots and to reduce that of the shoots (Table III). Its effect on the total phosphate taken up by the plants was smaller and varied depending on the experimental conditions. These results are similar to those of previous workers.[2,7] The same effect of iron was observed whether it was applied simultaneously with phosphate or in a previous period. However when iron was absorbed subsequently to phosphate little interaction between the ions was apparent. Phosphate affected the distribution of iron to only a small extent.

The mechanism responsible for the retention of phosphate in roots was examined by extracting the plant roots with buffer solutions of decreasing pH. Treatment with iron caused a considerable increase in the fraction of recently absorbed phosphate which was not extracted until the pH was lowered below 3 (Fig. 5). By contrast, the extent to which iron was extracted by the buffer solutions was little affected by the level of phosphate supplied, and a large fraction always remained in the tissues until the pH was lowered below 3. Two alternative interpretations of these results may be considered:

(*a*) The retention of phosphate is due to its precipitation as ferric phosphate in an essentially inorganic reaction.

(*b*) Phosphate and iron are associated in an organic complex.

The view that a simple inorganic mechanism is operative has been suggested.[7] Such a reaction would however be expected to reduce the labile iron in the plant to the same extent as the phosphate. The present results however show that the interaction of iron and phosphate reduced the number of gram atoms of phosphate extractable below pH 3 by an

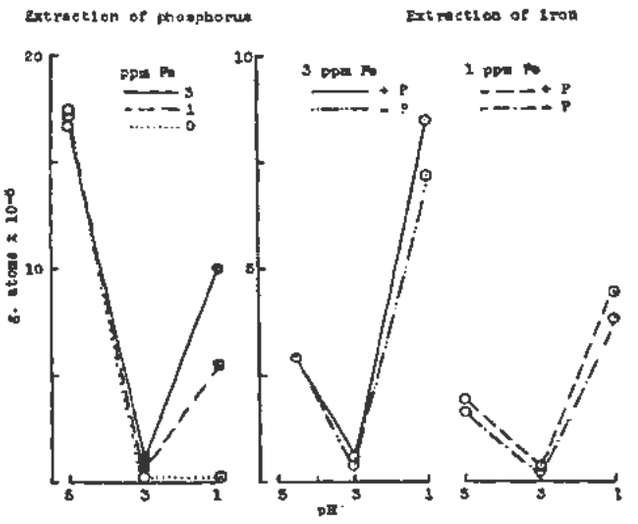

Figure 5. The extraction of phosphorus and iron in buffer solutions of decreasing pH from the roots of barley plants which had been treated for 24 hr with 3, 1, and 0 ppm Fe and 10 and 0 ppm P combined factorially

amount which was more than eight times greater than the corresponding effect on iron. This suggests that the iron is present in some organic combination that is hydrolysed below pH 3, and it is with this organically bound iron that the phosphate reacts. The atom ratio of phosphate to iron in the postulated complex, calculated as: gm atom increase in P extracted below pH 3 induced by Fe/gm atom Fe extracted below pH 3 in presence of P was 1.22 and 1.15 when 1 and 3 ppm Fe respectively and 10 ppm P were present in the external solution. These values are not significantly different from 1, and are compatible with the formation of a complex $R\mathrm{FeH_2PO_4}$.

The Effect of Aluminium on the Absorption of Phosphate

Since the activity of aluminium ions is high in many soils in which phosphate is relatively unavailable to plants, its effect on the absorption of phosphate by barley was examined. No evidence of direct interaction between the two ions was observed. Effects on the absorption and translocation of phosphate occurred only when the treatment with aluminium was sufficiently prolonged or the level of aluminium sufficiently high for effects on growth to be induced. The effects of aluminium on the absorption of iron reported by previous workers[8,9] likewise appear to have occurred only when aluminium toxicity was induced.

Comparison of the Absorption of Phosphate by Barley and Rye

The absorption of phosphate by rye and barley was compared in water culture over a wide range of phosphate concentrations at pH 6. The results (Fig. 6) support the suggestion in section 3.6 that when the external phosphate supply is very low the absorption of rye is markedly greater than that of barley. The resultant interaction between plant species and concentration with respect to absorption is highly significant ($P < 0.001$). In other experiments iron was found to cause greater retention of phosphate in the roots of barley than of rye. Thus if significant quantities of soluble iron are present in soils from which absorption is low this effect may enhance the differences between the two species.

CONCLUSIONS

The point of special interest in these results is that the labile phosphate content of different soils bears no consistent relationship to the amounts of phosphate which plants absorb. Soils containing large quantities of labile phosphate from which plants are able to absorb only very small quantities have been shown to sorb large quantities of phosphate. Initially the sorbed phosphate is in a largely labile form though in the course of time it may be rendered non-exchangeable. These results indicate that the description "available" cannot be applied to any chemically distinguishable soil phosphate fraction. Furthermore it appears that the inability of plants to absorb phosphate in the highly sorbing soil is not initially attributable to the precipitation of phosphate in insoluble forms but rather to the lowering of the "phosphate potential" of the soil.

Different genera of plants differ in their ability to absorb phosphate from dilute solutions and to extract phosphate from strongly sorbing soils. Rye and cabbage are superior to barley in this respect. Apart from this effect another physiological mechanism which may affect absorption of phosphate is the absorption of iron. In water culture it has been shown that phosphate can be retained in plant roots complexed with iron and possibly associated with organic compounds. Strongly sorbing soils are frequently characterized by high activities of iron and

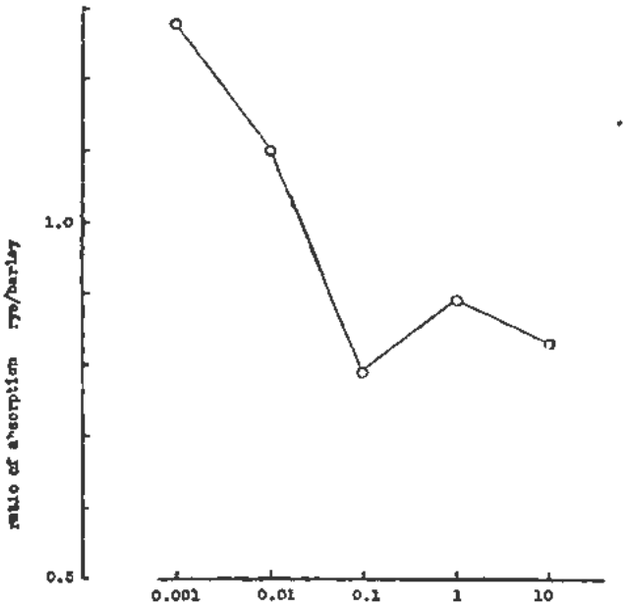

Figure 6. Comparison of the phosphate absorption of rye and barley grown for 24 hr in solutions of varying phosphate concentration

the investigation of the interaction of iron and phosphate in plants grown under such conditions should be of considerable interest.

It is apparent that labile phosphate measured by isotopic exchange or dilution methods is not a universally applicable method for determining the ability of soils to provide phosphate to plants. In consequence the description of Larsen's parameter as the *A* value may be misleading. None the less the value of tracer methods for certain purposes is apparent. The linearity of the relationships which both *E* and *L* bear to plant absorption in fractions of the same soil enriched with phosphate to varying extents, indicates that the absorption of phosphate from soils of similar sorbing capacity can be inferred from measurements of labile phosphate. For the same reason it should be possible to assess the relative merits of different fertilizers by comparing their effects on the labile phosphate in a test soil, provided the fertilizers differ only in the availability of the phosphate ion.

In conclusion the relative merits of *E* and *L* value procedures for measuring labile phosphate may be compared. The study of the factors which cause *E* and *L* values to differ in certain soils may well yield valuable fundamental information. However the two methods normally give sufficiently similar results for them to be regarded as alternative procedures for estimating the phosphate responses likely to occur under field conditions. They must be judged therefore in terms of convenience and economy of effort. The measurement of *L* values involves the growth of plants under carefully controlled conditions and since the method is applicable only when *L* bears a linear relation to plant absorption it normally provides no more information than can be obtained in exhaustion experiments of the conventional type. Only in highly phosphate sorbing soils, in which intraspecific differences occur, does the obtaining of unique information appear likely to repay the complication of the method. In contrast the determination of *E* values is a simple laboratory procedure, readily organised in a routine scale. It holds promise of greatly simplifying the evaluation of soils in which the determination of labile phosphate can provide valuable information.

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The Use of Tracer Atoms in Studying the Application of Fertilizers

By V. M. Klechkovski, USSR

With the increase in output and use of fertilizers, growing importance is attached to researches made with the aim of appraising the efficiency of different methods of applying fertilizers, and of establishing the most effective ways of using them in agriculture.

A scientific basis for methods of applying fertilizers can be obtained by making a careful study of the biological characteristics of different agricultural plants, their relations to the conditions of nutrition at different stages of growth and development, and by studying the effect of fertilizers on the metabolism of the plant and the formation of the crop. In doing this, considerable importance must also be attached to the properties of the soil and to ascertaining the behaviour of the fertilizers in the soil, since the nature of the interaction between the fertilizers and the soil has a great influence upon the efficiency of different methods of applying fertilizers.

The great variety of the soil, climatic and other conditions of agriculture in the USSR necessitates elaborating different systems of using fertilizers, due consideration being given to the properties of the soil and the biological characteristics of the plants. The use of tracer atoms, in addition to other methods of studying the nutrition of plants and the effect of different fertilization practices on quantity and quality of the yield of farm crops, considerably promotes the solution of this problem.

One of the chief advantages of using the tracer technique in solving problems of fertilization is the possibility of keeping under direct observation both the nutrient which the plant utilizes from the applied "labelled" fertilizer, and the nutrient obtained by the plant from normal soil constituents or from fertilizers that had been applied previously.

Furthermore, by using a labelled isotope it becomes possible to trace how the plant assimilates any given part of the fertilizer, for instance, when combining different forms or different methods and dates of applying fertilizers. All this makes the tracer technique extremely valuable in obtaining clear and prompt replies to a number of practical questions, connected with the appraisal of different ways of applying fertilizers under certain soil and climatic conditions and in conformity with the characteristics of different agricultural crops.

Numerous field and pot experiments carried out by experimental institutions in the USA, Canada, the USSR and other countries have secured comprehensive data on this matter.[1] In this paper, we present some of the results obtained in the USSR in recent years from a study of methods of applying phosphate fertilizers, made with the help of tracer atoms.

ASSIMILATION OF PHOSPHORUS BY PLANTS

Influence of Grain Size and Density of Distribution of Fertilizer

Farm crops have a higher phosphorus requirement during the early stages of their growth. A deficiency in phosphorus at the beginning of growth exerts an unfavourable effect upon the whole of the subsequent development of the plant and, consequently, upon the yield. Hence, the great efficiency of such methods of applying phosphoric fertilizers that improve the supply of phosphorus to the plants at the beginning of their growth and development. One such method is row fertilization, i.e. the application of fertilizers to the soil, when seeding with the help of a combined drill which places the fertilizer locally in the soil in the direct proximity of, or at some (small) distance from, the seeds.

The use of granulated neutralized superphosphate, which possesses good physical properties, facilitates the best possible application of the fertilizer in the rows when sowing. Another advantage of granulated fertilizer is that it can be applied not only by means of a combined drill, but in a simpler way, by being sown together with the seeds by an ordinary row drill.

In soils with a higher capacity for the absorption of phosphates, the application of granulated fertilizers reduces the volume of the soil in direct contact with the fertilizer and promotes the preservation of the availability of the phosphorus of the fertilizer by the plants. This is an additional factor ensuring the increased effectiveness of granulated superphosphate both when applied in the rows, or in other ways.

An increase in the size of the grains of superphosphate makes it possible to utilize to the fullest extent those advantages of granulated fertilizer which result

Original language: Russian.

from reduced absorption of phosphorus by the soil. With an increase in the size of the grains, however, when using the same level of fertilization, the density of their spacing in the row is reduced. In order to establish the proper size of the grains, therefore, it is important to know to what extent the local distribution of the fertilizer, concentrated in a smaller number of large grains, can ensure uniform nourishment of plants located at various distances from the grains of the fertilizer. The answer to this question could be obtained only with the help of radioactive isotopes. In field experiments (in podzol of the Moscow Region), with labelled granulated superphosphate[2] applied in the rows when sowing oats, grains 2×2 mm and 4×4 mm in size were compared. With one and the same level of fertilization (10 kg P_2O_5 per hectare), the density of spacing of the small grains in the row was several times as great as the density of spacing of large grains. In the experiments with the large grains, the position of the grains in the row was fixed with a small wooden peg and when choosing plants for sampling, separate specimens were taken from the soil near and between the grains. Furthermore, in order to determine the extent of phosphorus uptake from the fertilizer into plants differently located with regard to the grains of fertilizer, samples were taken from the part of the row which had not been fertilized, at different distances from the last grain. In the experiments with the 2 × 2 mm grains, average samples, taken from different parts of the row, were analyzed.

A periodic determination of the content of labelled phosphorus in the plants showed that during the first half of the growing stage, prior to earing, the movement of phosphorus from the 4 × 4 mm grains of fertilizer varied depending upon the position of the plants in the row. In samples taken from places between the grains, the content of labelled phosphorus was considerably less than in plants growing near the grains. It was only by the beginning of the earing stage, 1.5 months after the seedlings had been planted, that the diversity in the content of labelled phosphorus in plants growing in different spots in the row disappeared, and the content became practically equal in all plants, irrespective of their position in the row.

Similar data were obtained from experiments with buckwheat; in this case, the movement of phosphorus from the labelled fertilizer into plants, differently spaced in the row in relation to the 4 × 4 mm grains of fertilizer, remained uneven during the whole period of the experiment, until the buckwheat began to flower. However, the most striking difference was observed at the beginning of the growing stage. Diminution in the size of the grains of superphosphate to 2 × 2 mm created conditions ensuring a more uniform supply of phosphorus to all the plants, thanks to the denser spacing of grains in the row. By the end of the first month of vegetative growth, the content of labelled phosphorus in specimens from an experiment with 2 × 2 mm grains applied was approximately half the amount, found in an experiment with 4 × 4 mm grains in plants, growing in the immediate proximity of the grains, but it was considerably higher than in plants growing between the large grains in the row.

The use of organo-mineral granulated superphosphate leads to a decrease in the amount of phosphorus contained per grain, compared with grains of the same size produced without the admixture of an organic substance. Therefore, with the same amount of phosphorus and the same size of the grains, in the case of organo-mineral fertilizer the grains are spaced more densely in the soil, which leads, as experiments with labelled superphosphate have shown, to a more uniform flow of phosphorus into the plants. When applying granulated phosphoric fertilizer in the rows at a low rate, the above fact is of particular importance and is one reason why experiments with organo-mineral fertilizer, give better results than those with granulated superphosphate not containing an organic substance.

The advantage of small grains of superphosphate, resulting from their denser spacing in the soil becomes most noticeable at the beginning of the growing stage, when the root system of the plants is but poorly developed. With time, as the root system grows and penetrates a larger volume of soil, the significance of the dense spacing of grains in the soil decreases and greater influence is exercised by the factors causing the soil to fix the phosphorus of the fertilizer unevenly. In the later stages of growth, therefore, we observe, as a rule, a better utilization of phosphorus by the plants from larger grains of fertilizer.

As an illustration of this general regularity we present the following data from a pot experiment made with millet in soil cultures on the podzolized soil of the Moscow Region[3] (Table I).

While during the first month after germination a larger amount of labelled phosphorus was found in specimens obtained from the experiment with small grains, at later dates of sampling the large grains showed a greater advantage.

A determination of the total content of phosphorus in plants, obtained from experiments with labelled phosphates, makes it possible to establish what portion of the total amount of phosphorus contained in a plant is made up of labelled phosphorus, taken from the fertilizer, and what portion is made up of

Table I. Amount of Labelled Phosphorus Absorbed by Plants from Fertilizer (mg P_2O_5/100 plants)

Time of sampling (days after germination)	*Grains 2 × 2 mm*	*Grains 4 × 4 mm*
10	1.5	0.6
20	5.0	1.2
30	19.5	10.8
41	28.2	33.0
57	99.8	164.0

soil phosphorus (not labelled). Such determinations have shown that the amounts of phosphorus, taken by the plants from the soil in cases when phosphate fertilizer had not been applied, and in other cases when fertilizers had been used, proved to be unequal in most cases. Therefore, the usual method of determining the difference in the amounts of fertilizer phosphorus utilized by plants, by means of chemical analyses of plants grown in soil without fertilizer and in fertilized soil, cannot be regarded as safe in determining of the factor of utilization of fertilizers by plants.

The influence of the fertilizer on the amounts of phosphorus assimilated by the plants from the soil depends upon various factors. On the one hand, if the plant is provided with readily available phosphorus from the fertilizer, the utilization of the less available phosphates of the soil is reduced. On the other hand, since the action of the fertilizer markedly improves the growth of the root system, this also promotes a fuller utilization of soil phosphates by plants, even if they are less available than the phosphates supplied by the fertilizer. At different stages of plant growth, the relative role of these factors may differ. This can be illustrated by the data from the above mentioned experiment with millet, in which the following change in the assimilation of soil phosphorus by plants under the influence of a fertilizer was observed (Table II).

During the first month of growth, plants in fertilized soil assimilated less phosphorus from the soil, whereas during the following month the use of soil phosphorus (not labelled) was almost trebled under the influence of the fertilizer. In the experiments cited, this effect was observed in almost the same degree, irrespective of the size of the grains and the density of their spacing in the soil. In the course of time, only a slight tendency towards a greater change in the influence of the fertilizer on the assimilation of soil phosphorus by the plants was noted in the experiment with the large grains.

Table II. Absorption of Soil Phosphorus by Plants (mg P_2O_5/100 plants)

Time of sampling (days after germination)	*Without fertilizer*	*Treatment with the application of labelled superphosphate*	
		2 × 2 mm grains	*4 × 4 mm grains*
30	10.3	7.7	7.2
57	24.9	64.1	69.7

Effect of Depth of Application and Different Methods of Applying Fertilizers

To obtain high yields of farm crops of superior quality, the plants should be provided with an increased phosphate nutrition not only at the beginning but also throughout their growing stage. For instance, to obtain a high yield of cotton, phosphorus nutrition is of particular importance during the time of fruit formation, for sugar beet, during the time of intensive root growth and sugar accumulation, etc. All this accounts for the great practical importance of methods of regulating the phosphate nutrition of plants by different ways of applying fertilizers, by combining the main fertilizers, applied at given depths, before sowing, with row fertilization at the time of sowing and, in some cases, with additional fertilization during the growing stage. The tracer technique proved to be highly suitable for observing the rates at which phosphorus moves into the plant from the fertilizer, depending upon the time and method of its application.

Table III. Relation between Amount of Phosphorus Absorbed by Oats from Fertilizer and Depth of Application of the Fertilizer (mg P_2O_5/100 plants)

Time of sampling (days after germination)	*Depth of application of superphosphate*	
	7–8 cm	*18–22 cm*
7	7.1	0.7
14	10.5	11.5
45	60.7	313.7

One of the chief factors influencing the movement of phosphorus into the plant from the fertilizer, applied before sowing, is the depth of application. Very clear data, illustrating the relationship between the rate of movement of phosphorus into the plant from the fertilizer and the depth at which the fertilizer was applied, were obtained in field experiments with oats, carried out at the Field Experimental Station of the Timiryazev Agricultural Academy[4] (Table III).

As can be seen from this example, in the first days after germination the plants assimilated much more phosphorus from fertilizer applied at a small depth (7–8 cm). But, even within a fortnight after sprouting, the difference in the rate of the flows of phosphorus into the plant from fertilizers applied at depths of 7–8 cm and 18–22 cm practically disappeared, and in the following stages of growth the advantage was obviously with the relatively greater depth of application.

These observations make it possible to explain the reasons for the better effect of the deeply applied main fertilizer, as well as cases of positive action of phosphorus fertilizers applied at a slight depth by a cultivator before sowing. If fertilizers are applied only before sowing, and not in combination with row fertilization while sowing, and the soil is known to have a low content of readily available phosphates, then the good effect of superphosphate applied at a slight depth by a cultivator is chiefly due to the fact that this form of application of the fertilizer provides the plants with better phosphorus nutrition at the beginning of vegetation. If, however, the phosphate requirements of plants during the initial stage of growth are met by means of a fertilizer applied in the rows at a low rate when sowing, then better conditions for the positive action of the main fertilizer are

created when it is applied deeply by ploughing it under. Thus when the main fertilizer, applied to the layer of the soil where favourable conditions for the development of the root system are preserved throughout the growing season, is combined at sowing time with a row fertilizer ensuring better plant nutrition at the beginning of vegetation, such a combination creates the most favourable conditions for the plant to utilize both the nutrients from the fertilizer and those from the soil.

The following data were obtained from a field experiment with oats, when different methods of applying fertilizer and a combination of the main fertilizer with row fertilization were compared (Table IV).

The use of the tracer technique in studying problems of fertilization makes it possible not only to distinguish in the total quantity of the element contained in the plant that portion which was obtained from the fertilizer and that from the soil, but to continue this differentiation still further, in order to observe how the plant utilizes this or that portion of the fertilizer; for instance, how it assimilates the phosphorus of the main fertilizer if the latter is applied without combination with the row fertilizer, or, how this process takes place when in addition to the main fertilizer a local application of fertilizer has been used when sowing. The results of such experiments may be illustrated by the following data, obtained when studying the methods of applying phosphoric fertilizer to potatoes.[4]

In this experiment, labelled superphosphate was applied in two ways: at a depth of 20–22 cm at the rate of 3 centners per hectare, and half as much (1.5 cntn per hectare) applied in the hills when planting potatoes. In the experiment an application of three centners of labelled superphosphate per hectare at a depth of 20–22 cm was also tested, and besides this, while planting the potatoes ordinary (unlabelled) superphosphate was applied in the hills. The following results were obtained after determining the total quantity of phosphorus contained in the plants and taken from the labelled fertilizer (Table V).

Applying superphosphate to the hills while planting potatoes considerably increased the quantity of phosphorus assimilated by the plants from the soil (treatments 1 and 2). By comparing treatments 3 and 4, we also see that applying unlabelled superphosphate in the hills has resulted in a fuller utilization of the labelled superphosphate by the plants when it was applied with the main fertilizer at a depth of 20–22 cm. A determination of the total weight of plants and tubers shows a better effect of fertilizers, when a local application at planting time (in the hills) that meets the plant requirements in phosphorus at the beginning of the growing stage is combined with the application of the greater part of the fertilizer by ploughing it under.

The above examples show that the data on the flow of phosphorus into the plant from the fertilizer, that were obtained with the help of tracer atoms, make it possible to gain a clearer idea of the advantages or disadvantages of different methods of applying fertilizers to the soil, and to give judicious grounds for the advisability of combining different methods of applying fertilizers with the aim of creating optimal conditions of plant nutrition.

Depending on the soil conditions and the characteristics of the plant, the role of local row fertilization in the general system of applying fertilizers is not always the same, and, to furnish grounds for practical measures in this field, further investigations are necessary. Thus, the use of the tracer technique in studying the role of row fertilization, when cultivating cotton under irrigated conditions in the grey-

Table IV

Treatment	Amount of labelled superphosphate			Phosphorus absorbed by the plants 45 days after germination (mg/100 plants)		Weight of 100 plants (gm)
	at depth of		in rows with seeds	from fertilizer (labelled)	from soil (not labelled)	
	7–8 cm	18–22 cm				
1	3 centn/hect	–	–	60.7	1563	193
2	–	3 centn/hect	–	313.7	1719	270
3	–	2.5	0.5 centn/hect	406.3	2248	323

Table V

Treatment	Rate and method of applying labelled superphosphate	Rate and method of applying unlabelled superphosphate	P_2O_5 assimilated by the plants (mg/2 plants) on 25 July		Weight of tubers (gm)
			from labelled fertilizer	from soil	
1	Without fertilizer	–	–	358.8	456
2	1.5 centn/hect in hills	–	81.9	500.3	790
3	3 centn/hect at depth 20–22 cm	–	96.7	594.8	949
4	3 centn/hect at depth 20–22 cm	1.5 centn/hect in hills	130.5	633.9*	1106

*Total from soil and from fertilizer (not labelled) applied in the hills.

earth soils of the Uzbek SSR,[3] revealed interesting facts indicating that with different methods of applying superphosphate in the rows, while sowing cotton seed, the individual plants prove to be far from being uniformly supplied with phosphorus nutrition from the fertilizer. It was found that a considerable number of the plants in the plots, where row fertilization with labelled superphosphate was used, took a quite insignificant part, if any, in the consumption of labelled phosphorus when the superphosphate was applied in the rows by the ordinary methods. Only when resorting to the method of dusting the seeds with phosphorus fertilizer, prior to sowing, were all the plants found to participate in the consnmption of labelled phosphorus. One of the possible reasons of the poor utilization of the row fertilizer at the beginning of the growth of the cotton plant is, perhaps, the relatively high content of phosphorus in the soils of the cotton-growing areas, resulting from intensive fertilization with phosphates applied at fairly high levels in the course of many years. Under these conditions, a very large share of the phosphorus consumed by the plants is derived from the residue of previously applied fertilizers, and a smaller share from the fertilizer directly applied to the plant in the year of sowing. Another cause of the same phenomenon, closely connected with the first, is the higher capacity of carbonate soils to absorb the applied phosphates, that greatly reduces their mobility in the soil; on this account it is less probable that the roots of the plant would come into early contact with the locally situated sources of fertilizer phosphates absorbed by the soil.

Effect of Applying Fertilizer During the Time of Vegetation

Top dressing with fertilizer during the growing season of plants is directed toward increasing the plants' nutrition by applying this or that element at certain stages in their life when they make the greatest demands upon the conditions of nutrition and respond positively to the application of fertilizer, the yield being improved both in quantity and quality. If we approach the significance of top dressing from this point of view, it becomes exceedingly important to know at what period after the application of fertilizer to the soil its intensive consumption by the plaots begins.

If the fertilizer is soluhle in water and not absorbed by the soil, as, for instance, is the case with nitrates, the nutritive element of the fertilizer begins to move rapidly into the plant soon after the fertilizer has been applied to the soil during the growing stage, provided that the soil contains sufficient moisture to ensure that the fertilizer becomes dissolved and moves towards the roots. The case is somewhat different with such fertilizers as phosphates which are absorbed by the soil and almost deprived of the capacity of travelling any distance. Therefore, between the application of a phosphate fertilizer into the soil and the beginning of intensive consumption of phosphorus by the plants, a certain time elapses, in the course of which a sufficiently full contact is established between the fertilizer, absorbed by the soil, and the roots of the plant. In order to choose the proper date for the application of top dressing under these conditions, it is important not only to know the plant requirements of the given element at certain periods of its life but also to have some idea as to how soon the plant will begin assimilating the applied fertilizer. When the ordinary chemical methods of analysis are followed it is rather difficult to obtain an answer to this question. The use of tracer atoms greatly facilitates the task.

Experiments with sugar beets[3] have shown that, when applying labelled superphosphate to the rows during the vegetative season at a rate of 30 kg P_2O_5 per hectare at a distance of 10 cm from the plant row and at a depth of 10–15 cm, small amounts of labelled phosphorus will be already found in the plants within twenty-four hours after side dressing. However, fairly large quantities, of the labelled phosphorus begin to move into the plant approximately only a fortnight after side dressing. The most intensive flow of phosphorus from the fertilizer into the plant, when side dressing was applied at different dates, was observed during the interval of two to four weeks after the application of the fertilizer. Comhined application of nitrogen and potassium increased the utilization of phosphorus from the fertilizer by plants. As an example, we cite the following data of an experiment carried out in the Moscow Region (Table VI).

Similar results were obtained from a number of other experiments with sugar beets. For instance, when side dressing is applied at a later period (55 days after germination) the content of labelled phosphorus in the plants increases as follows:

Days after application of fertilizer	1	8	16	30	57
Content of labelled phosphorus in mg P_2O_5 per 100 plants	25.4	354.8	730.9	3042.4	2957.8

The portion of the phosphorus in the plant, assimilated from the fertilizer applied at the growing stage, was found to be rather small in these experiments, not exceeding 10–11 per cent of the total phosphorus content of the plants. Under less favourable conditions of soil moisture, the interval of time between side dressing and the beginning of intensive consumption of phosphorus from the fertilizer by the plants becomes even more prolonged and the portion of the phosphorus assimilated from the fertilizer, in relation to the total content of phosphorus in the plant, decreases.

In experiments with cotton plants, carried out in the Uzbek SSR in serozem soil, a study was made of the flow of labelled phosphorus into the plant from a fertilizer applied during the growing season

Table VI

Days after side dressing (side dressing applied 54 days after germination)	Content of labelled phosphorus in sugar beet plants (mg P_2O_5/100 plants)	
	P	NPK *
1	3.3	7.3
4	23.9	59.1
7	103.4	144.7
16	500.5	574.4
32	1512.0	2559.6
78	2071.6	4075.4

* Experiments: P—only labelled superphosphate applied at the rate of 30 kg P_2O_5/hect; NPK—superphosphate applied at the same rate, NH_4NO_3 in amount of 30 kg N/hect and KCl at the rate of 30 kg K_2O/hectare.

of the cotton plants, at different intervals after sowing.[5] By these experiments it was established that a fairly long interval of time elapses between the date of applying the fertilizer and the beginning of the consumption of labelled phosphorus by the plants; the length of this interval varied from 20 to 42 days.

One of the most effective methods of increasing the yield of winter grain crops (wheat and rye) is top dressing in early spring. The chief role here belongs to nitrogen fertilizers, since in most cases, in early spring the soil contains few nitrogenous compounds that would be available to plants, and the microbiological activity resulting in the mobilization of soil nitrogen is not yet sufficiently intensive. For this reason, the winter crop plants, although weakened during the winter and showing an intensive growth in spring, respond readily to top dressing with nitrogen in early spring.

Potassium and phosphorus fertilizers are mostly applied to winter crops prior to sowing, or at low rates, in the rows, when sowing. In many cases, however, an increase in the yield of winter crops was observed that was due to the influence of phosphorus and potassium fertilizers applied with nitrogen by top dressing in early spring. Top dressing of winter crops with an inorganic fertilizer (NPK) is particularly effective when these crops are planted in unfertilized fallow land and are sown without row fertilization. According to the data from the field experiments[6] carried out on a mass scale in the nonblack earth zone of the USSR, top dressing of winter crops with superphosphate in spring results in an increase of the grain yield that is little inferior to that obtained from nitrogen.

Since in top dressing with phosphates the phosphoric acid of the fertilizer is absorbed by the uppermost layers of the soil, the question arises as to what extent the plant utilizes the phosphorus in the fertilizer if this method of application is practiced. The answer to this question has been provided by field experiments with labelled phosphates.

Observations of the assimilation of labelled phosphorus by plants of winter wheat and rye have shown that absorption of phosphorus from a top-dressed fertilizer applied in spring continues for a long period including the stages of heading and florescence.[3] When this method of application was used, it showed that the amounts of phosphorus obtained by the plants from the fertilizer were relatively small, approaching the average values which were observed when using the method of broadcasting the fertilizer on the soil prior to sowing. In soils with a smaller content of phosphorus, the utilization of a top-dressed fertilizer by winter crops is greater than in soils with a higher content of assimilable phosphorus compounds. With the use of a labelled isotope, it has been established that the movement of phosphorus into the plants from top-dressed fertilizer, applied to winter crops in spring, takes place not only from the soil through the roots but, partly, through the foliage as well. If when applying top dressing, care is taken to prevent particles of the fertilizer from coming into contact with the leaves, a smaller amount of labelled phosphorus is absorbed by the plant than when a part of the top-dressed fertilizer happens to get on the foliage. This difference is particularly noticeable at the beginning of the growing season, during tillering and shooting, and becomes somewhat less noticeable by the time of florescence.

Field experiments with labelled phosphates have shown very clearly the great influence exercised by jointly applied fertilizers upon the rate of the flow of phosphorus into the plants, in particular when applied during the growing season. Joint application of phosphorus and nitrogen fertilizers when top dressing winter crops, as well as when side dressing sugar beets led to a considerable increase in the utilization of phosphorus by the plants. This relationship is particularly characterictic when fertilizer is applied during the growing season. In experiments with fertilizers applied in the row when sowing, an inverse relationship was noted, i.e., a somewhat retarded flow (at the beginning of vegetative growth) of phosphorus into the plant from the superphosphate, when the latter was jointly applied with nitrogen or with nitrogen and potassium fertilizer.

Influence of Liming and Joint Application of Phosphoric and Organic Fertilizers

The tracer technique was successfully applied to a study of the influence of liming the soil and joint application of organic phosphorus fertilizers and lime upon the absorption of phosphorus by plants. In acid soils, the efficiency of phosphorus fertilizers is considerably lowered, due to the absorption of

Table VII. Amount of Phosphorus Absorbed by Wheat Plants from the Row and Main Fertilizers with Local and Complete Liming of the Soil (mg P_2O_5/pot)

Days after germination	Liming of top 5-cm layer of soil only		Liming of the whole soil	
	from row fertilizer	from main fertilizer	from row fertilizer	from main fertilizer
10	2.1	0.1	1.7	4.4
22	5.2	0.5	6.2	28.5
32	5.8	1.6	10.0	42.4
68	9.2	1.5	23.4	83.0

phosphates by the soil and the conversion of the phosphoric acid of the fertilizer into forms only slightly available to plants. Besides lowering the acidity of the soil, liming also reduces the binding action of the sesquioxides upon the phosphoric acid, which has a positive effect upon the efficiency of phosphorus fertilizers. Furthermore, under the influence of liming the increased efficiency of phosphates is due to still other factors. Liming acid soils, and especially, soils with a higher content of mobile aluminium, creates more favourable conditions for the development of a heavy root system, capable of utilizing more fully the nutritive substances contained in the soil in non-mobile forms and, in particular, the phosphorus compounds of the soil and the phosphates absorbed by it.

The action of lime upon the soil is also connected with the biological processes taking place in the soil, since the elimination of surplus acidity promotes a better development of microorganisms, accelerating both the processes of mobilization of the organic compounds of elements of plant food contained in the soil and the processes of biological absorption of nutritious substances.

Experiments carried out with the help of tracer atoms have quite convincingly shown the importance of liming acid soils for increasing the utilization from the fertilizer phosphorus by plants, when various methods of applying phosphates and lime are practiced.

Pot experiments with wheat were carried out to study the influence of liming upon the assimilation of the phosphorus from a labelled fertilizer, applied locally both in the row together with the seed and at a depth of 12–14 cm from the soil surface in the pot (main fertilizer). The hydrolytic acidity being taken into account, lime was either applied only to the top 5-cm layer of the soil in which the seeds and the phosphorus row fertilizer were placed, or to the whole bulk of the soil in the pot. Labelled phosphorus was applied in the form of superphosphate; in the experiments with simultaneous application of row and main fertilizer, the labelled fertilizer, was applied by the one method while the unlabelled superphosphate by the other. In this way, we determined the influence of partial and complete liming of the soil upon the utilization of a definite portion of the fertilizer by the plants, depending upon its location in the soil in relation to the limed layer.

The results of the experiments have shown that liming of the surface layer of the soil creates favourable conditions for the plants to assimilate the phosphorus from the row fertilizer, applied to the layer of the soil that is subjected to liming and has practically no influence upon the extent of the utilization of phosphorus from the main fertilizer, applied at a great depth. By the same experiments it was further shown that the utilization of phosphorus from the row fertilizer by the plants was also augmented if not only the top layer, to which the fertilizer was applied, but the whole bulk of the soil was limed as well. As an illustration of the results of these experiments, we present the following data (Table VII).

The better utilization of the phosphorus from the row fertilizer as compared with that from the main fertilizer when liming the top layer of soil, as well as the extensive movement of phosphorus into the plant from the main fertilizer with total liming, may be explained by the greater availability of the phosphorus of the fertilizer to the plants in limed soil. The increase in the amount of phosphorus utilized by the plants from row fertilizer in the treatment with liming all of the soil in the pot is due to other factors, e.g., to the creation of more favourable conditions for the growth and development of the root system and to the improvement of the general condition of the plants. An increase in the utilization of phosphorus from row fertilizer when the whole soil in the pot had been limed was also observed in the treatment in which only row fertilizer was applied (without combining it with the main fertilizer) (Table VIII).

One of the ways of increasing the efficiency of phosphorus fertilizers, especially, in soils of a high capacity for binding the phosphates, is their joint application with organic fertilizers. The use of tracers in studying the question of the influence of joint application of phosphoric and organic fertilizers upon the assimilation of phosphorus by the plants has

Table VIII. Amount of Phosphorus Absorbed by Wheat Plants from Row Fertilizer (mg P_2O_5/pot)

Days after germination	Liming top layer of soil only	Liming all soil in pot
10	2.2	2.7
22	6.5	11.3
33	7.4	9.4
68	12.9	24.6

Table IX. Absorption by Plants of Labelled Phosphorus from Superphosphate Applied in a Mixture with Humus

Treatment	Days after germination			
	7	21	31	42
	Amount absorbed, thousands of cpm/plant			
3 centn/hect labelled superphosphate applied at a depth of 7–8 cm	0.7	3.6	3.7	5.9
The same in mixture with 2 ton/hect of humus	0.3	2.3	9.3	11.2
3 centn/hect of labelled superphosphate, applied at a depth of 18–22 cm	0.07	4.2	12.8	30.4
The same in mixture with 2 ton/hect of humus	0.02	2.3	17.1	40.9

permitted the establishment of a number of interesting facts. It has proved, that in most cases, joint application with organic fertilizers increases the utilization of the phosphorus of superphosphate by the plants. Under certain conditions, however, cases are noted when the plants utilized the phosphorus of labelled superphosphate from the organo-mineral mixture in a less intensive way at the beginning of the growing stage, this utilization being increased at later periods. As an example, we present the following data obtained from a field experiment with oats (Table IX).

These data show that, in combination with organic fertilizer, the phosphorus of superphosphate is utilized by plants to better advantage not at the early stage of growth, but at the later dates, and when deeply applied. This makes it advisable to test a system of applying phosphoric and organic fertilizers in which, for ensuring increased phosphoric nutrition of the plants at the beginning of the growing stage, a small amount of granulated superphosphate is applied in the rows, while the mixture with an organic fertilizer is applied by plowing it deeply under before sowing.

"FOLIAR FERTILIZATION" OF PLANTS

The fact that the elements of ash nutrients may move into plants not only through the roots but also through the foliage has long been known. The first experiments in foliar nutrition of plants with iron were made almost a century ago. Numerous studies were carried out later on, which showed that not only iron but other elements of mineral plant food as well could be assimilated by the leaves.

The tracer technique has afforded new possibilities in studying this problem and has promoted researches aimed at the elaboration of practical methods of additional control of plant nutrition by means of the so-called "foliar fertilization", i.e., by spraying the leaves with a fertilizer solution.

It has been proved that cotton plants, sugar beets, sunflowers and other species assimilate nutritive substances, applied in this manner, not only under artificially created conditions, when the movement of these substances through the roots is excluded, but also under normal conditions of growth and development, e.g., when most of the nutritive elements enter the plant through the roots and absorption through the leaves presents a source of additional nutrition.

Without using the tracer technique, it is practically impossible, under such conditions, to establish to what extent the additional nutritive elements applied through the leaves are assimilated by the plants, and how they are distributed in the plants.

Extensive studies on "foliar fertilization" of cotton plants with phosphorus were carried out in the Uzbek SSR[7]. One of the chief aims of these experiments was to study the method of foliar fertilization with phosphorus as a means of preventing abscission of the ovaries of cotton plants. The positive results of these experiments have served as a ground for the organization of large-scale field tests of the method of foliar phosphate fertilization of cotton plants, which are now being carried out over an area of many thousands of hectares.

Experiments with sugar beets have shown that additional application of phosphorus to the foliage at the period of sugar accumulation has a positive effect upon the quality of the yield of this crop, increasing the sugar content of the roots. In recent years this method of foliar fertilization of sugar beets has also been tested on a large scale over an area of many thousands of hectares in the USSR.[8]

When observing with the help of tracer atoms the way in which plants assimilate phosphorus (and other elements) in foliar fertilization, we are able to find the most expedient solution of problems that arise in connection with introducing this additional method of controlling plant nutrition and metabolism into practice with the aim of improving the quantity and quality of the yields of farm crops.

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Determination of the Availability of Soil Phosphates and Fertilizers with the Aid of Radioactive Isotopes of Phosphorus

By A. V. Sokolov, USSR

The efficiency of different kinds of phosphorus fertilizers is determined in greenhouse and field experiments. In these experiments various phosphoric fertilizers are introduced into different vessels or experimental plots. At the completion of the experiment, yields are estimated and phosphorus uptake is determined. Experiments are carried out with non-phosphorus fertilizers, with standard fertilizers and the new fertilizer on the basis of which the comparative assimilability of the new fertilizer is evaluated. The use of labeled phosphorus fertilizers enabled us to develop the method of so-called selective phosphate uptake by means of which the degree of assimilation of different kinds of phosphorus fertilizers can be studied. Some results of work done by this method, begun in 1950, have already been published.[1,2,3]

According to this method the kinds of fertilizer to be compared are introduced, not into different vessels, but into a single vessel. In this way the plant is, so to say, requested to decide which of the two available forms it prefers to take up from a single vessel in identical conditions. With two kinds of phosphates in the soil, one less and the other more mobile, the latter is more dissolved and more likely to be assimilated by the roots of the plant.

The determination of the kind of phosphorus taken up by the plant is based on the fact that one of them is tagged, while the other is not. If the standard fertilizer is labeled, several different forms of non-labeled phosphates may be compared with it. But, of course, for soil cultures it is preferable to have replicated experiments, viz, first with the standard form labeled and the other form not labeled, and then *vice versa*.

The method of selective uptake permits the determination of differences in availability of phosphates commonly considered as equivalent. Thus, for example, experiments on thick chernozem and on podsolised soil showed differences in the availability of bisuperphosphates and the precipitates.

In the first variant of these experiments labeled precipitate was used. In the next variant, half of the phosphorus used was in the form of the labeled precipitate and the other half, in that of the non-labeled bisuperphosphate, while in the third variant, on the contrary, half of the dose was in the form of labeled bisuperphosphate and half, in that of non-labeled precipitate; in the fourth, only labeled superphosphate was used.

The results of this experiment are presented in Table I. The activity of the crop for the first variant of the experiment is taken as 100.

It will be seen that with combined application of the precipitate and superphosphate, the comparative availability of the fertilizer was different in the podsolised soil and in the thick chernozem. In the experiment on thick chernozem, the bisuperphosphate was absorbed more readily, while on the podsolised soil the result was opposite.

The two forms of phosphate, introduced in a single vessel, might interfere with each other. If so, the results obtained would not give a true picture of the availability of either of them. This suggestion was checked up in the vegetation experiment. Monocalcium phosphate was employed as the labeled standard fertilizer. Granulated superphosphate and fused magnesium phosphate were studied, being introduced in a half-dose together with labeled monocalcium phosphate. Different ways of application of labeled phosphate were used, preventing to a different degree its interaction with the phosphates under study. The results of the experiment show that the way of packing, and hence of possible exchange reactions between the phosphates is of no significance (Table II). In all instances, the phosphorus supply from labeled bisuperphosphate in the sierozem increased when it was competing not with fused phosphate but with granulated superphosphate; whereas with podsolised soil, granulated superphosphate offered greater competition to bisuperphosphate than did the fused magnesium phosphate. This points to the negative influence of graoulation of phosphates on their availability in sierozem and to its positive influence in acidic soils.

The application of labeled phosphorus makes it possible to determine the utilization coefficient of phosphorus of the fertilizer, without taking recourse to the differential method; according to which this coefficient is determined as follows. The phosphorus content is determined in plants that have received phosphorus fertilization and in those grown in unfertilized soil. The difference indicates the amount of phosphorus received from the fertilizer. Let us assume that wheat received 60 kg of P_2O_5 with superphosphate applied, and 50 kg of P_2O_5 without

Original language: Russian.

Table I. Comparative Assimilation of Phosphorus of the Precipitate and of Bisuperphosphate (Plant, oats; strain, "Pobeda")

No. of variant of experiment	Labeled phosphate		Non-labeled phosphate		Comparative activity of crop	
	Fertilizer	Dose	Fertilizer	Dose	Thick chernozem	Podsolised soil
1	Precipitate	1.0	—	—	100	100
2	Precipitate	0.5	Bisuperphosphate	0.5	39	56
3	Bisuperphosphate	0.5	Precipitate	0.5	53	48
4	Bisuperphosphate	1.0	—	—	96	89

the application of superphosphate; then the difference in the phosphate, amounting to 10 kg P_2O_5, is accepted as the dose of phosphorus received from the fertilizer. The dose of superphosphate was 50 kg P_2O_5; hence, the coefficient in this case is 20%. Certainly, it is tacitly assumed that the phosphorus uptake from soil is the same in both fertilized and unfertilized ground.

But as we have seen (Tables I and II), phosphate utilization depends on the presence of other phosphates in the nutritive medium. Eventually, the application of soluble phosphates causes a sharp decrease in the utilization of soil phosphates, which becomes different in fertilized and unfertilized vessels. This is graphically illustrated by the results of an experiment carried out with different soils, viz., podsolised soil, thick chernozem and typical sierozem (Table III). It thus appears that all three possibilities occurred, i.e., the administration of the phosphorus fertilizer decreased, increased, or did not alter the utilization of soil phosphates by the plants.

Thus, the assumption that soil phosphates are always utilized alike, both with fertilization and without it, is not valid. Hence, there is no ground for determining the actual phosphorus supply from the fertilizer by subtracting the amount of phosphorus in the plant grown without fertilization from that in the plant grown with fertilization.

The actual supply of phosphorus from the fertilizer as determined with the aid of labeled fertilizer may be substantially different from the arbitrary value computed from the difference. This is obvious from the results of greenhouse experiments in which samples of soil were taken from experimental plots that had been treated for several years with different kinds of phosphorus fertilizers. The soils of these plots contained different amounts of assimilable phosphates, since in the field experiment plot 33 did not receive any phosphoric fertilizers, plot 2 received one dose, plot 5 a double dose, while the next plot was limed (Table IV).

The higher the amount of available phosphorus in the soil, the greater the amount of phosphorus (labeled) taken up by the plants from the fertilizers, in comparison with the amount determined by difference.

The method of selective phosphate uptake can be applied not only to evaluate the availability of phosphorus fertilizers, but also to estimate that of soil phosphates. On the assumption that the degrees (coefficients) of utilization of labeled fertilizer phosphorus and of the available soil phosphorus are similar it is easy to compute from the dose of labeled phosphate introduced into the soil and the uptake of labeled and non-labeled phosphorus by the plant, the total amount of available soil phosphates.

The introduction of soluble phosphates into the soil alters the composition of soil phosphates and the larger the fertilizer dose, the stronger is its influence on both the qualities of the soil phosphates and on their assimilation by plants. The following method is therefore suggested for the determination of the amount of assimilable soil phosphates by means of labeled phosphorus. The least possible amount of labeled phosphate (no more than 1 mg P_2O_5 kg soil), containing 10 to 100 μc of radioactive phosphorus

Table II. Effect of Introduction of Granulated Superphosphate and Fused Magnesium Phosphate on the Phosphorus Supply from Labeled Bisuperphosphate

Mode of administration of labeled bisuperphosphate	Fertilizer added	Supply of labeled phosphorus, mg P_2O_5/vessel	
		Podsolised soil	Sierozem
In solution, mixed with all the soil	Granulated superphosphate	33	45
	Fused phosphate	36	40
In solution, mixed with half of the soil	Granulated superphosphate	30	49
	Fused phosphate	37	44
Pulverized, mixed with all the soil	Granulated superphosphate	31	—
	Fused phosphate	37	—
Pulverized, mixed with half of the soil	Granulated superphosphate	32	32
	Fused phosphate	39	24

Table III. Experiment on 1952 Wheat. Effect of Applying Fertilizers on the Uptake of Phosphates from the Soil

Mode of administration of phosphate	Phosphate uptake from soil, mg P_2O_5		
	Podsolised soil	Sierozem	Thick chernozem
Without phosphorus	75.5	166.3	227.4
In a layer	96.8	104.2	187.0
Throughout vessel	68.7	113.7	227.1

isotope, is dissolved in the total amount of water introduced in the packing of the vessel. This solution is added to the soil sample, being carefully mixed with it, and left for three days. Then it is again carefully mixed and transferred to the experimental vessel. With such a procedure, the introduction of labeled phosphate does not influence the content of different fractions of assimilable phosphates in the soil.

After completion of the experiment and determination of total phosphorus of the crop and of its activity, the content of the assimilable soil phosphates is estimated from the formula $x = (P \times 100)/k$ where P is the phosphorus uptake by the plants, and k the per cent of P^{32} used by the plants.

The amount of assimilable phosphates determined in this way is however arbitrary, since it is based on the false assumption that the labeled phosphates and assimilable phosphates of the soil are used to the same degree. The soil contains many different kinds of assimilable phosphates, and their utilization by plants depends not only on their properties but on their content in the soil as well as on the experimental conditions. The difference in utilization of phosphates of fertilizers and of soil phosphates at the beginning and toward the end of the experiment, as noted both by Soviet and foreign students, also indicates the arbitrary character of the results obtained.

The application of common methods, viz. the introduction into the soil of labeled phosphates, for determining the amount of assimilable soil phosphates, as done by Larsen[4] and Fried and Dean,[5] yields still more arbitrary results, since with considerable amounts of labeled phosphates the plants may primarily take them up, as they are more mobile. The formula used by Larsen, Fried and Dean for computation is also based on the assumption that soil phosphates are utilized in proportion to the use of introduced labeled phosphates.

The radioactive phosphorus isotope is used for determining the amount of assimilable soil phosphates and in chemical analyses of the soil as well. It is well known that, upon isolation of assimilable phosphates from the soil by different extraction methods, exchange reactions occur between phosphate ions of the solid and liquid phase of the soil, resulting in a secondary dissolution and precipitation of phosphates. A balanced concentration of phosphates in the extract is considered to be the result of this dissolution and precipitation, viz. of an exchange between the solid and liquid phases, in the broadest sense of the word. The addition of a radioactive isotope makes it possible to determine the amount of substance in the solid phase that can participate in the exchange reactions between the solid and liquid phase.

Upon equilibrium a similar ratio should exist between the amounts of P^{32} and P^{31} isotopes in the solid and liquid phase, that is,

$$\frac{P^{32}\ \text{solid}}{P^{32}\ \text{liquid}} = \frac{P^{31}\ \text{solid}}{P^{31}\ \text{liquid}}$$

The slight amount of added labeled phosphate should not disturb the equilibrium ratio between the phosphates in soil and in solution.

The presence of "exchange" phosphates in the soil in the same sense as the presence of "exchange" bases absorbed by negatively charged colloid particles of the soil raises some doubts. Yet there is no doubt that in the soil there are present exchange forms of phosphates, i.e., phosphates capable of being forced into the solution by other phosphates. But these exchange phosphates may be quite different in their availability for plants. This is evidenced from the stability of phosphate fixation by soils.

These experiments were performed in the following way (Table V).

One hundred mg P_2O_5 of labeled monocalcium phosphate, and in other variants of the experiment

Table IV. Greenhouse Experiment on the Application of Labeled Phosphates in Podsolised Soil

No. of field experiment	Way of introduction of phosphates into vessels	Increase of crop, gm	Phosphorus taken up from the fertilizer, in mg P_2O_5, as determined by the	
			Method of labeled fertilizer	Differential method
Plot 33	In a layer	25.4	78.0	84.2
	Throughout vessel	7.2	29.5	32.6
Plot 2	In a layer	14.1	79.4	58.8
	Throughout vessel	5.5	40.6	36.2
Plot 5	In a layer	7.3	62.4	3.8
	Throughout vessel	5.1	43.8	18.2
Limed plot	In a layer	4.0	74.7	33.7
	Throughout vessel	4.6	57.4	30.2

Table V. Fixation of Phosphates by the Soil. (Phosphorus content of different extracts in mg P_2O_5/kg of soil and supply of phosphorus from the fertilizer)

Soil	Mg P_2O_5 introduced	Extracts: Carbonic acid, Total	Carbonic acid, Labeled	Acetic, Total	Acetic, Labeled	Phosphoric acid (exchange phosphates)	Labeled phosphorus taken up by wheat crop
Thick chernozem	100*	25	20	61	47	56	20.1
	100* + 900	442	43	734	63	61	—
Typical Sierozem	100*	32	23	68	61	64	19.2
	100* + 900	585	61	824	78	47	—
Podsolised soil	100*	6	4	35	22	53	12.0
	100* + 900	217	21	447	44	51	—
Podsolised sandy-loam	100*	4	3	51	34	53	3.8
	100* + 900	207	18	622	55	42	—
Red soil	100*	—	—	2	1	47	1.3
	100* + 900	4	—	87	7	49	—

* Labeled P.

100 mg of labeled and 900 mg P_2O_5 of non-labeled phosphate were introduced into the soil and after a thorough mixing were left for two weeks. Analyses of the soil were made after the F. V. Chirikov method. The vegetation experiments were made with wheat. Introduction of non-labeled phosphate increased the passage of labeled phosphate into the solution. This was the case irrespective of the time of introduction of labeled and non-labeled phosphates. Hence, the degree of phosphate fixation by soil depends on the total amount of introduced phosphates. Carbonic acid and acetic extracts distinctly illustrate the differences between the soils with regard to phosphate fixation. The results obtained with these extracts agree with those obtained with the plants, as regards the amount of labeled phosphorus taken up by wheat.

The extraction of labeled phosphate by exchanging it for non-labeled phosphate was made by means of phosphoric acid. The figures obtained with this extract have no bearing on the availability of soil phosphates for plants. When the phosphates fixed by red earth are no longer available and barely soluble, the content of exchange phosphates is high. The phosphoric acid extract was made with $^1/_{15}$ molar buffer solutions of sodium diphosphate and potassium monophosphate (after Sörensen) at pH 7.0. A 5-gm sample of soil and 125 ml of the buffer solution were stirred for two hours and then the P^{32} content of the filtrate was determined. The results obtained refute the idea that "exchange" phosphates are the assimilable phosphorus in all soils.

SUMMARY

The method of selective uptake of phosphates as developed in this paper makes possible a comparison between different forms of phosphate and an estimate of their availability under absolutely identical conditions. Differences between phosphate forms escaping common methods of study can thus be established.

In applying the method of selective uptake for determining the availability of soil phosphates, minimum doses of labeled phosphorus should be used, not more than 1 mg P_2O_5 per vessel to avoid any influence on the content of available soil phosphates. The assimilable phosphates of the soil are determined by the formula $x = (P \times 100)/k$, P being phosphorus uptake by the plant, and k the percentage of P^{32} utilized by it.

The application of the common vegetation test, in which large amounts of labeled phosphates are introduced, does not give a true picture since in such tests the composition and degree of utilization of the soil phosphates are different from those obtained in non-fertilized soil.

The mobility of phosphates in the soil depends on the dose. The introduction of large doses of phosphates ensures a comparatively large solubility in carbonic acid and acetic extracts. The phosphates introduced into the soil are largely in the exchange form and may be withdrawn by phosphate extracts, whose analysis data do not agree with those on the assimilation capacity in plants.

The determination of the coefficient of utilization, based on data on phosphorus uptake by the plants from fertilized and unfertilized soil, lacks any scientific ground since utilization of soil phosphates may be different with fertilized and unfertilized soil. The phosphorus compounds of the soil vary in their availability. The amount and extent of their utilization are changed by phosphorus fertilizers introduced into the soil. Hence, it is necessary to check all the current concepts on the coefficient of utilization of phosphorus fertilizers with the aid of labeled fertilizers.

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Co^{60} in the Study of the Role of Cobalt as a Micro-Element in the Nutrition of Plants

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In the drive for abundance of agricultural products carried on by our country which is building communism, great importance is attached to chemical aspects of land husbandry—the wide-scale application of mineral and organic fertilizers and other chemical methods of soil improvement. Chemical methods provide particularly effective means of increasing the productivity of agricultural crops. It is therefore necessary to fully utilize all possible ways of applying fertilizers. Apart from fertilizers already being used on a considerable scale, it is necessary to introduce into practice also those that at present are seldom or not at all utilized.

This first of all refers to microfertilizers, of which we now apply boron, copper and manganese. Research findings, however, state that respectable increases in crops grown under our conditions of soil and climate can be produced by various other micro-elements as well. Therefore research institutions of the Soviet Union are faced with the task of conducting a thorough study of the influence on agricultural crops of fertilizers containing these minor elements, and of determining the optimal conditions of their application.

One of the minor elements of this kind whose application might greatly facilitate the work of collective farms in increasing crop productivity is cobalt. Many investigations have been conducted on the subject of the significance of cobalt for agriculture. The result has been the elucidation of a number of points having serious bearing on cattle-breeding. These are the works of V. V. Kovalsky and V. S. Chebaevskaya,[1] Y. M. Berezin,[2] B. Malashkaite,[3] M. Rambidi.[4] Studies have also been made of various problems referring to the application of this micro-element in medicine.[5,6,7] In connection with studies on the importance of cobalt for medicine and cattle-farming several reports have been published on its occurrence in various plants and soils.[8,9,10,11,12,13] However, in spite of the large amount of work on this subject, the literature shows that some workers still hold the incorrect opinion that cobalt has no effect on the growth, development and yields of agricultural plants, and that therefore cobalt fertilizers have no significance for agriculture.[14,15]

This erroneous concept has been refuted by the works of the Institute of Socialist Agriculture of the Academy of Sciences of the Byelorussian SSR and the Laboratory of Soil Liming of the All-Union Research Institute of Fertilizers, Soil Management and Soil Science of the Lenin Academy of Agricultural Sciences in USSR.

As far back as 1942, in a greenhouse experiment carried out by the author together with O. E. Kedrova-Zikhman,[16] in conditions of limed sod-podzol soil, cobalt gave a considerable increase in the total crop, as well as intensifying rubber-accumulation in the crop of kok-saghyz roots.[16]

The results of subsequent works conducted by ourselves and L. N. Protashchik, beginning in 1947, showed that the findings of the above experiment with kok-saghyz were applicable to a number of other crops—clover, flax, winter wheat, and showed that the effect of cobalt on plants grown on acid sod-podzol soils to a great extent depends on liming. When such soils are limed, cobalt produces a considerable increase in the crops, but when this element is applied to non-limed soil, its effect is only slightly positive, entirely absent, or sometimes even negative.[17]

The result of vegetational and field experiments was to elucidate a number of other points concerning the effect of cobalt on plants. It was established that the positive effect of cobalt is more apparent in regard to reproductive than to vegetative organs; that cobalt may considerably accelerate the maturing of seed; that the influence of cobalt may bring about an increase in the fat and fibre content of linseed and in the sugar content of sugar beet; that the levels of cobalt recommended for application to increase the current year's crop (1–1.5 kg cobalt/hectare), may have considerable after-effects on plants sown in the following year.[17,18]

Greenhouse and field experiments of recent years conducted by ourselves together with R. E. Rosenberg in the Institute for the Improvement of Marshlands of Byelorussian SSR Academy of Sciences, showed also, that cobalt has a positive effect on agricultural crops even on lowland peat-bog soils without lime treatment.[18]

In recent years the positive effect of cobalt on grapes was noted in experiments carried out by O. K. Dobrolyubsky.[19] In addition, the positive

Original language: Russian.

influence of Co^{60} radiations on plants has been noted in recent studies by Acad. P. A. Vlasyuk.[20]

Beginning in 1951, the author with A. N. Kozhevnikova conducted a series of experiments on the application of the radioactive isotope Co^{60}. In 1954, this work was also taken up by A. F. Agafonova. The object of these investigations was firstly, to establish with the help of Co^{60} the effect of cobalt on soil and plants with regard to liming, and secondly, to study the effect of radioactive radiations of the cobalt isotope on agricultural plants.

These works consisted of greenhouse experiments followed by laboratory analysis, such as calculation of activity. The experiments were conducted in Mitcherlich vessels of the usual size. The soils used were the sod-podzol of the Forest Reserve of the Timiryazev Agricultural Academy, and that of the Central Experimental Station of the All-Union Research Institute of Fertilizers, Agrotechnique and Soils. The first soils to be taken, were sand-loam, while the other two kinds were medium clay loam. Agrochemical characteristics of these soils are given in Table I.

For the greenhouse experiments of the first two years in which we tested the comparative effect on agricultural crops of normal and radioactive cobalt, we took the soil of the Forest Reserve of the Timiryazev Academy, which we placed in each vessel in quantities of 6 kg dry weight. The main experimental crop was barley. The effect of cobalt was tested in this experiment on limed and non-limed soil. Lime was applied as a chemically pure preparation of calcium carbonate calculated for singular hydrolytic acidity. All vessels were likewise fertilized with other nutritive elements: nitrogen, phosphorus, and potassium, 0.5 of active substance per vessel. In addition to non-radioactive cobalt, some of the vessels, according to the plan, were treated with radioactive cobalt at two levels, corresponding to 160 μc and 480 μc. Both common cobalt and its radioactive isotope were applied in the form of a nitrate. In the main part of the experiment, cobalt was applied at levels of 6 mg Co, while in the variant with the higher dose—at a level of 20 mg per vessel. Each experiment was repeated four times.

Table I. Agrochemical Soil Assay

Agrochemical items	*Soil from forest territory of T.A.A. II quarter*	*Soil from C.E.S. Baribino I*	*Soil from C.E.S. Baribino II*
pH of salt extract	4.3	4.3	4.2
Hydrolytic acidity, me/100 gm soil	4.8	4.0	6.5
Exchange acidity, me/100 gm soil	—	0.4	0.5
Sum of absorbed alkali, me/100 gm soil	6.9	6.4	5.7
Degree of soil saturation with alkalis, %	59.3	65.0	46.5
Al (after Peive) mg/100 gm	10.1	—	—
Al (after Sokolov) mg/100 gm	—	3.2	3.9
Humus (after Tyurin) %	—	2.9	2.0

The crop figures of one of these experiments conducted in 1951 are given in Table II.

The data show that cobalt had a favourable effect on the barley crop on limed soil and acted negatively on non-limed soil. Co^{60} intensified both the positive and the negative action of cobalt. On non-limed soil there was no increase of total crop and grain crop under the influence of cobalt. In most cases there was even a marked decline.

In subsequent work we studied the transformations of cobalt compounds in the soil, their entry into plants depending on the lime treatment of sod-podzol soils. Some of the results obtained are given below.

In one of the experiments the effect of common cobalt was compared with that of a mixture of common cobalt with its radioactive isotope, Co^{60}. The soil for this experiment was taken from the Central Experimental Station in Baribino I. The cobalt was applied at two levels, 7 mg and 20 mg per vessel. In the vessel with Co^{60}, the latter's content corresponded to 160 μc and 480 μc. The effect of non-radioactive cobalt and that of Co^{60} was compared on limed and non-limed soil. Therefore some of the vessels, according to the experimental plan, were supplied with chemically pure $CaCO_3$ in a dose corresponding to single hydrolytic acidity. Yields from this experiment are given in Table III.

As seen from the data in the table, the total yield and the yield of grain was considerably greater on lime-treated soil. The effect of radioactive radiations was likewise favourable in all cases. So, the results of this experiment demonstrate that the application of Co^{60} at suitable levels, leads to the radiations exercising a favourable effect on the growth, development and yield of plants.

To explore the transformations of cobalt in the soil and the influence of liming on the mobility and accessibility of cobalt compounds for plants, we conducted an assay of the radioactivity of the liquid accumulating in the saucers under the Mitcherlich vessels when the soil is watered. For this purpose, the soil in the vessels after the crop had been harvested was watered until one liter of filtrate had been collected in the saucers. This liquid was evaporated to 50 ml, of which 10 ml were taken for assay, evaporating it till dry and measuring its activity, after which its cobalt content was assayed. The results of these tests are given in Table IV.

The data of the table show that the cobalt content of the filtrate varied considerably, depending on the quantity of the element introduced into the soil and on liming.

From the non-limed soil (when the lower level, 7 mg per vessel, was applied) only about thirty per cent less, and not three times less cobalt passed into the filtrate, than when the highest dose (20 mg per vessel) was applied. This relationship between the quantity of cobalt introduced into the soil and the

Table II. Effect of Non-radioactive Cobalt and Co^{60} on Barley Crop (6 mg Co/vessel). Greenhouse Experiment, 1951

Experimental conditions	*Experimental variables*	*Total yield*		*Yield of grain*	
		Gm/vessel	*%*	*Gm/vessel*	*%*
Without lime	Without Co	25.2	100	7.9	100
	With non-radioactive Co	20.2	80	6.0	77
	With Co^{60}	21.8	87	8.2	104
With lime	Without Co	34.6	100	13.1	100
	With non-radioactive Co	38.6	111	15.3	117
	With Co^{60}	43.8	126	17.6	135

Table III. Barley Crop in an Experiment with Common Cobalt and Co^{60} Greenhouse Experiment, 1952

Experimental variables	*Without lime*				*With $CaCO_3$*			
	Total yield		*Yield grain*		*Total yield*		*Yield of grain*	
	gm/vessel	*%*	*gm/vessel*	*%*	*gm/vessel*	*%*	*gm/vessel*	*%*
Common Co lowest level	32.5	100	10.4	100	51.2	100	16.7	100
Co^{60} lowest level	41.5	129	15.7	151	55.2	108	22.6	135
Common Co highest level	31.6	97	10.0	96	46.8	91	16.4	98
Co^{60} highest level	39.1	123	14.9	143	55.2	108	22.2	132

quantity remaining in the filtrates, after application of various doses, shows that most of the cobalt enters into the absorbing complex of the soil. As a result of this, the passage of the cobalt from the soil in the vessels to the filtrate is determined not only by its total content in the soil, but by the dynamic equilibrium existing at every given moment between the cobalt cations of the adsorptive soil complex and the cations of the soil solution. In addition, the passage of cobalt from the soil to the liquid must also be influenced by its adsorption into the roots of higher plants and by its consumption by micro-organisms.

When the soil was limed and the cobalt applied at the higher level, a considerably smaller amount of cobalt passed into the filtrate than from non-limed soil, while with the application of the lower level of cobalt only slight traces of it were detected in the liquid. This may be explained by the conversion of the soluble cobalt compounds of the soil into a non-dynamic and barely soluble condition in the filtrate, which is actually a diluted soil solution. It also follows from the table, that the influence of lime makes the soluble cobalt compounds assume a less mobile state, than they had when adsorbed by the particles of the adsorbing complex.

To investigate the effect of liming on the passage of cobalt from the soil into plants, and the distribution of cobalt in the different organs of the plant, assays were made of the activity of the barley crop. For this purpose, the weight of the separate organs in the crop was calculated per vessel. Then the dry mass of the separate organs was reduced to ash and the latter's activity was gauged by the Geiger-Müller counter. By assaying the results of these measurements and the content of Co^{60} in the cobalt preparation used for the experiment, we established the cobalt content of the ashes and the plant itself. Reliable results were obtained only for variants with the higher level of Co^{60}. The data of these tests are given in Table V.

The data of Table V show not only that barley may grow and develop normally at very high levels of radioactive cobalt (up to 480 μc) but that all organs of the plant may increase in weight.

Under the influence of liming the cobalt content of the plant as a whole and that of its individual parts fell sharply. This means that the cobalt compounds having passed into a less mobile state at liming had become less available to the plants but had not yet become absolutely unavailable to plants and were to a certain extent absorbed by the latter. The table also demonstrates that the plant's organs (in this case, barley) vary considerably in their cobalt content. Especially large is the amount accumulating in the roots. Least of all is the percentage of cobalt in seed. The percentage of the cobalt content both of the whole plant and of its separate parts may fluctuate between very broad limits, depending on the content of this minor element in the nutritive medium. The cobalt content of plants may be many times more than the quantity needed for their normal development. This particularly refers to the leaves.

The results of the latter experiment were fully confirmed in a greenhouse experiment likewise con-

Table IV. Activity and Cobalt Content of Filtrate. Greenhouse Experiment, 1952

Experimental variables	*Activity, cpm/l*	*Cobalt content, mg/l*
Co^{60} lowest level	510	0.00179
$CaCo_3$ + Co^{60} lowest level	Within limits of background	—
Co^{60} highest level	755	0.00265
$CaCo_3$ + Co^{60} highest level	415	0.00145

Table V. Weight and Activity of Separate Organs of Barley. Greenhouse Experiment, 1952

	Experimental variables	Leaves	Stalks	Pellicles and awns	Grain	Roots	The whole plant
Weight, gm/vessel	Co^{60}	9.8	8.7	5.6	14.9	6.1	45.1
	$CaCo_3$ + Co^{60}	14.0	12.5	6.5	22.2	8.1	63.1
Activity cpm/20mg dryweight	Co^{60}	882	2334	1116	1001	6220	11,559
	$CaCo_3$ + Co^{60}	26	79	46	38	771	960
Cobalt content, %	Co^{60}	0.000420	0.000634	0.000275	0.000080	0.006200	—
	$CaCo_3$ + Co^{60}	0.000010	0.000015	0.000011	0.000004	0.000823	—

ducted with barley in 1953, but on another type of sod-podzol soil with a heavier texture, obtained from the Central experimental station in Baribino II of the All-Union Institute of Fertilizers, Soil Management and Soil Science. The findings of this experiment are not given in the present paper.

Apart from experiments on barley, to explore the above mentioned problems we conducted a series of experiments with other crops, considerably varying in their biological features: white mustard, turnip, radish, red-beet, French beans. The soil for these experiments was taken from the territory of the Central Experimental Station in Baribino I.

In these experiments we were obliged to apply Co^{60} at a rather high level amounting to 342 μc, since, when the level was lower, assays of activity often gave obscure results. When this level was applied, although there were a number of occasions when the crop yield was lowered, the activity and therefore the cobalt content of the plants could be measured with sufficient accuracy.

Table VI. Effect of Non-radioactive Cobalt and C^{60} on White Mustard Crop. Greenhouse Experiment, 1954

Experimental variables	Total yield		Yield of seed	
	gm/vessel	%	gm/vessel	%
Control	1.4	100	0.15	100
Non-radioactive Co	2.9	204	0.16	107
Co^{60}	0.9	63	0.08	53
$CaCo_3$	38.8	100	9.31	100
" + non-radioactive Co	40.9	106	9.57	103
" + Co^{60}	34.0	87	10.31	111

One of the experiments (in Mitcherlich vessels) was conducted with white mustard. The effect of cobalt was investigated on limed and non-limed soil. The cobalt was applied at levels of 21.6 mg per vessel; some of the vessels contained Co^{60} in addition to common cobalt. In all other respects the conditions of this experiment corresponded to those established for the experiment conducted in 1952. Yields are given in Table VI.

The data in the table show that in non-limed soil the plants developed very feebly; hence the data obtained did not admit any definite conclusions. On limed soil, however, the plants developed normally; in the presence of lime, the radioactive cobalt isotope gave a somewhat larger yield of seed than non-radioactive cobalt applied at the same level. The yield of the whole crop was however less when Co^{60} was applied.

Different parts of the plants from the harvested crop varied in their radioactivity (Table VII). The data show that in all the organs of mustard cultivated in limed soil with the low concentration of cobalt compounds accessible to the plant (but sufficient for their normal development), the activity, and therefore the cobalt content of non-radioactive and radioactive cobalt is many times less than in plants cultivated in non-limed soil with a high concentration of cobalt accessible to plants.

Table VII. Activity of Different Organs of White Mustard, cpm/100mg Dry Weight

Plant organs	Co^{60}	$CaCO_3$+Co^{60}
Seed	117	17
Leaves	1010	22
Stalks	771	25
Pods	904	178
Roots	1220	297

Table VII likewise shows that the cobalt content in various organs of white mustard (as with barley) is different and may vary within broad limits, depending on the amount of cobalt compounds available to the plant. Both in non-limed and limed soil the greatest amount of cobalt had accumulated in the roots of mustard and least of all in the seeds. In plants cultivated in limed soil, with sufficient cobalt compounds available to the plant, the organs stand in the following order as regards cobalt content: roots, pods, stalks, leaves, seeds.

Another greenhouse experiment for estimating the comparative effect of common and radioactive cobalt was conducted with turnips. These experiments were carried out in Mitcherlich vessels under the same conditions as the one with white mustard. The results obtained are given in Table VIII. The data show that on non-limed soil non-radioactive cobalt had no marked effect on the turnip crop, whereas on limed soil it produced a certain increase in the total yield, and in the yields of roots and foliage. But under the influence of the radioactive cobalt isotope the total yield and the yield of foliage fell,

Table VIII. Effect of Common Cobalt and Co^{60} on Yield of Turnips. Greenhouse Experiment, 1954

Experimental variables	Total yield		Root yield		Dry weight of foliage	
	gm/vessel	%	gm/vessel	%	gm/vessel	%
Control	174	100	97	100	13.4	100
Non-rad. Co	181	104	101	105	13.7	103
Co^{60}	145	90	98	101	11.5	86
Lime	195	112	104	107	15.9	119
Lime + non-rad. Co	206	119	109	113	18.2	136
Lime + Co^{60}	169	97	100	103	11.7	88

both on limed and on non-limed soil, while the yield of roots was not noticeably affected. It might be supposed that the level of Co^{60} used in these experiments was too high.

The above experiments clarified certain points concerning the effect of cobalt on plants, but they had no bearing whatever on the influence of cobalt on the physiological processes concerned. For this reason, we assayed the rate of respiration and catalase activity in turnip leaves. The results obtained are given in Tables IX and X.

Table IX. Effects of Common and Radioactive Cobalt on the Rate of Respiration in Turnip Leaves

Experimental variables	Co_2 output, mg/hr/gm fresh weight
Control	0.64 ± 0.02
Non-rad. Co	0.79 ± 0.03
Co^{60}	0.84 ± 0.07
Lime	0.68 ± 0.12
Lime + non-rad. Co	0.73 ± 0.17
Lime + Co^{60}	0.70 ± 0.04

Table X. Effects of Non-radioactive Co and Co^{60} on Catalase Activity in Turnip Leaves

Experimental variables	Catalase activity
Control	8.50 ± 0.20
Non-rad. Co	7.48 ± 0.02
Co^{60}	8.25 ± 0.02
$CaCo_3$	8.10 ± 0.30
" + non-rad. Co	8.65 ± 0.20
" + Co^{60}	8.55 ± 0.25

As shown by the data of Table IX, under the influence of cobalt, the rate of respiration of plants cultivated on non-limed soil increased noticeably, the positive effect of the radioactive isotope manifesting itself rather more strongly than that of common cobalt. On the other hand, on limed soil both the influence of common and of radioactive cobalt caused merely a slight tendency towards acceleration of respiration. Apparently a much smaller amount of cobalt entered the plant from the limed soil, where the concentration of cobalt compounds available to the plant was lower than from the non-limed. Therefore the amount of cobalt available in the limed soil turned out to be insufficient to noticeably affect the rate of respiration.

The data of Table X show that on non-limed soil the cobalt exercised a depressing effect on catalase activity. This effect was more pronounced with common cobalt than with its radioactive isotope. On the contrary, on limed soil the effect both of common and radioactive cobalt was to cause a certain increase in catalase activity. This indicates that with respect to catalase activity, the level of cobalt applied in this experiment was somewhat too high.

Comparing the crop figure of the experiment with turnip with the data of that with respiration intensity and catalase activity of turnip leaves, we see that the optimum doses of cobalt needed for increasing crops, respiration intensity and catalase activity, do not coincide. When the applied dose of cobalt was too high for increasing yields and catalase activity, it was insufficient to increase the rate of respiration.

In addition, Co^{60} caused a reduction in the turnip yield, whereas with common cobalt this did not occur. However, in its effect on respiration and catalase activity, Co^{60} had roughly the same positive effect as common cobalt. Thus, the effect of cobalt on plants is of considerable complexity, and to a large extent depends on the conditions of cultivatiun.

The third experiment in which the effect of common and radioactive cobalt on plants was compared, was conducted with radish, likewise with the use of Mitcherlich vessels of the usual size, under the same conditions as in the experiment with white mustard and turnip. Yields obtained in this experiment are given in Table XI. The data cited above show that liming led to a considerable increase both in the total yield and in the yield of tubers alone. Cobalt in all cases, on limed and non-limed soil, had a negative effect on both crops. The negative effects were more pronounced in the case of Co^{60} than in that of non-radioactive cobalt.

Table XI. Effects of Non-radioactive Cobalt and Co^{60} on Radish Crops. Greenhouse Experiment, 1954

Experimental variables	Total yield		Yield of tubers	
	gm/vessel	%	gm/vessel	%
Control	173	100	92	100
Common Co	157	92	83	91
Co^{60}	129	75	60	65
$CaCo_3$	274	159	159	179
" + common Co	226	130	135	147
" + Co^{60}	176	102	113	122

Data on activity in various parts of the plant are given in Table XII. They show that radish plants, like all other experimental plants grown on non-limed soil, accumulated cobalt in much greater quantities than plants growing on limed soil. The data also show that there is great disparity in cobalt content between the various parts and organs of radish. The cobalt content in them may fluctuate very broadly. In plants grown on limed soil, with no cobalt surplus but with sufficient cobalt to ensure a normal crop, the greatest concentration of cobalt is found in the fine rootlets. Much less cobalt is to be found in various parts of the edible stalks, still less in the leaves and least of all in the veins and petioles of the leaves.

On the other hand, plants grown on non-limed soil accumulated the greatest concentration of cobalt compounds in the fine rootlets and in the lower leaves. Noticeably less cobalt was found in the middle leaves and still less in the upper. The cobalt content of the various parts of the stalk and the veins and petioles of the leaves was even less than in the rootlets and leaves.

Analysing the data on the activity of the radish in our experiment, we may note the following characteristic features concerning the distribution of cobalt between the various parts and organs of radish. Firstly, in cultivation on limed soil with a low but sufficient concentration of cobalt compounds available to the plant, an especially high cobalt concentration was found in the fine rootlets compared with that in the other organs. Secondly, especially intensive accumulation of cobalt was found in the radish leaves when there was a high concentration of cobalt in the nutritive medium and the soil had not been limed. Thirdly, with a high concentration of available cobalt in the nutritive medium, its content in the leaves is considerably higher than in the edible stalks whereas with a lower though sufficient concentration of cobalt in the nutritive medium, on the contrary, more cobalt is concentrated in the stalks than in the leaves.

Table XII. Activity of Various Parts and Organs of Radish in imp/min per 100 mg of Dry Substance.

Organs and parts of the plant	Co^{60}	$CaCO_3 + Co^{60}$
Fine rootlets	823	218
Radish tip	415	47
Middle part of radish	292	62
Top part of radish	331	74
Leaves, lower pair	918	37
Leaves, middle pair	748	42
Leaves, upper	569	30
Nerves and pedicels of lower pair of leaves	273	26
Of middle pair	432	28
Upper leaves	192	34

CONCLUSIONS

Our findings on the cobalt nutrition of plants studied with the help of Co^{60}, and on the effect of radioactive cobalt on plants, allow us to draw the following conclusions.

In acid sod-podzol soils, the amount of cobalt available to plants is quite considerable, therefore the application of even small doses of cobalt fertilizer to these soils does not usually produce a noticeable increase in yields, does not increase the crop at all, or even acts negatively.

When sod-podzol soils are subjected to liming, the mobile cobalt compounds are transformed into a less soluble state less available to plants, as a result of which the nutritive conditions of the soil with regard to cobalt are sharply worsened. When soluble cobalt compounds are applied under such conditions, the former also become less accessible to the plants, but, in spite of that, the nutritive condition of the soil in regard to cobalt becomes markedly improved. Therefore cobalt fertilizer in conditions of limed sod-podzol soils, produces a considerable increase in the productivity of agricultural crops.

When cobalt is applied as a soluble salt, apart from being converted into a less mobile state under the influence of lime, a considerable proportion of this minor element is absorbed by the soil (by its colloid complex) as an outcome of cation exchange. This process occurs both in limed and non-limed soils.

When cobalt compounds are converted under the influence of lime into a state less available to plants, a much smaller quantity of the element enters the plant than on soil that has not been limed. This refers both to the plant as a whole and to all its separate organs.

The cobalt content of various parts of the plant is unequal and varies widely with the content of cobalt compounds available to the plant in the nutritive medium. The cobalt content is highest in the roots, and, in case of excess cobalt nutrition, in the leaves. It is lowest in the seeds. With a suitable level of Co^{60}, the radioactive radiations may have a positive effect on the growth, development and crops of plants (barley, white mustard). Excessive doses, on the other hand, may have an injurious effect.

Cobalt may have both a positive and a negative effect on the rate of respiration and the catalase activity of the leaves of plants (turnip), depending on the concentration.

The use of the radioactive cobalt isotope Co^{60} has greatly assisted studies of the transformations of cobalt in the soil in connection with liming and has shed light on the entry of cobalt into plants and its distribution among various plant organs. This isotope must be widely employed in further investigations of the interaction of cobalt with the soil and the effect exercised by this element on the growth, development, and metabolic processes of plants.

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Application of the Isotope Method to the Study of Adsorption of Electrolytes by the Soils in Connection with Land Improvement

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The use of the isotope method has made it possible to solve many theoretical as well as practical problems that arise in the study of the processes of electrolyte adsorption by the soil which are becoming especially vital today in view of the widespread application of chemistry in agriculture, and soil-improvement work carried on in the USSR. First and foremost among these problems are the following: the mechanism of the soil's interchanging adsorption of cations; the laws governing this adsorption; the mechanism of the soil's adsorption of anions; the conditions making anions available to plants; development of accelerated methods of investigating the aforementioned and other soil-chemical problems. This report outlines some of the results of Soviet scientists' researches with the application of the isotope method.

THE LAWS GOVERNING THE PROCESSES OF INTERCHANGING ADSORPTION OF CATIONS IN THE SOILS

As is known, the basic principles of interchanging adsorption of cations by the soils were established in the works of K. K. Gedroitz. The quantitative regularities of interchanging reactions in the soils have been a subject of intensive investigation by a number of authors in the USSR, as well as in other countries, for the past 20-25 years. The results of the immense research work of K. K. Gedroitz were also used to determine the corresponding mathematical sequence. As a result of the efforts of many researchers in the USSR, we can consider as solved, now, the problem of the application of equations derived from the law of mass action to the interchanging adsorption of equivalent cations in experiments where the constant ionic force in solutions is observed and the accessory reaction which could lead to hysteresis phenomena is absent. However, up to now, the question of the conditions of the application of equations derived from the law of mass action to the interchanging adsorption of different valence cations by the soil is open to debate. There can be no doubt as to the urgency of solving this problem.

The literature we have on this problem is contradictory to a great extent because in the corresponding experiments no attention was paid to a number of physico-chemical requirements (for example, the calculation of coefficients of the activity of ions participating in interchanging reactions, in the application of solutions of different concentrations; on observing the constants of ionic force in solutions, etc.). In those few cases when these demands were complied with (see works of P. B. Nikolsky, I. N. Antipov-Karatayev and their colleagues), the results of the experiments on exchange reactions of cations having different valences were satisfactorily covered by the equalization of the law of mass action in its classical form:

$$\frac{G_L^{1/e}}{G_M^{1/m}} = K_1 \frac{C_L^{1/e}}{C_M^{1/m}}$$

in conditions of constants of ionic force in solutions when

$$K_1 = K \frac{f^{1/e}}{f^{1/m}} = \text{const.}$$

(In these formulas G is the quantity of adsorbed ions; C, concentration of ions in equally weighted solutions; K, constant of exchange; f, coefficient of activation.)

On this basis, it is possible to determine the quantitative significance of constant interchange of cations for the soils and clays as the fundamental quantities for the characteristics of the soils.

Their use in various soil improvement calculations and for the prognosis of the character of interaction, for example, of fertilizers and the soils, would be of great practical significance.

Thus, the determination of the constants of the exchange of cations in the soils acquires significance of principle. Yet, the existing ways of determining them by the usual analytic methods are extremely cumbersome. The isotope method makes it possible to simplify and greatly accelerate all the analytic aspects of the methods in studying exchange reactions in the soils. In this way we can quickly get the

Original language: Russian.

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necessary physico-chemical characteristics for the principal types of soils. Relevant experiments were conducted in the physico-chemical laboratory of the Soils Institute of the Academy of Sciences of the USSR (Y. A. Polyakov) on comparable determination of constants of the exchange of monovalent cations (K^+, NH_4^+) with bivalents (Ca^{++}) in two ways: the usual analytic method and, parallelly, the isotope method with the application of Ca^{45} as a tracer of a displacement solution (carrier). The black-earth soil of the Kutnetsky experimental station was used, samples of which were separately saturated with ammonia and with potassium.

EXPERIMENTAL METHODS

A number of solutions of the mixture $CaCl_2$ + NH_4Cl (or + KCl) possessing constant ionic force $I \cong 0.1$ but with different correlations of ions of ammonia (potassium) and calcium, were made up on the basis of the Lewis equation

$$\left[I = \tfrac{1}{2} (Z_1^2 C_1 + Z_2^2 C_2 + \ldots) \right]$$

Equal batches of soil saturated with ammonia (or potassium) were treated with these solutions. Ions of ammonia (or potassium) and calcium were determined in the filtrate after 48 hours of interaction. On the basis of the data obtained the exchange constants were calculated by application of the equation:

$$\frac{G_{NH_4^+}}{\sqrt{G_{Ca^{++}}}} = K \frac{C_{NH_4^+}}{\sqrt{C_{Ca^{++}}}} \text{ or } \frac{G_{K^+}}{\sqrt{G_{Ca^{++}}}} = K \frac{C_{K^+}}{\sqrt{C_{Ca^{++}}}}$$

The application of the isotope method eliminated the lengthy analytic measurements of interchanging ions. In these experiments, definite quantities of chlorine Ca^{45} were added to the above-mentioned solutions possessing constant ionic force. The quantities of adsorbed calcium were determined according to the changing activity of Ca^{45} in the solutions after the establishment of equilibrium. The possibility of this determination is based on the well-known Berthelot-Nernst principle of equal distribution of the indicator between the solid phase and the solution in heterogenous reactions. This determination with the application of radioactive indicators was extensively used in the works of Academician B. G. Khlopin and his collaborators. The quantities of exchanging ammonia (potassium) in the solid phase were determined by calculations based on the principle of equivalence of the exchange displacement of ammonia by calcium and the quantity of the adsorption capacity of the soil. The equal quantities in the solutions of ammonia (potassium) and of calcium were likewise determined by calculations.

In this way, by applying the isotope method, all the analytic operations boil down to two determinations of the activity of Ca^{45}—in the initial solution and in the equilibrium solution. These calculations are, of course, very quickly accomplished. An example of the calculation of the data obtained is illustrated by Table I.

The average value of the exchange constant of the same pair of ions (NH_4^+ and Ca^{++}), which was determined by the usual analytic method, was found to be $K_{Ca^{++},\ NH_4^+} = 0.187$. The reversibility of the exchange reaction was also tested. Actually, the hysteries phenomena were not discovered (Table I).

Analogous studies on the exchange of K^+ by Ca^{++} in the same black-earth soil, completely saturated with potassium, gave the following value of the constant of interchange,

$$K_{Ca^{++},\ K^+} = 0.236.$$

One can determine, in condition of complete reversibility the exchange reactions of three pairs of ions (in this case NH_4^+, K^+ and Ca^{++}) in the same soil on the basis of constants

$$K = \frac{G_{NH_4^+}}{\sqrt{G_{Ca^{++}}}} \cdot \frac{\sqrt{G_{Ca^{++}}}}{G_{NH_4^+}}$$

for two pairs of ions (in this case NH_4^+ and Ca^{++} and K^+ and Ca^{++}). It is possible to determine the constant of interchange for the third pair (in

Table I. Determination of the Exchange Constants of the Adsorption of Ammonia and Calcium in the Black-earth Soil with the Use of Solutions Having Constant Ionic Force. (Data of Y. A. Polyakov)

No. of experiment	*Activity of initial solution, cpm*	*Activity of equal solution, cpm*	*Quantity of carrier Ca^{++} in equal solution in meq*	*Adsorbed quantity (Ca^{++}) in the soil in meq*	*Quantity of NH_4 in eq. solution in meq*	*Adsorbed quantity NH_4 in soil meq* $K = \frac{G_{NH_4}}{\sqrt{G_{Ca..}}}$	*Constant of exchange ammonia with calcium* $\frac{\sqrt{G_{Ca..}}}{G_{NH_4}}$
2/35	3202	2713	3.520	0.980	1.480	0.156	0.199
3/37	2846	2183	3.070	0.930	1.930	0.206	0.194
4/39	2490	1852	2.605	0.895	2.395	0.241	0.172
5/41	2134	1560	2.195	0.805	2.805	0.331	0.195
6/43	1779	1193	1.679	0.821	3.321	0.315	0.137
7/45	1432	910	1.280	0.720	3.720	0.416	0.150
8/47	1067	634	0.892	0.608	4.108	0.528	0.156
9/49	711	405	0.570	0.430	4.430	0.706	0.183
							$K_{cp} = 0.178$

Note: The general content of Ca^{++} in the initial solution did not exceed 5 meq in the employed volume.

this case NH_4^+ on K) by applying the equation

$$\frac{K_{Ca^{++},\ NH_4^+}}{K_{Ca^{++},\ K^+}} = K_{K^+,\ NH_4^+}$$

This calculation gives the value $K_{K^-,\ NH_4^+} = 0.733$. The determination of this quantity by analytic methods in special experiments of exchange of ammonia ions by potassium ions gave the value of the constant as $K_{K^-,\ NH_4^+} = 0.730$. In this way, the values obtained by both methods were satisfactorily similar.

Thermodynamic Characteristics of Exchange Reactions in the Soils**

The value of the exchange constants for the aforementioned three pairs of ions in the same black-earth soil depending on the temperature of the experiments (the range of temperature varied from 15° to 70°) are shown in the researches of Y. A. Polyakov. It was found that actually the reactions of exchange adsorption do not depend on the changes of temperature and that the quantities of the exchange constants practically remain constant, and that is why the change of ΔH^0_{298}, which is determined by the equation of Vant-Hoff $d \ln K/dT = \Delta H/RT^2$, is equal to zero.

Further, the quantitative change of free energy can be determined by the constants of exchange by the equation $\Delta F^0_{298} = -RT \ln K$.

This calculation showed that in all cases of research the obtained quantities have a negative sign (for the system $G_{NH_4^+,\ Ca^{++}}$ $\Delta F^0_{298} = -1039.7$; for the system $G_{NH_4^+,\ K^+}$ $\Delta F^0_{298} = -186.2$), testifying that the formation process of the adsorptive combinations $G_{Ca^{++}}$ and G_{K^+} takes place spontaneously and that these compounds are more stable than the adsorptive $G_{NH_4^+}$

References to the inverted character of the reaction of exchange $G_{Ca^{++},\ NH_4^+}$ which one comes across in literature are unfounded (V. R. Williams).

The quantities (ΔS^0_{298} were calculated on the basis of the data of change of free energy of the system (ΔF^0_{298}) according to the formula $\Delta S^0_{298} = -\Delta F^0_{298} K$. The entropy of formation of $G_{Ca^{++}}$ and G_{K^+} was positive and had a small absolute value (for the system $G_{NH_4^+,\ Ca^{++}}$ $\Delta S^0_{298} = 3.49$ for the system $G_{K^+,\ Ca^{++}}$ $\Delta S^0_{298} = 2.86$ and for the system $G_{NH_4,\ K^+} \Delta S^0_{298} = 0.625$, that is why these systems can be considered as being close to the isoelectric systems. However, the reactions for formation of adsorptive compounds of calcium and potassium with displacement of ammonia ions take place with an increase of entropy. Consequently, the compounds formed, according to this thermodynamic characteristic are more stable than the initial compound ($G_{NH_4^+}$).

In concluding this part of the report it should be stated that the advantage of the isotope method of research of exchange adsorptive reactions in the soils is due to its high sensitivity which makes it possible to use systems which are close to natural systems in their concentration of salts (with weak concentration of salts in the soil's solutions). The usual research method does not make this possible.

THE POSSIBILITY OF APPLYING THE ISOTOPE METHOD IN DETERMINING THE CAPACITY OF THE CATION ADSORPTION OF THE SOILS

It is known[1] that in an homogeneous environment only those atoms participate in the reactions of isotope exchange which are able to break away from the molecule or from the radical, as a result of dissociation into ions, or as a result of hydration, of oxidation and of recombination. In other instances, isotope exchange does not take place.

As stated above, the exchange adsorption reactions with electrolytes are adequately studied in such heterogeneous systems as clays and soils. The reactions of isotope exchange take place here too and, consequently, it is possible to use this reaction for methods of study.

It is highly important to use the method of isotopic exchange for the accelerated determination of the adsorptive capacity of the soils, clays, sedimentary rocks, the results of weathering of rocks and so farth in relation to cations. For this purpose it would be sufficient to prepare so-called monocation samples from the objects to be investigated and to treat them with salt solutions of corresponding cations of the same named marked with their radioactive isotopes in order to effect the isotopic exchange.

It is expedient preliminarily to saturate the soils with such cations as calcium and sodium, which possess the long-lived isotopes Ca^{45} and Na^{22}. To determine the capacity of exchange of soils, it is necessary to use the salt solution of the cation of the same name (calcium or sodium) marked with its isotope. During the interaction of the salt with the soil, the isotope exchange of the adsorbed stable isotope with its radioactive isotope in the solution is quickly effected. The distribution of the isotope's activity takes place according to Nernst's principle of distribution, i.e., proportionately to the general content of the exchanging cation in the solid phase and to its general quantity in the equilibrium solution. The last mentioned quantity is constant in the experiments and equals the given initial quantity of cations in the solution. That is why, knowing the activity of the equilibrium solution and the activity of the solid phase (which is determined by the difference of the activity of the initial solution and the equilibrium solution) one can determine the capacity of interchange of the soil. Thus, it is sufficient to determine the quantity of the activity of the initial

** In determining the quantities of ΔH^0_{298} and ΔF^0_{298} and ΔS^0_{298} in all instances of increasing these functions they have a positive sign. In these conditions the change of the heat content of the exothermic reaction has a negative sign ($\Delta H < 0$). In writing the law of the mass action the obtained products are the numerator and the initial products the denominator. Thus, to calculate ΔF^0_{298} and ΔS^0_{298} one should use not the quantity of K, given in the Table I, but its reciprocal, $1/K$.

Table II. The Comparative Data of Adsorptive Capacity of Soils Obtained by the Isotope (Ca^{45}) Method and by the Usual Colloid-chemical Method. (Data of S. G. Rydky and F. G. Yankowskaya)

Soils and clays	Impulses per min: in the equilibrium solution (b)	Impulses per min: difference between initial and equilibrium solutions (a-b)	Adsorptive capacity of soils and clays in milliequivalents per 100 gm: by the isotope method	Adsorptive capacity of soils and clays in milliequivalents per 100 gm: by the colloid-chemistry	Remarks
1	2	3	4	5	6
Usual black-earth soil (Kamennaya steppe), Ap.	1402	581	55.7	55.7	50 ml of 0.1 solution of $CaCl_2$ marked with Ca^{45} was used
Usual black-earth soil (Stalingrad region), Ap.	1154	591	45.4	45.9	
Sodium saline (Voronezhskaya region)	1447	536	50.2	53.5	
Thick black-earth (Streletskaya steppe)	—	—	42.1	42.6	
Dark-grey forest soil (Shipov forest)	—	—	60.8	63.6	
Grey forest soil (Moscow region)	—	—	16.9	17.0	
Brown forest soil	—	—	14.8	19.1	
Turf middle podzol soil (Moscow region)	—	—	9.1	10.4	
Integumentally agrillaceous (clayey) soil					
Moscow region	1513	510	13.4	13.7	
Bentonite clay	1294	689	76.6	75.4	

solution (a) and the activity of the equilibrium solution (b): then the activity of the solid phase of the soil will be equal to $(a-b)$, which is proportional to the adsorptive capacity of the soil. If the quantity of the cation-displacer in the equilibrium solution is known, and according to the method this quantity is equal to the quantity of the cation-displacer in the initial solution and depends on the conditions of the experiments; let it be equal to c. Then the adsorptive capacity of the given batches of soil will be equal to $x = [(a-b)/b]/c$.

According to these principles S. G. Rydky and F. G. Yankowskaya carried out their investigations on evolving the isotope method of determining the cation adsorptive capacity of soils in the laboratory of the soils department of the Moscow University under our direction.

At first, they used in their investigations the non-carbonate soils (black-earth and podzol soils). Batches of these soils were first saturated with calcium (treated with a solution of $CaCl_2$). The surplus of salt was washed off with water. The soils saturated with Ca were then treated with the solution of $CaCl_2$ to which was added the radioactive isotope of Ca (Ca^{45}), in static conditions The equilibrium distribution of the activity between the solution and the solid phase was quickly attained in one hour.

The usual colloid-chemical way of determining the adsorptive capacity of the soils was used parallelly with the isotope method when evolving this method.

As shown by the data given in Table II, the isotope method does not yield to the colloid-chemical method in the accurate determination of the exchange capacity of soils and clays and surpasses the colloid-chemical method in the speed of determination several times (Table II).

Very important is the application of the method previously described for determining the exchange capacity of the carbonate soils preliminarily saturated with calcium. However, one might suppose that the isotopic exchange would take place not only between the solution and the exchangeable calcium in the soil, but also between the solution and the carbonate calcium in the soil, which could, to a greater or smaller

degree, distort the quantities of the adsorption capacity of the soils being investigated. Special determinations of the quantities of the isotope exchange of the tracer solution of calcium chloride and suspensions of $CaCO_3$ of different origin (amorphous or crystalline samples of calcium carbonate) showed that even for a long interaction (up to 24 hours) these quantities did not exceed 0.15 milliequivalents to 1 gm of $CaCO_3$.

In experiments where various quantities of calcium carbonate were added to the soil, the method of isotopic exchange yielded quite satisfactory results, as can be judged from the data illustrated in Table III.

Thus, it can be considered that the developed isotope method of determining the exchange adsorptive capacity of soils and grounds is universal. This method can be recommended for extensive application in scientific-theoretical, as well as in practical investigations. S. G. Rydky and his collaborators are successfully developing the accelerated method of determining the adsorptive capacity by oxidized soils preliminarily treated with calcium carbonate in order to be saturated with calcium after which the isotope method is employed; it is likewise used to determine the adsorptive capacity of carbonated soils. After the completion of the experimental part of the research conducted by the above-mentioned authors in 1954, the work of Blume and Smith came out, on an analogous subject on the same problem,[2] and somewhat earlier, on a similar theme—the work of Smith, Blume and Whittaker.[3] More complicated variants of the isotope method are recommended in these works than in the above-stated recommendations of Soviet investigators.

Table III. Comparative Determination of Adsorptive Capacity of Soils Possessing Calcium Carbonate. (Data of S. G. Rydky and F. G. Yankowskaya)

Soils and variants	*Adsorption capacity of soils in milliequivalents/100 gm*	
	by isotope method	*by colloid-chemical method*
I. Thick black earth (Kursky):		
(1) Initial soil	37.8	37.8
(2) Same with 1 gm of $CaCo_3$ crystalline for 3 gm of soil	39.2 (from 37.9 to 41.0)	—
(3) Same with 1 gm $CaCo_3$ amorph.	40.2 (from 39.1 to 41.5)	—
II. Thick black earth (second example):		
(1) Initial soil	47.8	47.8
(2) Same with 0.01 gm $CaCo_3$	47.7	
(3) Same with 0.05 gm $CaCo_3$	47.6	
(4) Same with 0.1 gm $CaCo_3$	48.0	

THE APPLICATION OF THE ISOTOPE METHOD IN THE INVESTIGATION OF THE MECHANISM OF THE ADSORPTION OF ANIONS BY THE SOILS

As we know, the theory of anion adsorption is devoid of complete clarity. However, the urgency of solving this problem is fully evident.

It is natural that researchers devote the greatest attention to studying the phenomena of adsorption of phosphate ions by the soils. We also pay special attention to this phenomenon in our report. It is understood that the mechanism of interaction of the soil with phosphates cannot be described by the exchange adsorption of cations.

It is known that many researchers do not differentiate between the chemical precipitancy of phosphate ions in soils that take place with the formation of a new phase, and the reactions of the adsorption of these ions by clay minerals and by minerals with a 50 per cent increased oxidation in the soils. They consider the latter reaction a purely chemical act which takes place in stoichiometric relations.

Another group of researchers, on the contrary, isolates the fraction of exchange-adsorption groups of ions among the phosphate ions. The action of the exchange-adsorption groups of anions is considered similar to the cation exchange or close to this mechanism.

The correct understanding of the character and the mechanism of the adsorption of phosphate ions and of their action in the soils and the development of suitable methods of differentiated investigation of forms of phosphates in them, could serve as the key to the basis of suitable practical forms of the rational use of soil phosphates, as well as the phosphate fertilizers used in soils. The usual methods of investigating forms of phosphates, to which the development of agro-chemistry has devoted scores of years, has been of little benefit to us in solving this problem.

The modern development of the isotope method gives us grounds to hope for the closer approach to the correct solution of this complicated problem by applying this method.

Research of past years has shown that isotopic exchange of compounds of phosphorus in soils is realized by groups of ions PO_4. The volume of isotopic exchange in various soils is different.[4,5] Notwithstanding the fact that there is a fraction of phosphates in the soils which can be isotopically displaced by the solutions of P^*O_4, it is difficult to consider this fraction as an exchangeable adsorption form, i.e., on the same footing with anti-ions, because the soils, in the overwhelming majority of cases are charged negatively, and that is why the anions cannot be anti-ions. It is most probable that the fraction of phosphate-ions in the soils which is easily determined by the isotope method, forms hemosorptive-potential-determination of anion stratum on the surface of the colloid particles of the soil. The usual exchangeable cations are anti-ions for this stratum. The quantity of this fraction of phosphates must be greater in the amorphous compounds of soil (hydrates with oxidation increased by 50%). As to the crystalline

colloid clay particles of the soil, the quantity of the stratum there is comparatively small. The mechanism of formation of the potential-determining stratum of the phosphate ions on the surface of particles of soil must consist in the formation of difficult soluble compounds of ions according to the principle of Panet-Faians. That is why the displacement of the hemosorptive anions must proceed through different anions; the more so in accordance with how much more insoluble are the compounds formed by the displacers on the surface of the colloid particles.

For the purpose of all-round examination of these positions, S. N. Ivanov, Master of Sciences of the Soil Institute of Academy of Sciences of the USSR, conducted special investigations using the isotope method in the Agricultural Institute of the Byelorussian Republic. The results of these researches make possible the quite definite solution of some of the above-stated problems.

The Quantitative Laws Governing the Adsorption of Phosphate Ions by the Soils as the Exponent of Mechanism of the Process

It is known that the isotherm of the hemosorptive adsorption of ions, when the adsorption takes place as the adsorption of potential determining ions, can be described by the Fervei equation: $X = k_2 + k_1 \ln C$, where X is the quantity of adsorbed ions, C is the concentration of the ions in the equilibrium solution and k_2 and k_1 are constants.

S. N. Ivanov made special experiments to determine the isotherm of sorption of the phosphate ions by the turf-podzol soil and by the rush-sedge turf of the Byelorussian Republic and worked over the data obtained according to the Fervei equation (Table IV).

From the data illustrated in Table IV, it is clear that the quantity of sorption of P_2O_5 in the soil and turf found experimentally well agrees with the quantity calculated by the Fervei equation.

The Dynamics of the Isotope Desorption of the Adsorbed Phosphate Ions from the Soils and Its Comparison with the Isotopic Exchange of Phosphate Ions on the Chemical Precipitations

It is possible to determine the difference between curves of the isotope desorption of phosphate ions in their chemical stoichiometric compound and in their adsorption by using the isotope method.

S. N. Ivanov prepared fresh active (in P^{32}) chemical precipitations of iron phosphate and of aluminium phosphate and, parallelly, likewise prepared gels of hydroxides of iron and aluminium, on which were "placed" active phosphates by the sorption method. The curves of the isotopic exchange of the active phosphates with the inactive phosphates in dynamic conditions were determined on these preparations. The conditions of the experiments are identical in all respects: same activity of the batches of preparations; same volumes and concentrations of the desorption solutions of KH_2PO_4; same speed of the autofiltration. As can be judged by the curves of activity of consecutive filtrates (see Figs. 1 and 2), there is an essential difference between the isotopic exchange, which takes place in the stoichiometric chemical compounds of phosphates with iron and aluminium ($FePO_4$ and $AlPO_4$) and the action of sorption compounds of active phosphates with the gels of the hydroxides of iron and of aluminium. The first part of the curves of the isotope desorption of the phos-

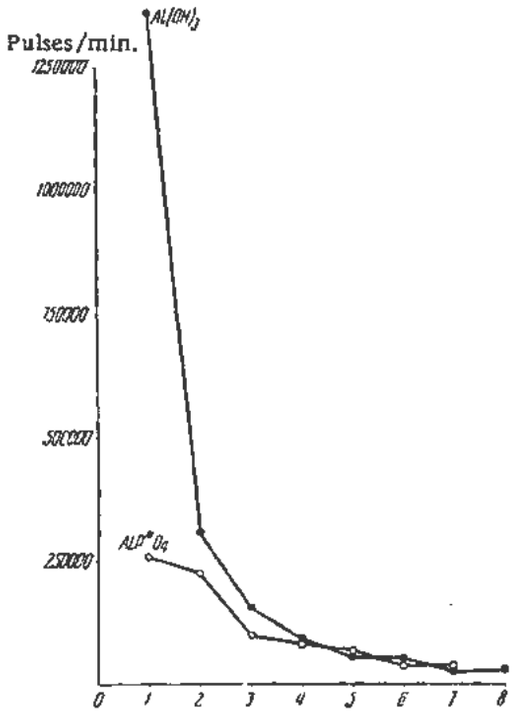

Figure 1. Ordinal numerals of consecutive extraction at 25 ml of the solution 0.1-N KH_2PO_4

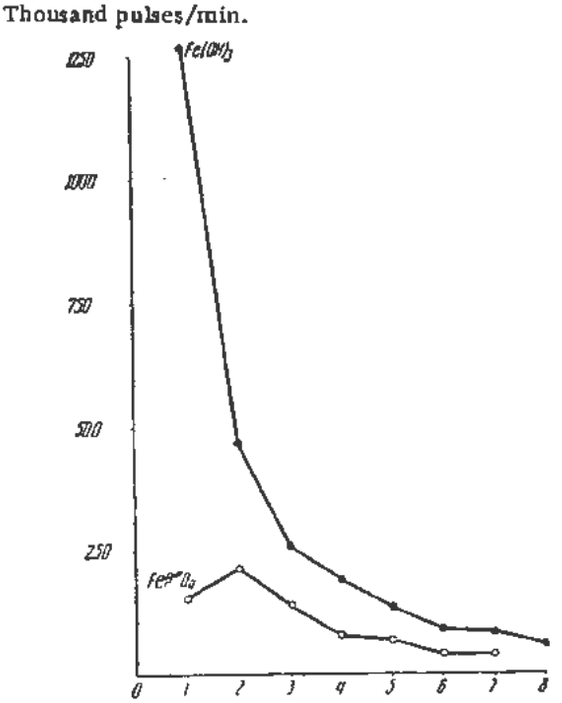

Figure 2. Ordinal numerals of consecutive extraction at 25 ml of the solution 0.1-N KH_2PO_4

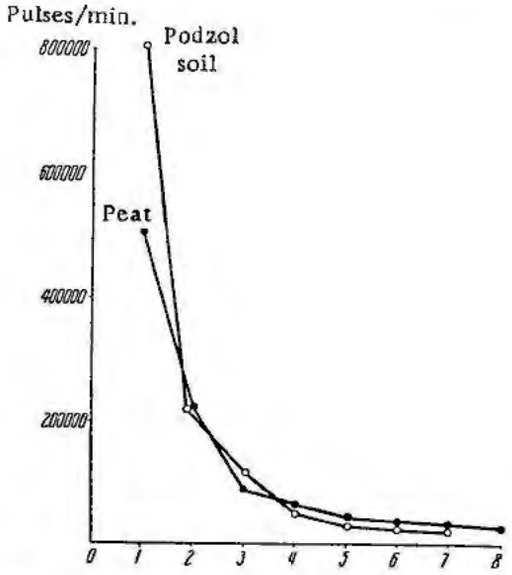

Figure 3. Ordinal numerals of consecutive extraction at 25 ml of the solution 0.1-N KH_2PO_4

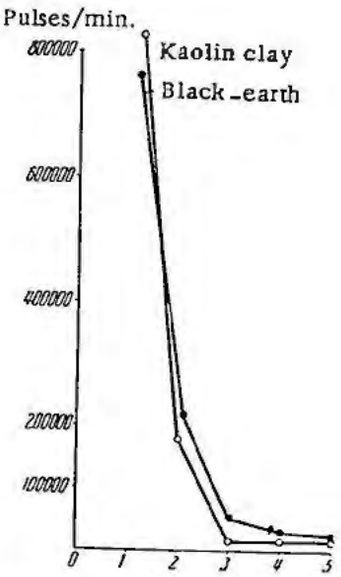

Figure 4. Ordinal numerals of consecutive extraction at 25 ml of the solution KH_2PO_4

phate ions on the last-mentioned objects drops abruptly, while for that of the first-mentioned (chemical) this drop is actually not expressed. This fact must be considered as indicative of the difference of the mechanism of desorption of the phosphate ions in both cases, consequently, it should be considered as indicative of differences in the binding forces in the chemical and sorptive compounds.

In the same dynamic conditions the experiments on the desorptive isotopic exchange of active phosphate ions, absorbed by soils and turfs, and the experiments on the desorption of phosphates in the gels of hydroxides of iron and aluminium, resulted in analogous curves. The same can be seen in the isotope desorption of adsorption active phosphates in kaolin clay and in black-earth soil (see Fig. 4).

In conclusion we can say that in the first stage of the adsorption of phosphates by the soils and turfs, the hemosorptive process with the formation of superficial chemical compounds really takes place.

Adsorption and Desorption of Phosphate Ions by Soils

The adsorption of phosphate ions by the soils (clay minerals, organic colloids, etc.) leads to the increase of the cation adsorptive capacity, because this adsorption takes place like the sorption of potential-determining ions. By special experiments, S. N. Ivanov showed that in soils and turfs the adsorption of anti-ions increases simultaneously with the adsorption of phosphates. Suppose that 5% of the concentrations of K^+ and Ca^{++} from the initial solutions KCl and CaCl are exchangeably adsorbed by the soils, then in equal concentrations of corresponding solutions of phosphates of potassium and of calcium, the exchange adsorption of these cations by the soil increases several times. Proof of the sorptive form of adsorption of phosphate ions by the soils similar to the adsorption of potential-determining ions is the well-known fact of increasing dispersion of the sorbents in treating them with phosphates of alkaline elements. In the experiments of

Table IV. The Isotherm of Sorption of Phosphate Ions by the Podzol Soil and by the Turf of the Byelorussian Republic and the Calculations of this Isotherm according to the Fervei Equation: $X = k_2 + k_1 \ln C$. (By S. N. Ivanov)

Sorbent	*Balanced concentration of phosphate ions in meq/l, C*	*Sorption quantity of P_2O_5 in mg/100 gm*		*% of digression of X_2 from X_1*	*Remarks*
		experimentally X_1	*by calculations X_2*		
Turf podzol middle-argillaceous soil	2.02	70.4	68.9	−2.1	$K_2 = 3100.2$
	5.41	108.7	105.6	−2.8	$K_1 = 1142.8$
	18.9	143.6	152.2	+6.0	
	42.4	178.2	182.3	+2.3	
	66.01	207.0	198.7	−4.0	
Rush-sedge lowland turf	6.04	574.6	564.5	−1.8	$K_2 = 299.2$
	16.3	1022.9	1057.2	+3.4	$K_1 = 85.72$
	29.5	1379.1	1351.6	−2.0	
	47.5	1602.3	1588.0	−0.9	
	88.6	1859.2	1897.4	+2.1	
	130.4	2109.7	2089.2	−1.0	

S. N. Ivanov the gels of hydroxide of iron increased dispersion to a great extent as the result of the sorption of potassium phosphate.

The desorption of phosphate ions by different anions takes place in general according to the principles of Panet-Faians, i.e., the anions on the surface of the colloid particles of the sorbents form less soluble compounds and desorb more P_2O_5 than those anions which form more soluble compounds.

In the experiments of S. N. Ivanov, the active phosphate ions sorbed by the sod-podzol soil were desorbed by other anions in the following order: Cl displaced 5.93% of sorptive P_2O_5 in the experiment; $FeCN_6$ displaced 13.2%; HCO_3 displaced 17.88%; humate of sodium displaced 21.40%.

From this, conclusions have been drawn on the possible regulation of the phosphate regime in the soils by forming a favorable organic substance for this regime in the soils.

The application of the isotope method in static equilibrium conditions does not permit the disintegration of phosphates in the soil, into their chemical compounds, nor into their sorptive compounds. In these conditions the amorphous compounds of phosphate ions in the form of active phosphates of iron, aluminium and potassium of the active phosphates in the soils and the gels of the hydroxide increased by 50 per cent, are completely subjected to the isotope exchange with the inactive phosphate ions. So, in the experiments of S. N. Ivanov the volume of the isotope exchange of AlP^*O_4 with KH_2PO_4 makes up 87.3%; with FeP^*O_4, 71.5%; and in the isotope absorption of P^*O_4 by the amorphous phosphate of potassium the volume of the isotope exchange reaches 79.4%. From this we can conclude, that in these conditions the isotope exchange does not limit itself by the outer sorptive strata, but, under the influence of self-diffusion, penetrates into the deep strata of the particles of the sorbent.

However, the use of the isotope method in its static modification permits to disintegrate the phosphate chemical compound into crystalline and amorphous forms. If the latter, as we have seen above are characterized by a high per cent of the isotopic exchange, then for the crystalline phosphates this process is limited. So, in the experiments of S. N. Ivanov on the initial stages of crystallization $Ca_3(P^*O_4)$ only about 20% of the volume of the isotope exchange with KH_2PO_4 is shown. In this case the isotope exchange took place, principally, on the surface of the object. This is also proved by the fact that according to the two-fold increase of the preparation batch, the general quantity of the displaced active phosphate ions is also increased twice over.

The crystal phosphates of calcium (Khibinsky apatites, phosphorate flour) are characterized by the small volume of isotope exchange (about 13%) with P^*O_4.

Thus, the isotope method of the differential study of forms of phosphates in the soils, applied in various modifications, permits a closer approach to the rational group of compounds of phosphorous in the soils.

SUMMARY

Some important results of the researches of Soviet soil specialists on the application of the isotope method conducted under the direction of the author in the past years were reviewed in this report.

Methods on determining the constants of the exchangeable cation adsorption by the soils with the application of radioactive indicators (Ca^{45}) were described. These methods greatly accelerate the investigations and increase the accessibility of these determinations for many soil laboratories which is of great importance for swiftly obtaining fundamental physico-chemical characteristics for the majority of types of soils.

Further, the new universal method to determine the cation adsorptive capacity of soils and grounds by using isotope exchange (with Ca^{45}) is described.

Finally, the data on researches on the use of the isotope method on the state and régime of phosphates in soils, are considered in great detail.

Thus, the isotope method permits investigation of a number of complicated soil-chemical problems and brings to a simple solution a number of debated and complex problems in physico-chemical soils.

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Utilization of Radioactive Isotopes in Resolving the Effectiveness of Foliar Absorption of Plant Nutrients

By H. B. Tukey, S. H. Wittwer, F. G. Teubner and W. G. Long,* USA

Future progress in world agriculture depends to an increasing degree upon exactness and efficiency in cultural operations, accurate evaluation of new procedures, and reduction of chance hazards imposed by environment. To this end, familiar and long established concepts in plant nutrition and fertilizer practices need to be re-examined in the light of new knowledge and new tools.

Roots are commonly accepted as the principal organs through which plants absorb nutrients, which are then transported to other portions of the plant, as stems, leaves, flowers, and fruits. That the reverse of this process could and does occur, namely, the uptake of nutrients by stems, leaves, flowers, and fruits has long been indicated by certain horticultural practices. Yet only recently has this truth been demonstrated experimentally.

Several factors have recently prompted interest in the possibilities of applying mineral nutrients to the above-ground parts of plants. Thus, current progress in the formulation of chemical fertilizers has been characterized by greater water solubilities and higher analyses of the major mineral elements utilized by plants. Coincident with the commercial availability of these materials has been the wide, general use of sprays and dusts as pesticides and herbicides, accompanied by remarkable engineering advances in spraying and dusting equipment. Finally, radioisotopes of the elements commonly applied as fertilizers have made it possible to follow and evaluate the absorption of nutrients by above-ground parts of plants in comparison to absorption by roots. A remarkable efficiency of uptake by leaves, and many important facts relative to mineral nutrition and fertilizer usage have been revealed.

ENTRY OF NUTRIENTS INTO DORMANT BRANCHES

Radioactive mineral nutrients applied to leaf, stem, and fruit surfaces are readily absorbed as measured by subsequent assay of non-treated parts. In fact, nutrients have been shown to enter even through the bark of dormant fruit trees. Cotton gauze was dipped into solutions of K^{42} potassium carbonate and P^{32} ortho-phosphoric acid, and the impregnated gauze wrapped around branches of apple trees (*Malus domestica*) and peach trees (*Prunus persica*).[9] Within 24 hours of application, during February and March even with temperatures below freezing, radioactivity was detected within the branches 18 to 24 inches both above and below the points of application. Similar applications made just as the buds were commencing to swell, showed the material to move in through the bark and up through the branches, and to concentrate near the buds, available for the flush of new growth.

A proved method is now available for treatment of wounded and injured fruit trees through application of nutrients to the trunk and branches during the dormant and early spring seasons, and the practices of correcting zinc deficiency and applying nutrients by dormant applications is rationalized.

Further, the surface area of the trunk, branches, and twigs of fruit trees is substantial. For a 25-year-old McIntosh apple tree, the figure is 800 square feet, equivalent to a wall 8 feet high and 100 feet long. It has been shown that as much as 3 pounds of high analysis fertilizer may adhere to such a surface area when applied as slurries or pastes.

UPTAKE AND MOVEMENT OF MINERAL NUTRIENTS APPLIED TO FOLIAGE

Although nutrients may enter woody plants through the bark, even during the dormant season, it is of more importance that they may enter through the leaves. It is now established that the surface of a leaf is not the impervious structure described in many text books. Instead, it is structurally well equipped to absorb materials through both the upper and lower surfaces.[6,7] In addition, the leaf area of a plant is considerable. Thus, it has been determined that a 12-year-old apple tree provides in its foliage, both upper and lower surfaces, an area of $^1/_{10}$ of an acre or approximately ten times the spread of the tree.

By isotope techniques, the highly mobile elements nitrogen,[10] phosphorus,[8] potassium, and rubidium applied to leaves are shown to be freely transported, both acropetally and basipetally, at a rate comparable to that which follows root absorption. Calcium,[1] strontium, and barium do not move from the absorbing plant part, and basipetal transport is negligible (Fig. 1). The patterns of phosphorus, potassium and calcium absorption and transport in the bean plant (*Phaseolus vulgaris*), as illustrated by the autoradiograms of Fig. 1, are duplicated without

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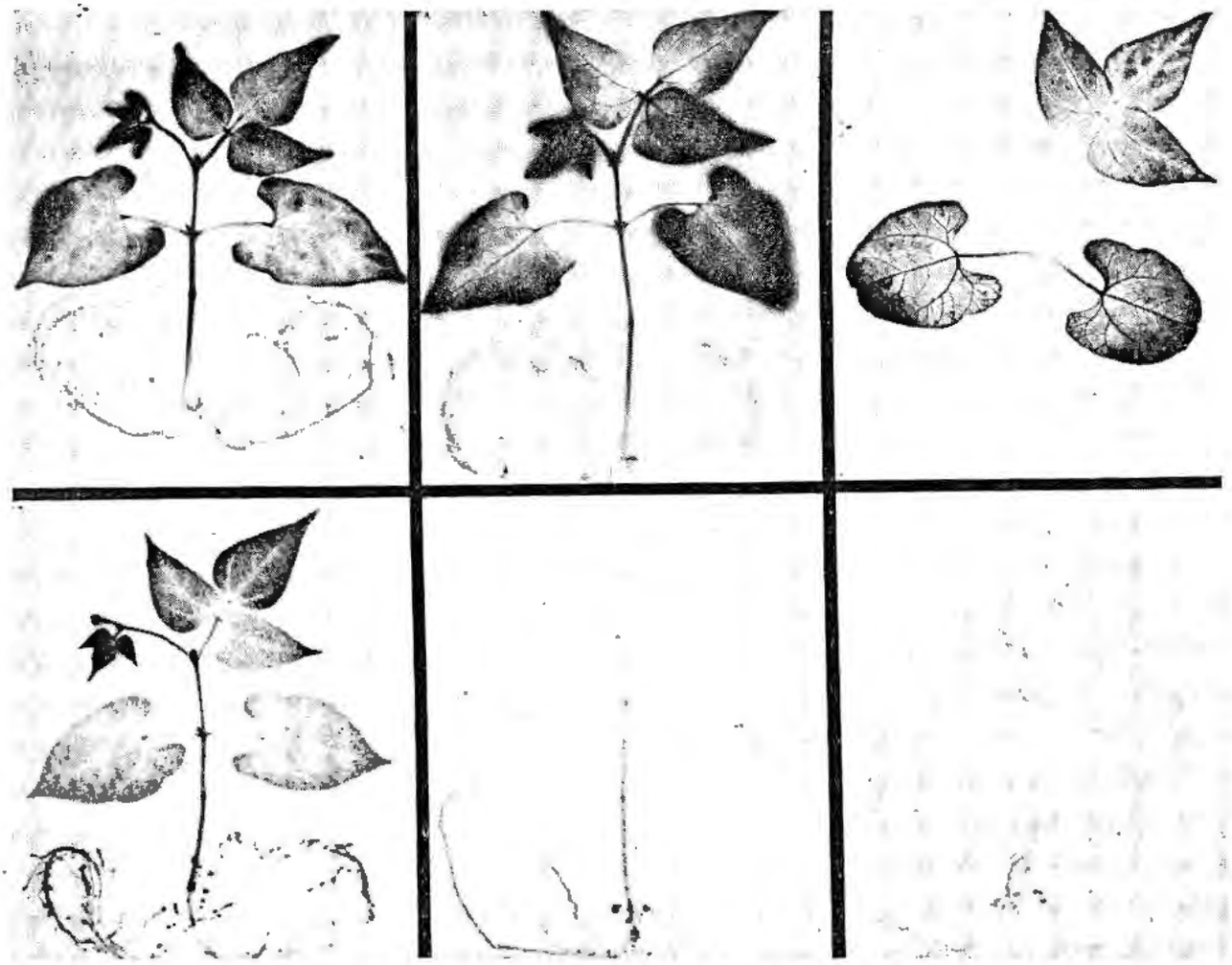

Figure 1. Comparative distribution of P^{32}, K^{42}, and Ca^{45} in the bean plant following treatment of roots and leaves. Top, foliar applications; bottom, root applications. Left to right, P^{32}, K^{42}, and Ca^{45}

exception in many other crop species and under many unusual conditions. Further, acropetal and basipetal movement of phosphorus is not restricted by reciprocal grafting of roots and tops of tomato (*Lycopersicum esculentum*) and tobacco (*Nicotiana tobacum*). In contrast, no basipetal movement of calcium has been detected in either intact or grafted plant segments.

The absence of basipetal transport of radiocalcium is depicted by the strawberry (*Fragaria spp.*). Following application to the roots of the mother plant, active upward transport occurs. Lateral movement then follows into the stolons and aerial parts of daughter plants. The almost complete absence of basipetal transport into the roots of daughter plants is striking (Fig. 2). Obviously as revealed by the radioisotope technique, the roots of the runner plants must depend upon their own absorption for a source of calcium. The apparent absence of basipetal transport of calcium in plants does not, however, exclude the usefulness of calcium as a foliage spray for the correction of certain nutritional disorders.[3] Radiocalcium is readily absorbed by leaves and fruit from external sprays, but there is no transport or redistribution from these organs to other plant parts.

The transport and distribution of radiophosphorus is similar within the plant following either foliar or root absorption. Accumulation occurs in rapidly growing meristematic regions[11] such as root tips, vegetative growing points, flowers, fruits and seeds, and even embryos within seeds (Fig. 3). The autoradiograms further suggest that foliar absorbed phosphate is metabolized and functionally serves the same needs as that absorbed by roots.

By using isotope techniques it has been demonstrated conclusively that with many herbaceous crops, foliar applied nutrients can make a significant contribution to the total nutrient needs of the plant. Numerous carefully controlled experiments have also shown that the percent of phosphorus utilized from applied fertilizer is highest in foliage sprays—up to 95 per cent of the fertilizers applied in some instances. In fact, the application of phosphorus to the leaves of plants represents the most efficient method of fertilizer placement yet devised.

For example, beans and tomatoes were grown in pot culture in the greenhouse on three soil types and at variable levels of phosphate in the soil. Measured

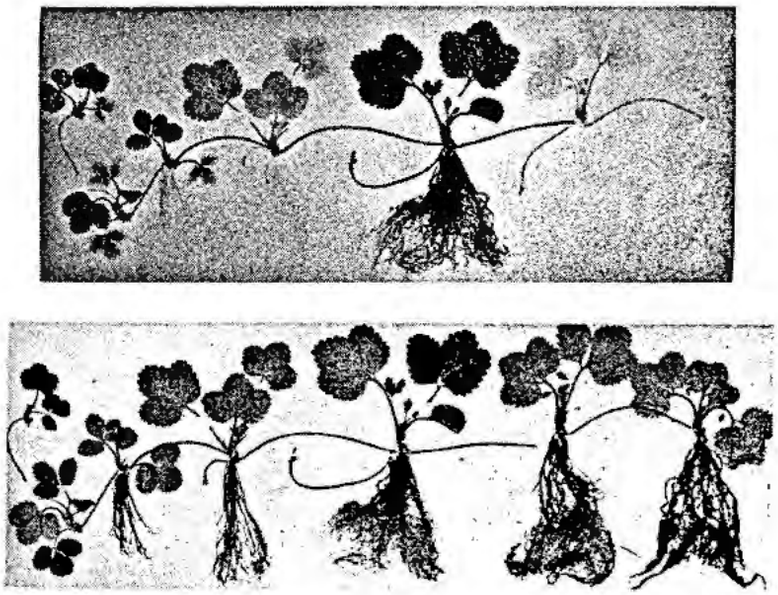

Figure 2. Autoradiograms showing the distribution of Ca^{45} in a series of strawberry runner plants following initial root absorption by center plant. Top, autoradiogram of treated plants, showing lack of appreciable movement of Ca^{45} into roots of runner plants; bottom, photograph of plants prior to treatment (leaves which appear dark are turned with upper surface towards camera)

quantities of P^{32} labeled phosphoric acid (0.3 per cent solutions) were applied to the soil in a band in the root area and as a foliage treatment during early flowering. Accumulation of the applied phosphorus in the developing fruit during a 3-week period from the two methods of placement was ascertained. In Table I are found the essential data for the two crops. The foliage applications were far more effective

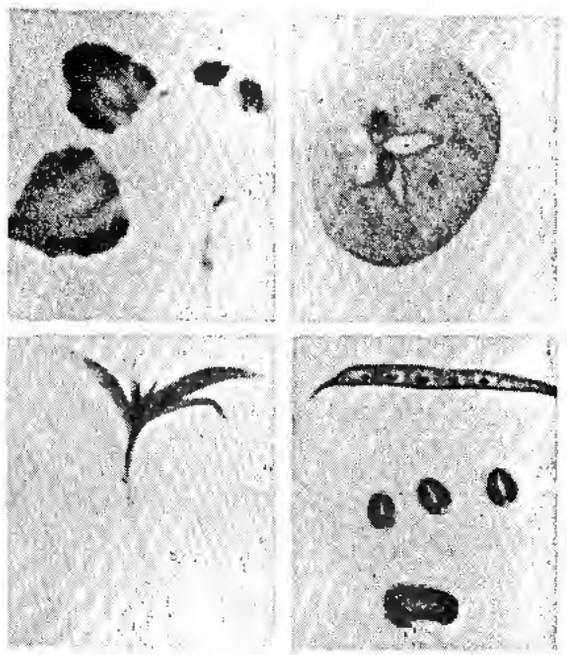

Figure 3. Autoradiograms showing the pattern of distribution and accumulation of P^{32} in the fruit and seeds of strawberry, apple, bean, and tip of a peach shoot following foliar application of P^{32} orthophosphoric acid[11]

Table I. Comparative Effectiveness of Foliage and Root Applications as Sources of Phosphorus for Developing Fruit of the Bean and Tomato

		Phosphorus accumulated in developing fruit		
Crop	*Part treated*	*µg*	*% of total*	*% of applied*
Bean	Foliage	91	1.10	6.98
	Roots	36	0.38	0.27
Tomato	Foliage	33	0.40	1.23
	Roots	24	0.27	0.18

for the micrograms of applied phosphorus accumulated in the developing fruit, for the per cent of the total phosphorus in the fruit derived from that applied, and for the per cent of the applied phosphorus found in the developing fruit. With the bean, the foliage was more than 25 times as efficient as the roots in phosphate uptake based on the percentage applied which accumulated in the fruit.

Typically, many crops, especially fruits and vegetable crops, during normal development pass through critical periods associated with flowering and fruiting when nutrient demands are high, and when availability from soil sources is low or at a minimum. It has been demonstrated by the isotope technique that during these critical periods leaves can supplement the function of the roots as nutrient absorbing organs. Up to 25 per cent of the phosphate needed in fruit growth can be supplied by properly timed foliage spray applications.

FACTORS WHICH AFFECT FOLIAR ABSORPTION

It may be anticipated that foliar absorption of nutrients will be affected by external and internal

factors. Temperature, light, the pH and the carrier of the treating solutions, and various additive chemicals may be important, as well as the species of plant involved, the morphological nature of the absorbing organ, and the nutritional status of the plant.[5]

Of the many phosphates (organic and inorganic) which have been tested, orthophosphoric acid at a concentration of 0.30 per cent (2.5 lb/100 gal water) was found most desirable for stimulating growth and early fruiting. Three cations (carriers) of phosphate have been compared with ortho-phosphoric acid with respect to the rapidity of uptake through the leaves of the bean plant. The three carriers included ammonium, potassium, and sodium, the first two being commonly found in the high-analysis fertilizers recommended for nutritional sprays, and sodium often accompanying natural phosphate deposits. The hydrogens of ortho-phosphoric acid were replaced by adjusting the pH of the treating solutions with the hydroxides of ammonium, sodium, and potassium.

Several important facts concerning phosphate absorption by leaves are evident from the data presented in Table II. All phosphate salts were absorbed less effectively than ortho-phosphoric acid to which only a slight amount of ammonium, sodium or potassium was added (pH 2–3). The slowest penetration through the leaf was with mono-potassium phosphate (pH 4–5). Of the three di-substituted phosphates at pH 7, the potassium salt was absorbed most rapidly. This has been confirmed in later experiments at a pH of 8; however, at pH 7 and 8 the rate of absorption was only half that of ortho-phosphoric acid (pH 2). The lack of stability of di-ammonium phosphate may account for its low rate of uptake at pH 7 and 8. A mixture of the mono-substituted phosphates and ortho-phosphoric acid (pH 2–3) resulted in 2-to 10-fold increases in uptake through bean leaves as compared with other inorganic phosphates.

Table II. Effect of pH and Accompanying Cation on Absorption of Phosphorus by Bean Leaves (μg in Roots, 6 Hours after Treatment)

	pH					
Cation	*2*	*3*	*4*	*5*	*6*	*7*
K	1.47	0.96	0.16	0.11	0.41	0.80
Na	2.03	2.97	0.31	1.59	1.21	0.25
NH_4	3.70	3.94	1.59	2.44	0.33	0.26

The effects of pH and ionic carrier on absorption of radiopotassium and radiorubidium by bean and tomato leaves were in marked contrast to those for radiophosphorus. Phosphate, citrate, and chloride were the accompanying anions, with pH levels of 2, 4, and 8. The absorption of rubidium was accelerated 10 to 20 times at a pH of 8 compared with a pH of 4 when phosphate was the carrier. In general for rubidium, chlorides or citrates were less effective than phosphates. However, at similar pH levels and with the same carriers, potassium uptake when applied as the citrate at pH 8 was twice that of any other form.

Leaf absorption of radiophosphorus and other nutrients is facilitated and possible leaf burning is avoided at recommended dosages if a wetting agent is added to the spray formulation. This results in a more uniform film of liquid on the leaves. Certain detergents, however, will reduce the speed with which phosphorus is absorbed. Young and rapidly expanding leaves are more efficient in absorption than are leaves fully matured, although all green leaves seem to absorb some radiophosphorus.

Both sides of the leaf blade, as well as the petiole, will absorb nutrients. When the primary leaf blade of the bean plant is treated with a radioactive phosphoric acid solution, and subsequent accumulation of radioactivity in the roots, is ascertained, absorption is slightly greater through the upper leaf surface. This is most evident when applications are made at the base of the leaf blade, as indicated in Table III. Since the frequency of stomata is seven times as great in the lower epidermis of the bean leaf,[2] it would appear that phosphate entry is not facilitated by these structures. Placement of the treating solution at the tip, along the outer margin, and in the center of the leaf along the midrib gave only a slight increase in absorption when compared to similar positions on the lower surface. On the other hand, application to the petiole of the leaf resulted in very rapid uptake. It was found, however, that the region of placement on the leaf surface was of no importance in determining the level of phosphorus which had accumulated in the roots by 12 hours after treatment.

Table III. Absorption of Phosphorus by Different Regions of Bean Leaves (μg in Roots, 2 Hours after Treatment)

	Surface of leaf	
Position on leaf	*Upper*	*Lower*
Tip	0.104	0.062
Margin	0.130	0.102
Midrib	0.148	0.104
Base	0.206	0.090
Petiole	0.516	

Furthermore, the opening and closing of stomata is of little significance, since foliar absorption is not confined to daylight hours, but is equally apparent during the night. Slight diurnal fluctuations are revealed, however, where continuous recordings of nutrient uptake have been made. Initially, light has a slight but significant effect in increasing the foliar absorption of phosphorus as is evident from studies on bean stem tissue presented in Table IV. This initial stimulation disappears under prolonged periods of light or dark; and the actual accumulation of foliar-applied phosphorus by roots and buds is favored by prolonged darkness. The depression of absorption (or possibly the movement from epidermal cells into the vascular system of the leaf) by dark may be duplicated by the addition of sucrose to the

Table IV. Effect of Light and Sucrose on Absorption of Phosphorus by Bean Leaves (μg in Stems)

Solution	Environment	Hours after treatment 3	6	18
0.2% H_3PO_4	Light	0.684	1.046	1.172
0.2% H_3PO_4	Dark	0.382	0.670	1.390
0.2% H_3PO_4 plus 5% sucrose	Light	0.346	0.614	1.142
0.2% H_3PO_4 plus 5% sucrose	Dark	0.334	0.566	1.070

Table V. Effect of Boron and Temperature on Absorption of Phosphorus by Bean Leaves (μg in Roots)

Treating solution	Hours after treatment	Temperature 14°C	21°C	25°C
Phosphate*	3	0.015	0.307	0.243
Phosphate plus boron†	3	0.013	0.202	0.183
Phosphate	6	0.433	1.040	0.560
Phosphate plus boron	6	0.230	0.975	0.625
Phosphate	12	1.230	1.675	0.738
Phosphate plus boron	12	1.225	1.162	0.868

* 0.2% H_3P_4.
† 0.005% boron.

treating solution. However, this does not greatly impair the ability of plants in the dark to accumulate foliar-applied phosphorus at some distal point. This occurs regardless of the inclusion of sucrose in the treating solution.

An effect similar to that obtained with sucrose occurs when boron is added to the phosphate solution (Table V). Not only did borate (0.005%) depress the initial absorption of phosphate, but this initial depression appeared to be independent of temperature. Maximum uptake of radiophosphorus occurred at 21°C with and without boron, and was less at both 14°C and 25°C. The similarity in levels of radiophosphorus in the roots after 12 hours at the two lower temperatures (14 and 21°C) with and without boron, suggests that absorption rather than translocation or accumulation is the limiting factor.

The many factors which have been shown to influence the absorption of foliar applied nutrients provide evidence for the existence of more than a mere passive entry through leaf surfaces into the plant. The effects of pH and cation carrier when considered in the light of the ionic species of phosphate and the degree of molecular dissociation which occur, suggest an exchange mechanism in the entry of foliar applied phosphorus. This is confirmed by the apparently competitive action of the borate ion. That the exchange is active rather than passive would seem to follow also from the temperature relationships already discussed. Ionic penetration of phosphate would preclude diffusion through the cuticle, and the now demonstrated widespread occurrence of epidermal plasmodesmata by Lambertz[6] provide most probable sites for protoplasmic exchange at the surface of the leaf.

FOLIAR APPLICATIONS OF C^{14} UREA

Urea is one of the most useful and well known nitrogenous fertilizers for leaf application. With some horticultural crops the entire requirement for nitrogen can be satisfied by a few appropriately timed sprays. Radioisotopes provide a specific and sensitive tool to ascertain the rate and extent of utilization, and to predict crop tolerance of foliar applications of urea nitrogen. The first step in the utilization of the nitrogen in urea by the leaves of plants presumably is hydrolysis by the enzyme urease, splitting the urea molecule and giving ammonia and carbon dioxide. As a measurement of urease activity, and thereby the rate of hydrolysis and possible utilization of urea applied to the leaves of horticultural plants, radioactive C^{14} urea may be employed and the rate of evolution of radioactive carbon dioxide determined.[11]

The apparent relative rates of urea hydrolysis (utilization) of foliar applied C^{14} urea by a number of vegetable and fruit crops have been determined and plotted against field and greenhouse tests of the tolerance of the foliage of the same crops to urea sprayed in pounds per 100 gallons of water.[11] From such studies it is possible to predict the tolerance of the leaves of various plant species to concentrations of urea applied as nutrient sprays. It is of interest that the leaves of plants which hydrolyze (utilize?) urea most rapidly—cucumber (*Cucumis sativis*) and bean—are those which are the most responsive to treatment and which show the lowest spray concentration tolerance. Conversely, crops showing the greatest tolerances—cherry (*Prunus cerasus*), peach (*Prunus persica*), celery (*Apium graveolens*), and potato (*Solanum tubersoum*)—are those which have the lowest rates of urea hydrolysis or utilization. Intermediate crops are apple (*Malus domestica*), strawberry (*Fragaria spp.*), grape (*Vitis labrusca*), tomato, raspberry (*Rubus spp.*), corn (*Zea mays*), and plum (*Prunus domestica*).

LEACHING OF NUTRIENTS FROM LEAVES

Since, as has been shown, nutrients may enter the leaf, it would seem plausible that under appropriate conditions they may be lost from the leaf. The leaching of nutrients from plants during times of heavy rainfall may be, indeed, as fully responsible for lack of productivity as a deficiency in sunlight. This hypothesis has been subjected to experimental evaluation by allowing plants to absorb isotopically labeled nutrients through their roots or through cut stems and then exposing them to simulated rainfall ("foliage leaching") from a mist atomizer in a propagation chamber.

Measured quantities of radiophosphorus were applied to the roots of bean, sweet potato (*Ipomaea batatas*) or poinsettia (*Euphorbia pulcherrima*). After 48 hours absorption, the foliage was leached for 48 hours, and no loss of P^{32} was observed. However, if stem cuttings of these plants were supplied P^{32} through the bases of the cut stems and

then subjected to foliage leaching, 1.5 to 12.8 per cent of the absorbed P^{32} was lost.

Results with radiopotassium and radiorubidium were different (Table VI). Following a 12-hour absorption of these ions, subsequent leaching for 4 hours removed up to 71 per cent of the K^{42} and 14 per cent of the Rb^{86}. The plants were grown either in nutrient solutions at full strength,[4] or at $^1/_{10}$ dilution. This loss was greatest from plants grown in the dark (covered with black cloth) at the full nutrient level. With those grown in daylight, the reverse was true. More potassium than rubidium was lost by leaching.

Table VI. Loss of K^{42} and Rb^{86} from Bean Leaves by Leaching for 4 Hours under Simulated Rainfall as Influenced by Light and Nutrient Intensity

Experimental conditions	*Per cent of absorbed ions lost*	
	Potassium	*Rubidium*
Daylight; full nutrient solution	5.1	4.9
Daylight; 1/10 nutrient solution	12.2	4.9
Dark; full nutrient solution	71.0	14.4
Dark; 1/10 nutrient solution	42.5	6.6

These data show that loss of nutrients by leaching from leaves does occur. The magnitude of loss with crops grown under field conditions is not known. The results suggest, however, that the leaching of nutrients from plants in humid areas or during periods of frequent heavy rainfall may be comparable to the losses of some ions from soil.

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The Use of Radioactive Isotopes to Ascertain the Role of Root Grafting in the Translocation of Water, Nutrients, and Disease-Inducing Organisms Among Forest Trees

By J. E. Kuntz and A. J. Riker,* USA

Forest trees constitute an important and replaceable natural resource. Intensified efforts are being made not only to improve existing stands, but also to provide reforestation. Thus, factors influencing tree development have special interest. An understanding of the movement of materials within the sap streams of trees helps to explain their responses to nutrients, silvicides, and pathogenic micro-organisms. The function and frequency of natural root grafting among forest trees influence the translocation of water, nutrients, and disease-inducing organisms from one tree to another. Experiments with radioactive isotopes have clarified these relationships.

The purpose of the present study was to determine the rate of movement and the distribution of chemicals in the sap streams of certain forest trees, the environmental conditions which directly affected such movement, and both the incidence and the role of root grafting among forest trees. This information has helped to interpret the movement of materials within and between oak trees infected by the oak wilt fungus, *Endoconidiophora fagacearum* Bretz.

MATERIALS AND METHODS

Naturally occurring northern pin oaks (*Quercus ellipsoidalis* Hill) and bur oaks (*Q. macrocarpa* Michx.) were used in most studies. Many were scrub oaks of stump sprout origin, growing in mixed oak and pine stands on relatively infertile Plainfield sand in central Wisconsin, USA. Other forest tree species both in natural stands and in plantations also were studied.

The radioactive isotopes employed were sodium iodide-131 and rubidium-86 carbonate. Both had satisfactory gamma radiation, were readily soluble in water, could be detected easily at low concentrations, and had half-lives of 8 and 19.5 days, respectively. Thus, no disposal problem occurred. Standard precautions were taken in handling these materials. Radioactivity was detected with a portable, Geiger-type monitoring instrument with a usable sensitivity of approximately 0.05 milliroentgens per hour. The working background was 0.05 to 0.07 mr/hr. An activity twice this background was set as a minimum positive reading.

Treatments of trees were made in various ways. In branch treatments, a cut branch was inserted immediately into a bottle containing the isotope. Roots were treated after excavation in a similar manner. Trunks were treated by fastening waterproof paper cones with asphalt cement to the main bole. A dilute solution of iodine-131 was poured into the cone and a half-inch chisel cut was made under the liquid through the last 3 annual rings. However, with rubidium-86, a 1.5 per cent KCl solution first was poured into the cone to reduce tissue adsorption or absorption of the rubidium. Uptake was rapid in the summer on sunny days. About one-half a millicurie of rubidium-86 in water then was added. Readings were taken immediately and at suitable intervals thereafter.

The movement of isotopes was checked by using 0.1 per cent aqueous solutions, respectively, of eosin, safranin O, and fast green as well as a concentrated sodium arsenite solution containing an excess of sodium hydroxide.

EXPERIMENTAL RESULTS

The normal rate of upward movement of the isotopes in the sap streams of oaks in full sunlight and with low relative humidity was between 1.5 and 3 feet per minute. Downward movement also was rapid. However, the extent of downward movement into the roots was limited except in those roots which were grafted to other roots from adjacent trees.

In northern pin oaks, the isotopes were distributed throughout the crown. Radioactivity was detected within 20 minutes in most branches, twigs, and leaves of oaks 35 feet high. In bur oaks and white oaks, the rate was comparable. However, radioactivity appeared only in narrow, vertical streaks which originated at the chisel cuts and in branches which had vascular connections with such trunk streaks. Thus, certain branches were radioactive; others were not.

The movement of isotopes in the sap stream varied considerably throughout a 24-hour period. Rapid movement occurred during the day from the time the

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sun first shone on dry leaves until the sun dropped below the crown canopy. At this time, light intensity diminished rapidly, temperature decreased, and relative humidity increased. A greatly reduced rate (0.05 to 0.1 foot per minute) occurred throughout the night. Comparable results were obtained when trees were covered with light-proof canvas. During daylight hours, dense clouds over the sky reduced movement to 0.4 feet per minute. Movement was only 0.2 feet per minute when the leaves were wet from dew, rain, or artificial sprays with water. No movement occurred at 0°C or below.

Seasonal variations in the rate of upward movement were examined through 3 calendar years. The average rate of movement remained uniformly high during the growing season until fall coloration appeared. During the dormant period and until the buds swelled in the spring, movement continued at a much reduced rate of 0.03 feet per minute. During this dormant period, movement was practically limited to the south sides of the trees. As leaves expanded in the spring, movement gradually increased to the normal summer high.

The downward passage of radioisotopes into the root collar and their subsequent detection in certain adjacent trees led to a study of natural root grafting among forest trees (Fig. 1). Radioactive potassium bromide-82, because of its extremely short half-life and its strong gamma radiations, proved to be especially effective in tracing movement through roots and root grafts. To insure maximum downward movement, trees were cut above the cone just prior to treatment. Because of the expense of isotopes and because of their rapid dilution in passage through trees, comparable studies also were made with dyes and poisons (Fig. 2).

In a representative experiment where trees were about 10 feet apart, treatment of 1 tree disclosed that 5 nearby trees were grafted to it. When these were treated, 21 additional trees proved to be joined. When these were treated in turn, 10 more trees were involved. Thus, after only 3 successive treatments, 36 trees were found to be grafted directly or indirectly. Occasionally, some trees near a treated tree were skipped; however, such trees usually became involved following successive treatments. From a series of comparable experiments, each tree appeared grafted to 3 or 4 adjacent trees. In one case, at least 8 trees within 52 feet of the treated tree were connected.

Among hundreds of trees investigated, root grafting was common among northern pin oaks, but was infrequent among bur oaks or among white oaks In only one instance was a graft discovered between different species of oaks; namely, a northern pin oak and a bur oak.

Roots of all sizes, ⅛-inch or larger, were found grafted together. Trees in dense stands were united much more frequently than were those in open stands. Washing and digging out of tree root systems

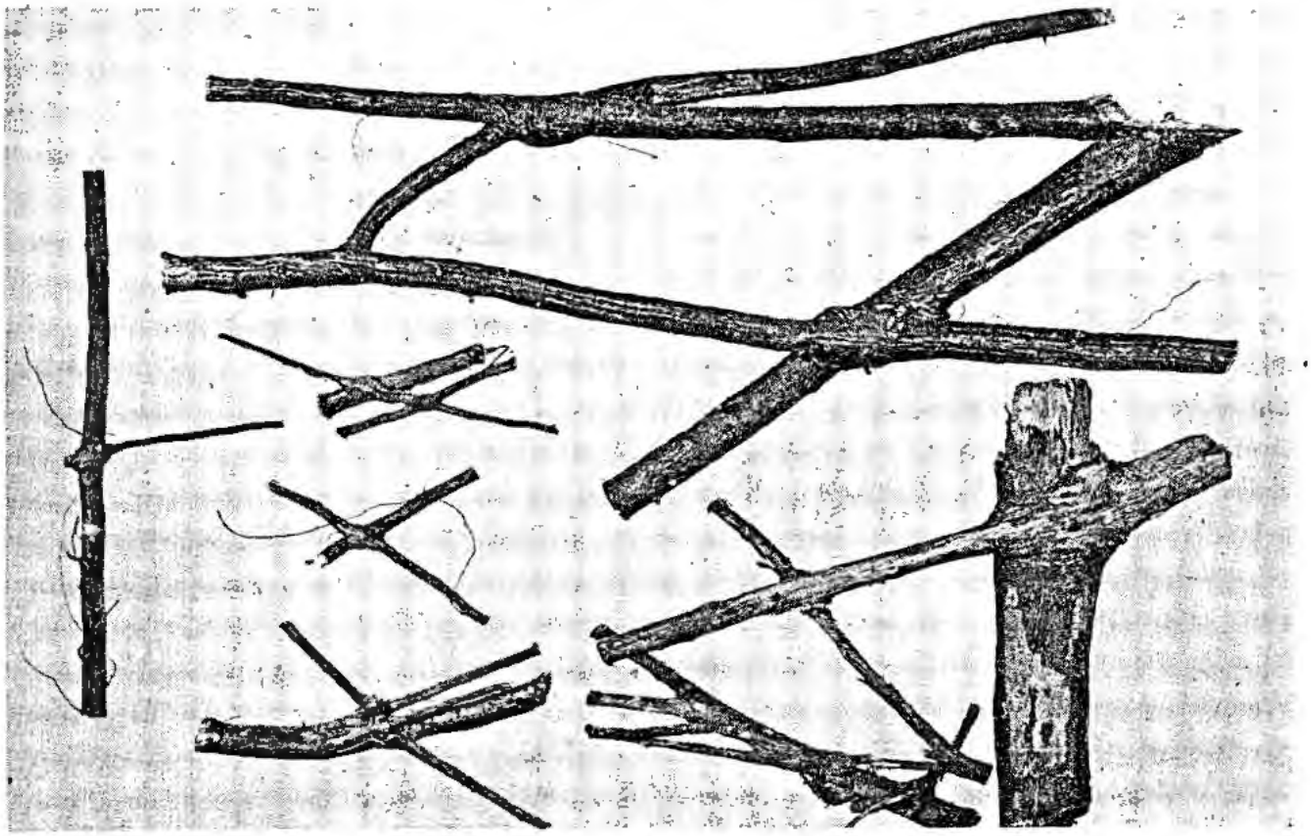

Figure 1. Representative grafts between different size roots of northern pin oak trees

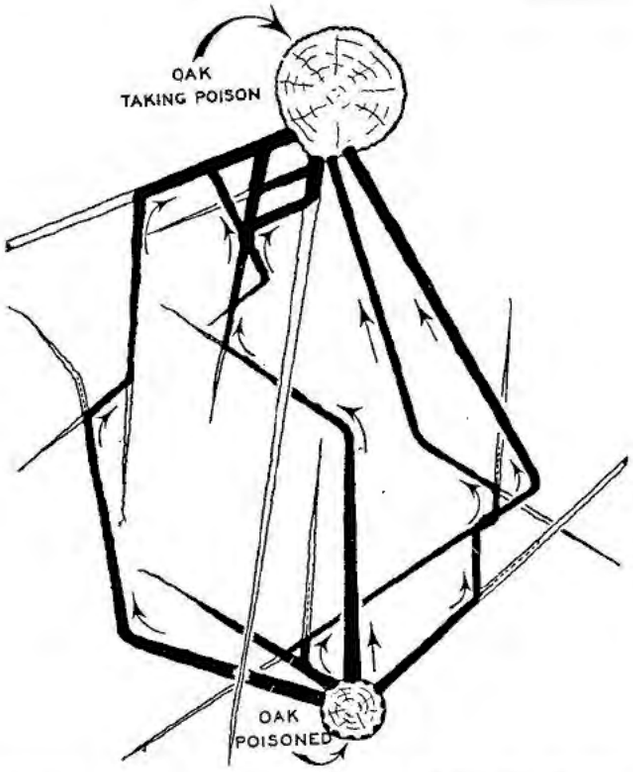

Figure 2. Diagram of the root systems of two northern pin oaks, showing the movement of sodium arsenite from one treated tree to a nearby tree: grafted roots through which the poison passed are solid black

confirmed these results. For example, when the root systems of 3 nearby northern pin oaks were partly excavated, 28 root grafts among the 3 trees were revealed (Fig. 3). Grafts between roots of the same tree were common. By means of root grafts, trees often "adopted" parts of the root systems of other trees which had been destroyed (Fig. 4).

The direction of sap flow between dominant and suppressed trees was determined in limited studies. While isotopes moved in both directions, they more often passed from the dominant to the suppressed tree. This phenomenon perhaps assists the survival of suppressed trees.

Root grafting within other given species was observed as follows: large-toothed aspen and red pine trees were grafted to a considerable extent; red oak, sugar maple, and white pine trees were grafted frequently; quaking aspen trees were grafted occasionally; white birch and jack pine trees were grafted seldom. No grafts were found among white spruce, black spruce, or balsam fir trees. Root grafting was common among 4-year-old red pine seedlings in nursery transplant beds. These results confirm the occasional reports in the literature concerning root grafting between trees.

In a similar manner, radioactive isotopes have clarified the manner in which the oak wilt disease develops and spreads from tree to tree in local areas. Oak wilt is a vascular-wilt disease which results when the fungus, *Endoconidiophora fagacearum* Bretz, invades the water-conducting vessels of oak trees. Sudden wilt, premature defoliation, and death of infected northern pin oaks usually follow. Bur oaks and white oaks more often show only wilting of the leaves on scattered branches and usually live for several seasons after initial symptoms. Oak wilt is the most serious disease threatening the vast hardwood timber forests of eastern United States.[3] Hence, oak wilt studies involving radioisotopes are of special significance.

The movement not only of solutions but also of particulate material was traced with isotopes. The rate and distance radioactive spores of the oak wilt fungus were carried in sap streams were measured. A concentrated spore suspension first was treated for 1 day with 1 mc of $Ag^{110}NO_3$. Next, the spores were washed repeatedly until the supernatant liquid had little radioactivity. The spores were resuspended and treated with 1 mc of NaI^{131}. The spores, now containing insoluble $Ag^{110}I^{131}$, were doubly radioactive. They again were washed repeatedly. Although their weight was increased, the radioactive spores were soon detected in the terminal branches of an oak 5 feet high. Fungus spores thus passed readily through the water-conducting vessels of oaks.

Studies of host responses to fungus invasion demonstrated that the upward movement of radioactive isotopes in the transpiration streams of infected northern pin oaks was reduced by 85 per cent 3 to 4 days before foliage wilt appeared.[1] A further decrease occurred during the development of moderate and severe wilt symptoms. Movement in tress with severe wilt often was reduced by 99 per cent. Similar reductions were found in the flow of water through branch sections cut from wilting trees. Microscopic examinations showed that tyloses and gums had formed in the xylem vessels throughout the outer annual rings of the sapwood. They were consistently associated with and preceded leaf wilt.[6] These results indicated that vascular plugging had effectively blocked water conduction. Wilt and death followed.

Critical differences in the reaction of other oak species to infection were detected immediately by means of radioisotopes. In infected bur oaks, radioisotopes moved normally until 3 days prior to incipient foliage wilt. After wilt development, movement continued only in certain unobstructed portions of the outer annual ring. Radioactivity was detected only in the symptomless branches. Microscopic examinations of the trunk and branches of infected bur oaks showed that vascular plugging had developed only in narrow, vertical sectors of the trunk and in branches having vascular connections with these sectors. Thus, as with infected pin oaks, radioisotopes demonstrated that fungus distribution, vascular plugging, and symptom development were closely associated.[4]

The continued downward movement of isotopes into and through grafted roots of diseased oaks indicated that vascular plugging was limited in the roots. Through roots isotopes moved even after aerial portions of the tree had died.

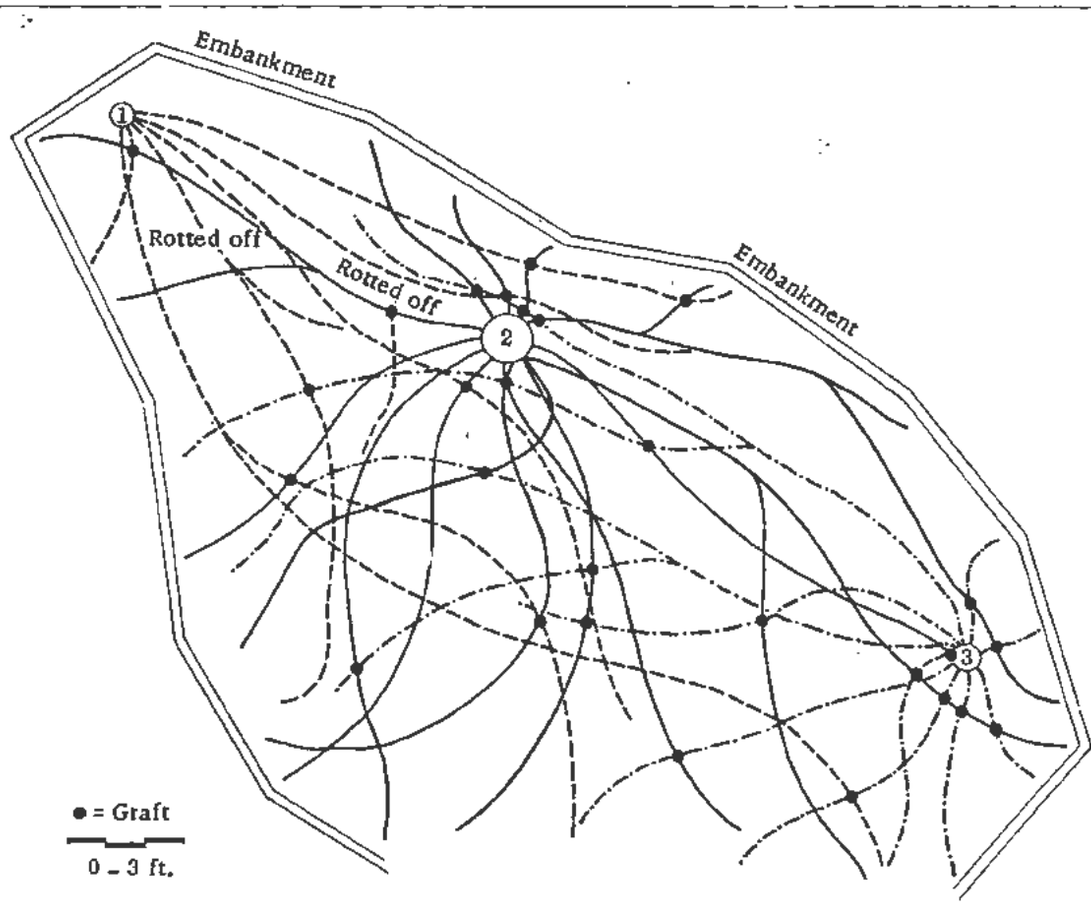

Figure 3. Diagram of the partly exposed root systems of three northern pin oak trees. Small solid black circles indicate 28 graft unions between roots of different trees: the numerous grafts between roots of the same tree are not shown

This basic information expedited effective control measures.[2,5] In lawn and park areas where individual trees were of great value, severing all root connections to a depth of 36 inches or more between wilting and adjacent healthy trees usually prevented further local spread. Since the oak wilt fungus frequently moved from infected trees soon after wilt development into nearby trees, severing root connections between 2 or 3 rows of trees appeared advisable. In woodland areas where individual trees were of relatively low value, poisoning healthy oaks within 25 feet of the wilted trees usually confined the infection. Root kill was essential. The width of the poisoned barrier depended on species, age, stand density, and accessibility.

DISCUSSION

Radioactive isotopes proved to be effective tools for studying the movement of materials within and between forest trees. Even at low concentrations, their presence could be detected immediately and for some time. As used, they caused no visible disturbance to the treated trees. They facilitated root-graft studies by indicating which roots should be excavated. Their chief disadvantages were their expense and their rapid dilution in moving through the trees.

In relatively dense stands of northern pin oaks in central Wisconsin, practically all trees appeared to be united to their neighbors through root grafts. Thus, trees of a forest stand might be considered as a united "community" rather than as independent individuals. Normally, such unions could be beneficial. However, when a disease such as oak wilt appeared, the fungus had direct vascular "pipelines" from tree to tree. Even-aged stands of the same species are especially vulnerable to such attack.

Determinations of the extent and functions of natural root grafts clarified, in part, the survival and persistence of suppressed trees, the increased growth of the trees remaining after a partial thinning, the longevity of certain stumps, and the vigor of many stump sprouts.

In oak wilt investigations, radioactive isotopes opened the way for studying certain host reactions to fungus invasion, the basic reasons for symptom development, and the manner in which the causal fungus spread from an infected tree to neighboring trees. Doubtless many of these findings may be helpful with other serious vascular wilts of forest trees.

SUMMARY

Radioactive isotopes were used to trace the movement of materials in the sap streams within and between both healthy and diseased trees. Iodine-131 and rubidium-86 proved satisfactory. Upward move-

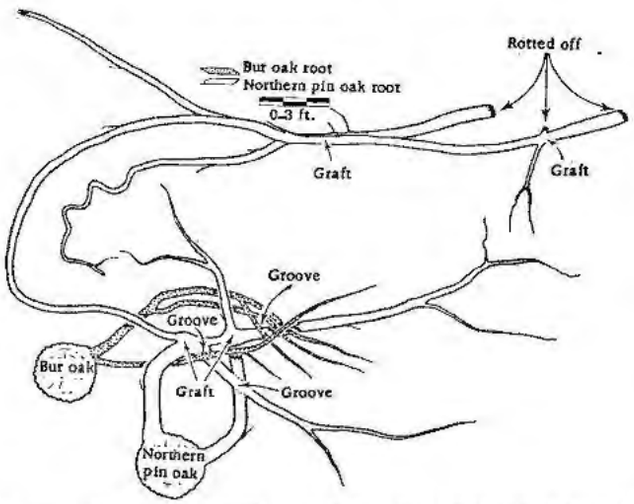

Figure 4. Diagram of several roots from a tree previously destroyed which have been "adopted" through root grafts by an adjacent healthy tree. Bur oak roots (stippled), though tightly intertwined and deeply grooved by mutual pressure with roots of the same northern pin oak, did not graft

ment in oaks under conditions favoring transpiration averaged 1.5 to 3 feet per minute. Very low light intensities, free moisture on the leaves, very cold temperatures, and the absence of functional leaves all greatly reduced movement. Little movement occurred in the dormant period. Downward movement into the roots was limited except in those roots which were grafted to roots of adjacent trees.

When dominant and suppressed trees were connected by root grafts, isotopes moved both ways but usually from the dominant to the suppressed trees.

The diffuse movement of isotopes throughout the trunks and the crown of northern pin oaks was readily traced. Such movement was strikingly different from the limited linear flow in the trunk and certain branches of bur oak trees.

In studies of the oak wilt disease, radioactive fungus spores were carried rapidly for some distance in the sap stream. The failure of iodine-131 and rubidium-86 to move in trees with oak wilt led to the discovery that the xylem vessels in aboveground parts were plugged with tyloses and gums. Critical differences between oak species were found. Few root vessels were obstructed, however. Thus, the isotopes helped to demonstrate a close association of fungus distribution, vascular plugging, symptom development, and the spread of infection from tree to tree through root grafts. This basic information advanced the development of practical control measures for oak wilt.

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The Utilization of Ionizing Radiation in Agriculture

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The ever-growing availability of the various sources of ionizing radiation makes it desirable to look for means of effective application of this type of radiation to food industry and agriculture. Research conducted in the Soviet Union in the sphere of application of ionizing radiation embraces: (*a*) sterilization and conserving of foods and extension of the period of storage of potatoes and vegetables; and (*b*) acceleration of seed germination and of the initial stages of development of agricultural crops, as well as raising their yields.

The present paper brings up for discussion the problem of the future prospects of utilizing the stimulating action of ionizing radiation for the growth and development of agricultural plants.

The possibility of using ionizing radiation for stimulation of the initial stages of plant growth was proved by Körnicke,[1] Evler,[2] Miege and Coupé,[3] Rochlin and Glechgewicht,[4] Doroshenko,[5] Stoklasa,[6] Chelhov,[7] Breslavets and Atabekova,[8] Breslavets and Afanasyeva,[9] Breslavets *et al.*,[10] Long and Karsten,[11] Frolov,[12] Atabekova,[13] Breslavets,[14] Drobkov,[15] Dietrich,[16] Sparrow and Christensen,[17] Bossi,[18] and many others.

In a series of studies by Yamada,[19] Nakamura,[20] Komuro,[21] Doroshenko,[5] Breslavets,[14] Grankhall *et al.*,[22] the stimulating effect was followed up to the ripening period, and higher crop yields were registered.

However, in view of the fact that in spite of similar treatment contradictory results were eventually obtained, the stimulating effect on the quality and the amount of yield still remains in dispute. Thus, Johnson[23] obtained no stimulating effect probably owing to low-precision dosimetry. Besides, his conclusions are based on too scanty material. The experiments by the same author (1948) on a decorative plant (*Kalanchoe tubiflora*) showed accelerated flowering and ripening of plants under the influence of radiation.

On the basis of experimental evidence obtained in twelve different species of plants—wheat, barley oats, peas, beans, horse beans, mustard, lettuce, esparcet and three pasture grasses—Schwartz *et al.*[24] denied the stimulating action of X-rays. In treating dry and sprouting seeds of wheat and peas with various doses of X-rays, A. V. Koltsov and A. I. Koltsov[25] did not observe any stable stimulating effect. Patten and Wigoder[26] subjected to radiation the seeds of beans, mustard and barley, but also failed to obtain any marked stimulation.

The contradictory results of a number of studies are largely due to a difference in the methods of treatment, to inadequate dosimetry in the past, to underestimation of radiosensitivity of different plant species, as well as to the conditions of the environment in which the treated plants developed. The possibility of obtaining a positive effect makes it expedient to continue research in this field.

Within recent years Breslavets, Berezina, Butenko, Vlasyuk, Drobkov, Zhezhel, Kuzin, Kuznetsov, Nichiporovich, Shirshov, Engel and many other Soviet scientists studied the action of ionizing radiation on growth and development of plants. It should be emphasized that some data obtained by Soviet scientists under practically similar experimental conditions are indicative both of a stimulating effect and the absence of a stable positive action. In the present paper we shall confine ourselves to those studies which conclusively enough demonstrate the presence of the stimulating effect and which seem to be of interest for the problem at issue.

The following methods of treatment were applied: (*a*) pre-sowing irradiation of seeds, (*b*) pre-soaking of seeds in solutions containing natural and artificial radioactive substances, (*c*) treatment of the soil with radioactive substances serving as microfertilizers, (*d*) continuous irradiation of growing crops with Co^{60} γ-rays. The results are briefly discussed below.

PRE-SOWING IRRADIATION OF SEEDS

Irradiation of seeds before sowing has great advantages as compared with other methods of treatment. Its chief merits are that (1) irradiation can be carried out in specially equipped places with subsequent transportation of seeds; (2) irradiation of material can be performed at a suitable time; and (3) there is complete absence of radioactivity both in the planted material and in the crops.

In a series of experiments conducted in 1953–54 the difference in radiosensitivity of several species of agricultural plants was tested. Radiation of dry seeds with X-rays was carried out at 170 kv, 6 ma, with a cardboard filter at a distance of 60 cm from the source of radiation (43 r/min). The subsequent procedure consisted in germinating irradiated seeds on filter paper in Petri dishes in duplicate or triplicate and measuring the length of the roots in the course

Original language: Russian.

of growth. One hundred irradiated seeds were sown in each Petri dish.

The following irradiation doses were used for short-time exposures of the seeds of a number of plants: rye, 750–1000 r; pea, 350–500 r; radish, 500–1000 r; cabbage, 1000–2000 r; and cucumber, 100–300 r.

Table I illustrates the results of measurement of root length of rye shoots for various radiation doses. Thus, the maximum length of the roots was registered in seeds irradiated with 1000 r.

In one of the experiments not only the length of the shoot roots was measured, but also their diameter in the region of tissue differentiation (Table II). It will be seen from the data that the maximum root diameter corresponds to the radiation dose of 750 r.

It is theoretically important that the increase in the root diameter is due not to the increase in size of the cells, but to the increase in their number (Table III). The increase in the number of cells is indicative of accelerated cell division as caused by irradiation.

Radish seeds were also studied. The conditions of irradiation were the same as for rye seeds. The stimulation of development is shown in Table IV.

Table I. Effect of X-irradiation on Length of Roots in Rye

Dose, r	Length of roots, mm	
	3rd day of development	4th day of development
Control	17.8	43.5
250	18.9	49.8
500	20.7	52.2
750	18.9	49.7
1000	24.1	55.6

Table II. Effect of X-irradiation on Root Diameter in Rye

Dose, r	3rd day of development Diameter, μ
Control	304
250	363
500	387
750	393
1000	359
2000	326
4000	317
8000	305

Table III. Number of Cells in the Subepidermal Layer of Roots after Irradiation with X-rays

Dose, r	Number of cells
Control	40
250	56
500	61
750	68
1000	57
2000	39
4000	40
8000	36

Table IV. Root Length of Radish Shoots after Irradiation with X-rays. Root Length, mm

Dose, r	Day of development		
	3rd	4th	5th
Control	27.2	37.6	50.0
250	27.4	50.7	63.9
500	32.6	64.0	72.2
1000	28.2	43.6	53.0
2000	17.2	34.0	48.0

Table V. Root Length of Pea Shoots after Irradiation with X-rays. Root Length, mm

Dose, r	Day of development			
	3rd	4th	5th	6th
Control	15.5	21.6	53.6	65.4
500	21.3	32.5	61.2	70.4
1000	22.4	28.6	49.7	57.1
2000	22.7	26.7	38.4	41.6

Table VI. Root Length of Cucumber Shoots Irradiated with X-rays. Root Length, mm

Dose, r	Day of development		
	3rd	4th	5th
Control	21.2	41.4	82.6
100	18.6	40.6	97.0
200	16.6	21.9	67.4
300	21.6	39.2	92.0
400	16.1	21.6	35.0
500	16.6	33.3	89.5
1000	13.2	15.7	49.9
2000	14.1	18.6	43.7
4000	17.1	29.4	59.1
8000	12.1	18.9	34.5
16000	11.1	19.2	26.6

Similar experiments with pea seeds gave the following results (Table V). It will be seen that the dose of 500 r calls forth a distinct stimulating effect.

The effect of X-ray irradiation of dry cucumber seeds was rather peculiar: while no clear-cut results were obtained in measuring the root length of the shoots of irradiated seeds (Table VI), an optimal dose of 300 r accelerated the growth of the first true leaves (Table VII).

It will be seen that radiation doses of 100–300 r stimulate the growth of the first true cucumber leaves.

However, the stimulating effect was not universal. Thus, irradiation of dry soaked seeds and shoots of soft wheat did not call forth any stimulation. The wheat did not react to low doses of radiation, while doses of 1000 r or more produced an inhibitory effect. Frolov's[12] experiments with another variety of wheat showed a stimulating action of X-rays and a higher yield. This fact points to the necessity of taking into account the radiosensitivity not only of individual species, but of different plant varieties as well.

Table VII. Length and Width of First True Leaves after Irradiation with X-rays (mm)

	1st true leaf		2nd true leaf		3rd true leaf		4th true leaf	
Dose, r	Length	Width	Length	Width	Length	Width	Length	Width
Control	31.9	—	86.6	76.0	59.0	62.3	no 4th	leaf yet
100	36.8	—	74.3	76.0	57.0	71.3	no 4th	leaf yet
200	30.3	—	79.3	73.3	42.6	40.3	no 4th	leaf yet
300	34.2	—	97.0	91.6	74.3	74.0	22.0	20.3
400	23.2	—	86.3	82.6	45.3	44.3	no 4th	leaf yet
500	30.4	—	86.6	76.0	44.3	37.3	no 4th	leaf yet

The acceleration of the initial stages of plant development is of primary importance since it may essentially influence the yield in the arid districts as well as in those where the sowing period is limited.

The acceleration of growth at the initial stage of plant development results in earlier ripening and a higher yield. This will be illustrated by a few examples:

Experiments with Radish

On January 1, 1954, in the Marfino State Farm, seeds of radish of the Saks variety were sown in the hothouse on an area of 12.5 m². Some of the dry seeds were irradiated with different doses of X-rays, the remainder were used as the control. The results are summarized in Table VIII. This little experiment, in which the plants were grown to commercial ripeness, illustrates the significance that may be acquired by this kind of radiation.

In another experiment carried out in the same State Farm on March 18, irradiated seeds of the "Moscow hothouse" variety were sown. The same radiation doses were used as in the first experiment. When treated with 1000 r, the radish ripened several days earlier than control specimens. The tubers grown were bigger in size (Fig. 1) and of prime quality, both as regards taste and succulence.

At the vegetable station of the Timiryazev Agricultural Academy in Moscow, irradiated radish seeds of the "White-pinkish" variety were sown in the field on 6 m² plots over an area totalling 72 m² (Table IX). The tubers grown from irradiated seeds were of prime quality, both as regards their taste and succulence.

Table VIII. Radish Yield as Influenced by X-rays

Dose, r	Yield of tubers from the test area, kg	Yield, %
Control	3.74	100
500	4.48	119
750	4.47	119
1000	4.98	133

Table IX. Radish Tuber Yield in the Field after Treatment with X-rays

Dose, r	Total weight of tubers obtained from a 6 m² plot, kg	Yield, %
Control	9.42	100
500	11.30	119
1000	12.27	140

Experiment with Cabbage

An experiment with cabbage of the "Kolkhoznitsa" variety was made at the Gribovo vegetable station. Dry seeds were subjected to irradiation with X-rays. Radiation doses of 1000 and 2000 r were tested which caused an acceleration of ripening and a certain increase in the yield (Table X).

Experiment with Peas

This experiment was conducted at a vegetable station of the Timiryazev Agricultural Academy in Moscow. It consisted in planting a variety of split peas on 6 m² plots, in triplicate. To estimate the yield, 50 plants were taken from each plot and the number of seeds produced by each plant was determined (Tables XI and XII). Any further increase in radiation doses resulted in reducing the yield.

Experiment with Rye

Irradiation of spring rye seeds with X-rays (doses of 750–1000 r) resulted in a 21–22% increase in the weight of 1000 grains.

Irradiation of soaked pea seeds with Co^{60} γ-rays (dose 250 r) likewise resulted in increasing the yield (Table XIII). These experiments prove that exposure of seeds to ionizing radiation before sowing may result in higher yields and accelerate the ripening of some crops.

Table X. Yield of Cabbage of the "Kolkhoznitsa" Variety Treated with X-rays

Dose, r	Yield, centner/hectare	Yield %
Control	222.4	100
1000	265.6	119
2000	266.3	119

Table XI. Harvest Yielded by Split Peas Treated with X-rays

Dose, r	Number of pods	Yield, %
Control	73	100
350	80	110
500	88	121

Table XII. Weight of Pea Pods and Seeds after Treatment with X-rays

Dose, r	Weight of 1000 pods, gm	Weight of 1000 seeds, gm
Control	3700	338
350	4290	407

Figure 1. Tubers of irradiated and non-irradiated radish

Table XIII. The Effect of Irradiation with Co^{60} γ-rays on Pea Yield

	Weight per plant			
	Seeds		Vegetation mass	
Exposure time, hr	gm	%	gm	%
Control	2.64	100	5.48	100
24	3.87	150	6.80	124
6	3.47	131	6.14	112
6	3.68	139	6.40	117
0.5	3.24	123	6.18	113
0.5	3.60	136	6.68	121

The use of X-ray equipment under rural conditions is not profitable. The most convenient source of radiation for this purpose is Co^{60}. It has been experimentally proved that there is no essential difference between the effect produced upon seeds by X-rays and by Co^{60} γ-rays while the use of cobalt as a source of radiation is by far more practical. A preliminary comparative study of short exposures to large doses of X-rays and of long exposures to small doses of Co^{60} γ-rays shows that by increasing the exposure time it is possible to reduce considerably the effective dose.

PRE-SOAKING OF SEEDS IN SOLUTIONS CONTAINING NATURAL AND ARTIFICIAL RADIOACTIVE SUBSTANCES

Extensive work in this direction was conducted in various research institutes of the Soviet Union. The object of study was the influence exerted by a number of radioactive isotopes upon the growth and development of plants, particular attention being paid to the use of non-separated mixture of the fission products of uranium. These studies demonstrated that prolonged action of diluted solutions of β- and γ-emitters is more effective than that of concentrated solutions with a lesser exposure time. The best results were obtained with solutions of a mixture of β- and γ-emitters with an activity ranging from 0.2 to 0.5 mc/l, the seeds being soaked for 24 hours. The experiments were made in a laboratory and verified in field conditions.

The following cultures were studied: peas, vetch, haricot, beans, soybeans, alfalfa, wheat, barley, oats, millet, buckwheat, sugar beet, flax, maize and tomatoes. A few examples are cited below:

The experiment with peas* was conducted on 5 m^2 plots and repeated six times. The seeds were soaked for 24 hours in a solution of non-separated fission products of uranium with activity of 0.5 mc/l. Control seeds were soaked in water. Analysis of the yield gave the following results (see Table XIV).

Table XIV. Effect of Radioactive Fission Products on Pea Yield

Yield indices	Excess over control, %
Weight of aerial organs	40
Number of pods	60
Number of seeds	100
Weight of pods	55
Weight of seeds	100
Yield of seeds in per cent of yield of aerial mass	40

As a rule, in the experiments with peas, vetch, beans and soya conducted on plots, flowering was found to take place 2–5 days earlier.

The average data computed from a number of experiments show that the weight of seeds of one plant exceeded that of control seeds as follows: peas, by 34%; vetch, by 57%; haricot, by 98%; beans, by 26%; and soya, by 53%.

In field experiments with leguminous crops the seeds were soaked for 24 hours in a solution of non-separated fission products of uranium with a concentration of 0.5 mc/l.

Numerical differences in the results obtained in repetitive experiments with leguminous crops are shown in Table XVI.

Wheat seeds were soaked in solutions containing artificial radioactive isotopes of phosphorus and calcium. The experiments were conducted on 125 m^2 plots in quadruplicate (Table XVII).

The results of 32 field experiments, carried out over a period of four years, with seeds soaked in a non-separated mixture of radioactive fission products (0.5 mc) are presented in Fig. 2. The experiments with different crops were conducted on plots with an area ranging from 2.5 to 300 m^2 (Fig. 2).

The experiments with soaking seeds were repeated on a production scale on an area of 290 hectares. The results obtained were as follows (Table XVIII).

The results obtained in 32 field experiments and 42 production experiments with seeds soaked in solutions of radioactive fragments conducted in the course of four years, confirmed the validity of the observed positive effects. The real positive deviation amounted, on the average, to about ± 16% for leguminous crops and ± 10% for cereals.

* Similar experiments with wheat gave a 16–20 per cent increase in the absolute weight of the seeds, the pre-sowing material being treated with a solution of radioactive fission products.

Table XV. Effect of Radioactive Fission Products on the Yield of Leguminous Crops

Crop	Area, m²	Number of replications	Variants	Yield		
				kg	centner/hectare	Per cent over control
Peas	3	8	Control	4.179	17.4	—
			Experiment	4.626	19.3	110
Peas	10	5	Control	4.620	9.2	—
			Experiment	5.048	10.1	109
Peas	90	6	Control	25.450	4.7	—
			Experiment	31.300	5.8	123
Peas	100	5	Control	56.500	11.30	—
			Experiment	66.100	13.22	117
Peas	300	5	Control	156.900	10.46	—
			Experiment	165.000	11.00	106
Beans	2.5	4	Control	2.450	24.50	—
			Experiment	3.290	32.90	134
Beans	2.5	5	Control	2.69	23.74	—
			Experiment	3.73	29.84	125
Beans	15	5	Control	12.9	17.20	—
			Experiment	16.7	22.27	130
Vetch	3	7	Control	4.28	20.40	—
			Experiment	4.80	22.89	112
Vetch	10	5	Control	3.97	7.95	—
			Experiment	5.09	10.19	128
Vetch	100	5	Control	25.6	5.12	—
			Experiment	27.8	5.56	109
Lucerne	50	4	Control	140.6	70.3	—
			Experiment	163.5	81.7	116
Haricot	3	6	Control	2.06	11.4	—
			Experiment	2.70	15.0	130

The above method of pre-treatment of seeds is of interest from the point of view of utilization of the radioactive waste of atomic production. The difficulties of its large-scale application involve the organization of adequate health protection against radioactive substances. As to the traces of radioactivity in the harvest and the radioactive contamination of the soil this question requires further inquiry. Analyses have shown that the activity of the agricultural products amounts to less than 10^{-9} c/kg.

TREATMENT OF SOIL WITH RADIOACTIVE SUBSTANCES AS MICROFERTILIZERS

The application of radioactive fertilizers has been often discussed in the literature. Some researchers[27,28,29,30,6] point to the positive effect produced by small concentrations of natural radioactive

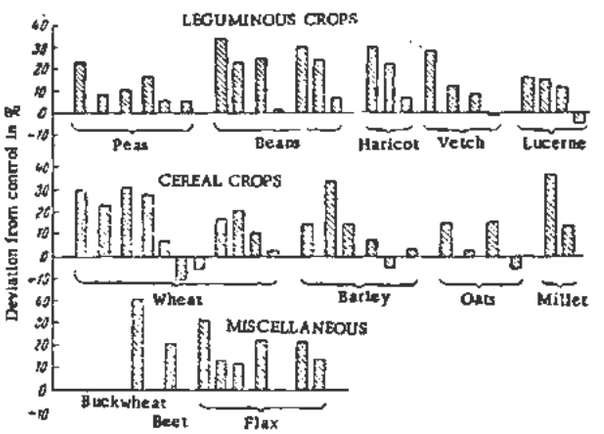

Figure 2. Effect produced by soaking seeds of various agricultural crops in solutions of radioactive fission products

Table XVI. Effect of Radioactive Fission Products on Replicated Yield of Leguminous Crops

Crop	Replications	Yield, kg/hectare	
		Control	Experiment
Russian black beans	1	22100	26600
	2	24000	28400
	3	23000	27000
	4	22900	26700
White beans	1	16400	16400
	2	24300	28400
	3	25800	24900
	4	14800	30200
	5	17550	24400
Beans (low-grade)	2	1975	2380
	3	1910	2420
	4	1630	2475
	5	1990	2240
Haricot	1	1110	1725
	2	1535	1608
	3	1440	1660
	4	1120	1293
	5	658	1120
	6	999	1610

Table XVII. Effect of P^{32} and Ca^{45} on Wheat Yield

Variants of experiment	Grain yield, centner/hectare	Per cent over control
Control (P^{31})	29.26	
Seeds treated with P^{32}, 50 μc/kg	32.45	110.9
Control (Ca^{40})	29.16	
Seeds treated with Ca^{45}, 50 μc/kg	32.23	110

Table XVIII. Effect of Radioactive Fission Products on Crop Yields

Crop	Yield, % over control (replications in different years)
Peas	110, 108
Vetch (for grain)	116, 110, 86, 105
Vetch (for hay)	103, 124, 122, 120, 115, 111
Lucerne	143, 123
Grass mixture (clover, lucerne, timothy)	108
Turnip	107, 108
Wheat	110, 132, 110, 105, 109, 99
	110, 118, 91
Oats	119, 102, 91, 102, 120

elements of radium, uranium, and thorium in doses varying from 10^{-12} to 10^{-9} c/l or c/kg of soil. No inhibitory effect was produced by these doses. Within the range of doses varying from 10^{-9} to 10^{-5} c/l, both a stimulating effect[28,30,31,32,33] and absence of such an effect[31,34,35,36] has been observed. The use of uranium and radium as α-emitters produced an inhibitory effect.[36] Higher doses cause, as a rule, an inhibition of growth and development of plants.

According to a report of the US Atomic Energy Commission,[37] the use of radium, uranium and an actinium-containing preparation as fertilizers produces neither a positive nor a negative effect.

Within the past few years extensive research has been carried again in the Soviet Union on natural radioactive substances (radium, uranium, thorium), radioactive fragments, artificial radioactive isotopes, as well as minerals and rocks with heightened radioactivity. In order to avoid contamination of soil, particular attention has been paid to the amount of fertilizers producing a slight increase in its natural radioactivity. No negative effect was observed in treating a number of crops with small doses of radioactive substauces (10^{-11} to 10^{-6} c/kg). The positive effect proved to be more stable in greenhouse experiments, while in field experiments it varied greatly according to the soil conditions and cultivation methods.

Thus, in water cultures of tomatoes an admixture of radioactive phosphorus caused a 25–38% increase in the yield (Table XIX).

In a series of eight-year experiments with the cultivation of potatoes, cabbage, oats, clover with timothy, spring wheat, sugar beets and sunflowers, the annual yield increases amounted to nearly 27% (10–68%). A single dose of 1×10^{-9} c/kg of radium and 1×10^{-4} c/kg of uranium was introduced into the soil. The results of the experiments are summarized in Table XX.

Table XIX. Effect of P^{32} on the Yield of Tomoto Fruits

Variants of experiments		Total weight, gm	Per cent over control
1st experiment	Control	265	100
	2.3×10^{-5} c/l	366	138
2nd experiment	Control	247	100
	2.3×10^{-5} c/l	311	126
	5.3×10^{-5} c/l	310	125

In field experiments with sugar beets carried out in different years with the application of radioactive fertilizers, there was an increase in sugar content varying from 0.2% to 1.5% and a 10–18% increase in the yield. Note that the results of the field experiments varied with the soil and vegetation conditions.

Many experiments conducted under laboratory and field conditions demonstrated a considerable stimulating effect of small doses of natural radioactive elements (uranium, radium, thorium), as well as of radioactive fission products, on reproduction, development and nitrogen-fixing capacity of *Bacillus radicicola* and *Azotobacter*. In some experiments the stimulating effect amounted to 60–100%.

The formation of root nodules was not observed in greenhouse experiments with peas, in which nutrient mixtures were used with specially purified admixtures of natural radioactive elements. The addition to the medium of radium or uranium salts in concentrations of 10^{-10} and 10^{-5} c/kg respectively resulted in abundant formation of nodules and a considerable increase in the average weight of the air-dry aerial mass, particularly in the weight of dry seeds (by 60–80%).

The stimulating effect of radioactive substances on the growth of tubercles was likewise observed upon application of artificial radioactive isotopes. A series of experiments demonstrating the influence of radioactive phosphorus on the development of tubercles on the roots of water and sand cultures of peas belonging to the "Capital" variety, may serve as an illustration (Table XXI).

Table XX. Effect of Natural Radioactive Substances on Crop Yields

Year	Crop	Yield, % over control: Radium 1×10^{-9} c/kg	Yield, % over control: Uranium 1×10^{-4} c/kg
1947	Potatoes (tubers)	124.2	137.3
1948	Cabbage (marketable)	113.3	138.3
1949	Oats (grain)	133.9	123.6
1950	Clover-timothy mixture (hay)	110.9	118.1
1950	Same, 2nd harvest	112.7	168.9
1951	Spring wheat (grain)	133.8	135.5
1952	Red beet (roots)	110.0	129.6
1953	Sunflower (silo)	111.3	122.8
1954	Potatoes	132.1	121.9
	Average for 8 years	120.2	132.9

Table XXI. Effect of P^{32} on the Development of Nodules

Doses of P^{32} in c/l	Number of nodules		Volume of fresh nodules		Air-dry weight	
	Pieces	%	cm^3	%	gm	%
Water cultures:						
Control	276	100	0.19	100	0.103	100
8.3×10^{-7}	329	119	0.22	116	0.155	150
8.3×10^{-6}	1122	406	0.44	233	0.358	347
8.3×10^{-5}	844	305	0.58	307	0.551	534
Sand cultures:						
Control	225	100	0.14	100	0.100	100
8.3×10^{-7}	275	122	0.13	94	0.125	125
8.3×10^{-6}	887	394	0.46	317	0.371	371
8.3×10^{-5}	476	212	0.61	417	0.582	582

The results of five-year large-scale production tests of the action of fertilizers consisting of natural radioactive rocks on various crops demonstrated a steady 15–25% rise in the yield. Moreover, the possibility of a positive effect being produced by other non-radioactive micro-elements contained in the specimens at issue is not excluded. However, a comparison of the results obtained with other data pertaining to the effect of radioactive elements corroborates the decisive role played by radioactivity in these experiments too.

Although the introduction of radioactive micro-fertilizers into the soil has justified itself in numerous experiments, it cannot yet be recommended for practical application, since the selective adsorption of the radioactive elements by the harvest and the effect produced upon the animal and human organism has not been sufficiently studied.

This method of application of radioactive fertilizers raises the extremely serious problem of protecting the working personnel. Until this is solved, this method cannot be called practical.

CONTINUOUS IRRADIATION OF AGRICULTURAL CROPS WITH COBALT γ-RAYS THROUGHOUT THE VEGETATION PERIOD

Research work in this field was started in 1954, and the results thus far obtained are, therefore, only preliminary. The effect of continuous exposure to weak external Co^{60} γ-radiation (activity of 1 curie) of the growing culture of sugar beet and buckwheat was studied in field conditions on a levelled-out plot. Observations were made along radial zones at various distances from the source. During the two months of the experiment the dose received by the plants varied between 0.9 and 116 r, depending on the distance from the plant to the radiation source. This experiment conclusively showed an acceleration of buckwheat growth. In the zones of maximum radiation (4.3–116.7 r) the treated buckwheat blossomed five days in advance of the controls. The number of plants in flower per zone (in per cent) on the 65th day of vegetation is given in Table XXII.

The buckwheat was grown for rutin and the green tissue was harvested at time of flowering. The harvest estimates are summarized in Table XXIII.

The experiment showed that plants of the first zone treated with 21.5 r during the period between sowing and the time of flowering increased the harvest yield by 45% as compared with the control.

Under similar conditions, plants of sugar beet were irradiated at a time when each rosette had 4–5 leaves. The result was an increase in sugar content varying from 0.6 to 1.2% in the zones with the lowest intensity of irradiation. Figs 3 and 4 show results of two repeated experiments with sugar-beet plants.

It will be noted that numerous experiments have been carried out by various authors on sugar beets in which the seeds were soaked in solutions of radioactive elements P^{32}, Zn^{69}, Ca^{45}, S^{35} and the plants receiving radioactive phosphorus, as extra nutrition via the leaves. In these cases as well as in greenhouse experiments with micro-quantities of uranium and radium salts, there occurred a 0.3–1.2% increase in sugar content above 16.8–19.4% control.

The results obtained are in conformity with the conclusions drawn by Granhall *et al.*,[22] who likewise observed a stimulating effect of small radiation doses.

The preliminary results obtained with small-dose irradiation of growing plants throughout the vegetation period open new prospects for further research along these lines, particularly with valuable crops

Table XXII. Effect of Gamma Radiation on the Initiation of Flowering in Buckwheat

Zone	Dose, r	Number of plants in flower
Control zone (outside of radiation)		6 ± 0.72
0 zone	116.7	51 ± 5.2
1st zone	21.5	41 ± 1.4
2nd zone	4.3	33 ± 1.78
3rd zone	1.7	10 ± 0.84
4th zone	0.9	7 ± 0.84

Table XXIII. Effect of Gamma Irradiation of Buckwheat on the Harvest of Vegetation Mass

Zone	Dose, r	Yield from one plot, kg	Per cent over control
0 zone	116.7	5003	112
1st zone	21.5	6323	145
2nd zone	4.3	5675	123
3rd zone	1.7	5047	110
4th zone	0.9	4515	99.3
Control	Natural conditions	4569	100

grown in the areas where acceleration of the harvest may prove of decisive significance.

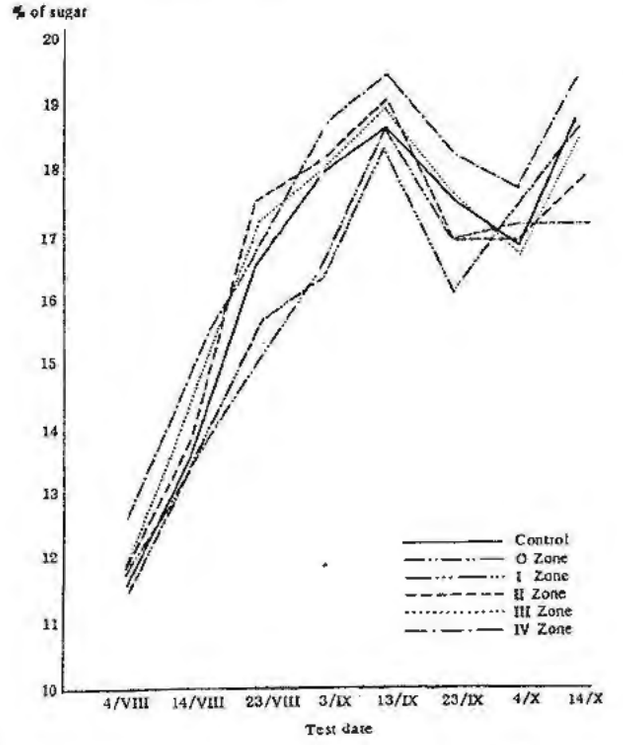

Figure 3. Sugar content in the tubers of irradiated and non-irradiated sugar beet plants

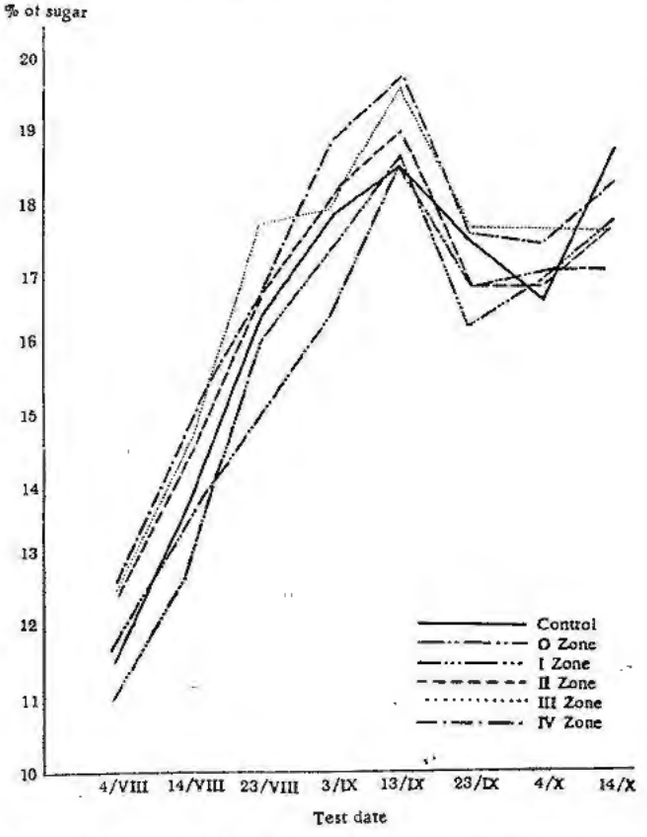

Figure 4. Sugar content in the tubers of irradiated and non-irradiated sugar beet plants

The above experiments demonstrated the possibility of raising harvest yields by using the stimulating effect of small doses of ionizing radiation. This makes it expedient to elaborate concrete methods of applying ionizing radiation for agricultural purposes. However, considerable fluctuations in the degree of effect as manifested by experiments conducted under different soil and climatic conditions, and insufficient elaboration of the theory of stimulation point to the necessity of combined efforts of all scientists for the solution of this problem.

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Use of Labeled Compounds in Weed Research

By A. S. Crafts,* USA

The mechanisms responsible for the movement of water, salts, and organic foods in plants have presented many difficulties in interpretation. The structure of the circulatory system of higher animals is quite obviously adapted to the function of distribution and tracers injected into this system are rapidly carried throughout the animal body. In plants the distribution of water and minerals absorbed by the roots takes place primarily through the xylem which comprises the woody cylinder of the plant body. And the primary distribution of organic foods apparently takes place through the phloem or inner bark. In addition secondary movements of minerals may occur through the phloem, secondary movements of foods may take place in the xylem, and a considerable quantity of water may move in the phloem in the assimilation stream.[1,2] The exact details of phloem structure that adapt this tissue to its function in transport are still not clearly understood.[3]

In the control of perennial weeds and woody plants by foliage sprays the toxic chemicals must be absorbed by the leaves or stems and transported via the vascular tissues to the roots. Lithium, potassium, radioisotopes, fluorescein, and many dyes have been employed as tracers in translocation studies (see reviews[2,4,5]). The herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) has proved to be an ideal translocation tracer. After application in small quantities it is readily absorbed through the cuticle of plant leaves. Once in the mesophyll cells it migrates to the vascular tissues of the leaf veins. In the phloem it is carried along with food materials to meristematic regions where the foods are being used in growth and respiration or are being stored. Its presence in growing tissues is soon evidenced by formative effects, production of malformed organs, proliferation of callus and in many instances injury and death of cells. This latter is the manifestation of its herbicidal properties.

The incorporation of C^{14} into the 2,4-D molecule marked a distinct advance in the use of translocation tracers in plants. The conformation of the 2,4-D molecule is apparently well adapted for rapid movement through plant cuticle. The parent acid molecule seems particularly well adapted for rapid absorption. Inside the plant, 2,4-D is so active physiologically that its formative effects on a sensitive plant like cotton can be detected following application of as little as one-tenth microgram. One microgram applied to a cotyledon of a cotton seedling causes obvious deformation of several of the newly formed leaves. When C^{14} is incorporated in the molecule it can be demonstrated by the autographing technic and shown to be present in the meristems responsible for the malformed tissues. In fact, high activity (8–9 mc per Mmol) 2,4-D can be used in doses so small that they cause little or no disturbance of normal plant function yet the treated plants after killing and drying can be autographed and shown to have transported the tracer in the normal manner. By killing and autographing plants at different stages in the process of translocation one can reconstruct the process as it has taken place in the test plants.[6]

After three years of study using C^{14} labeled 2,4-D we have eliminated many pitfalls in the method. These have included a rapid distribution of the tracer through the xylem resulting from quick killing with dry ice followed by thawing in the drying process. Freeze-drying eliminates this effect. Also included have been pseudo-autographs produced by naturally occurring plant constituents, autographs from volatile constituents in plywood used to back the films and a number of other effects.

We have adapted the method to studies on a wide variety of plants. So far the following greenhouse-grown plants have been utilized: bean, *Phaseolus vulgaris* variety red kidney; cotton, *Gossypium hirsutum* variety Acala; cucumber, *Cucumis sativus*; corn, *Zea mays*; oats, *Avena sativa* variety Kanota; wild morning glory, *Convolvulus arvensis*; zebrina, *Zebrina pendula*; ladino clover, *Trifolium repens*; blue oak, *Quercus douglasii*; live oak, *Quercus wislizenii*.

In the field we have used: wild morning glory, *Convolvulus arvensis*; blue oak, *Quercus douglasii*; live oak, *Quercus wislizenii*; willow, *Salix lasiolepis*; coyote brush, *Baccharis pilularis*; ceonothus, *Ceonothus cuneatus*; manzanita, *Arctostaphylos manzanita*; toyon, *Photinia arbutifolia*.

So far we have used the following labeled molecules: 2,4-D, urea, phenyl-acetic acid and benzoic acid. In the near future we will have employed 2,4,5-T, 3, para-chloro-phenyl, 1, 1-dimethyl urea, maleic hydrazide, and amino triazole. The acids are being applied as the parent molecules; we also contemplate using them in the salt and ester forms.

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Results of our work to date include the following general findings:

1. The herbicide 2,4-D can penetrate the plant cuticle, migrate across the mesophyll, and move in the phloem in quantities that are not immediately injurious to these tissues. The rates observed in the various stages of this transport agree with those cited by Day.[7]

2. The tracer is carried in the phloem and it moves only when and where foods are moving. These results agree with those of Rhorbaugh and Rice,[8] Weintraub and Brown[9] and Davis and Smith.[10]

3. The herbicidal properties (toxicity) of 2,4-D are expressed most prominently in young growing tissues. This agrees with van Overbeek's[11] conclusion.

4. The herbicide is broken down and lost from living plants presumably as a result of plant metabolism. Weintraub *et al.*[12] have reported on this phenomenon.

5. In killed, dried plants the radioactivity persists for three years or more with no evidence of loss.

6. Whereas the toxicity as expressed by killing of tissue may be localized in meristematic tissues, our recent experiments indicate that distribution may be more or less uniform along a stem (*Zebrina*) consisting of alternating intercalary meristems and mature regions. This indicates that it is the response and not the chemical that is localized.

From these generalizations a number of deductions directly relating to weed control may be made.

1. In entering the plant 2,4-D must pass a lipoid barrier. In some regions this is made up of the cuticle, in others of the cuticularized walls of the substomatal chambers, and in the case of all living cells, of the living cell membrane. For such passage the ester should be best, the acid next, and the salts least effective. Apparently certain adjuvants having surfactant properties facilitate this passage.

2. Once through this barrier, the herbicide must part from the lipoid phase and migrate through living cells to the phloem. Here the acid is superior to the esters. The heavy esters are more mobile than the short chain aliphatic esters.

3. Movement in the phloem is from regions of synthesis (mature leaves) to regions of utilization of foods (young leaves, flowers, buds, cambium, roots).

4. Response in roots (death) requires that the roots be growing and not simply storing food reserves.

5. Where plants are growing normally and particularly where roots are active, translocation of 2,4-D is active and distribution effective. Failure to kill apparently results more often from failure of the final response than from failure of translocation.

Normally plants growing in temperate climates go through an annual cycle made up of a period of dormancy, break of dormancy, spring growth, blossoming, maturation, and return to dormancy during which food reserves are stored. Because of the role of temperature, light, and soil moisture in this annual cycle of perennial plants it seems obvious that the time for successful treatment of such plants is critical. Under natural conditions in the west and the southwest where rainfall is distinctly seasonal it seems that the time for successful treatment of perennial weeds is limited to (1) a period in spring or early summer when growth is still active and soil moisture is available, and (2) a period in the autumn when growth is resumed and soil moisture is again available. In normal years these periods may last for weeks or months; in dry years they may be short or non-existent. This probably explains the failure of herbicide application to mesquite during certain years in Texas and Arizona. Where there are summer rains and where irrigation is practiced the favorable period may extend from spring through summer and into autumn. In the tropics the favorable period may be nearly continuous throughout the year where moisture is available for plant growth.

Althought these final conclusions draw upon research and deductions that have not involved use of the labeled herbicide, many physiological interpretations of our results have been quickly and positively verified by the tracer work. Such verification would have taken years of painstaking work if attempted by classical plant physiological methods. And the labeled materials are enabling us to plan and conduct studies that would be impossible by conventional methods. Thus they are making an outstanding contribution to the present day chemicalization of agriculture.

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Use of Isotopes for Determining the Availability of Chelated Metals to Growing Plants

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The availability of iron and zinc fertilizers to plants growing in soil has been greatly increased by combining these metals with chelating agents such as the amino acid ethylenediaminetetraacetic acid (EDTA). However, the availability of chelated iron and chelated zinc varies with the soil in which the plants are growing, the complexes being considerably more effective in some soils than in others. The reason for this variation constitutes the primary problem of the work to be reported, a problem in which the isotopic tracers carbon-14, zinc-65, and mixtures of iron-55, and iron-59 proved to be exceedingly useful.

The need for nutrients in a form that is readily available to plants is recognized to be of basic importance. Iron has been the most difficult one of the nutrients to supply to plants and a deficiency of this element is found in almost every major fruit growing area of the world. Previous to the development of chelated iron as a fertilizer in 1951 there were no practical means of correcting iron deficiency in many plants. Application of 100 pounds of iron sulfate to single orange trees growing in acid soils was insufficient to correct iron deficiency but application of only 10 to 20 grams of chelated iron greened the chlorotic trees.[3] Chelated iron is much more effective in acid soils than in alkaline soils and it is primarily in soil with high pH that difficulty with iron availability is encountered.[4]

Previously, it was believed that chelates other than those of iron would not be effective as sources of plant nutrients. Iron chelates are more stable than those of other metals so that when applied to the soil, rapid exchange between the metal in the chelate and iron in the soil was to be expected. However, it was found in the present work that in some soils, chelated zinc is much more effective as a source of this nutrient for plants than inorganic forms of zinc. Thus, the potentialities of metal chelates in plant nutrition are greater than was originally suspected.

The use of chelating agents for increasing availability of plant nutrients appears to be a very promising field of research. Investigations with chelated forms of copper, manganese, calcium, magnesium, and molybdenum, all of which are essential for growth of plants and often not available when applied as fertilizers, would seem to be desirable.

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MATERIALS AND METHODS

All the greenhouse and laboratory studies were carried out in triplicate and two or more determinations were made on each sample. The isotope measurements were made with a Geiger-Muller counter using end window tubes and a gas flow counter. Radioautographs were also found useful for showing the distribution of the isotopes in plants.

The carbon-14 analyses in plants were made by burning the dry tissue in a carbon train, precipitating the carbon-14 in $Ba(OH)_2$ and counting in a gas flow counter on samples of the $BaCO_3$ that had been collected and dried. Two methods were used for counting iron-55 and -59 in plants. One method was similar to that described by Peterson[6] in which dry plant samples were ashed and the iron extracted with cupferron and electroplated on copper disks. Another method was making counts on the plant ash. The latter method was used for the analysis of zinc-65.

The amount of the isotopes used varied for each experiment, and was determined by preliminary trials. In tagging the various compounds used in these studies the isotopes were first mixed with carriers which were iron and zinc sulfates for iron-55 and -59 and zinc-65 respectively and the disodium salt of EDTA for carbon-14. The chelates were prepared by adding an equivalent amount of a chelating agent to one of the metals to form a 1:1 complex. The carbon-14 EDTA used in these experiments had the carbon atoms adjacent to the nitrogen in the acetate groups tagged.

ADSORPTION OF CHELATES ON EXCHANGE RESINS

The chemical properties of chelates were studied to determine their relative stabilities. This was done by passing dilute solutions through ion exchange columns. Fifty milliliters of the chelates iron-hydroxyethylethylenediaminetriacetate (FeEDTA-OH), FeEDTA, and ZnEDTA, were passed dropwise through columns of a cation exchange resin IR-120 and through an anion exchange resin IRA-400. Iron was used at a concentration of 10 ppm and zinc at a concentration of 20 ppm and equivalent amounts of the chelating agents were added. Separate solutions of the chelates were labeled with iron-55 and -59, zinc-65, and carbon-14. Counts were made on two milliliters of the solutions that had passed through the columns and compared with those from

an equivalent amount of the solution that had not gone through a column.

All the FeEDTA passed through the cation resin without adsorption, but was completely taken up by the anion resin (Table I). However, all the iron-55 and -59 in the FeEDTA-OH was adsorbed on the anion resin and 76 per cent of it on the cation resin. This result suggested that under these conditions FeEDTA-OH is not so stable as FeEDTA even though the hydroxy chelate has been found to be more efficient in calcareous soils. Chaberek and Bersworth[1] suggested that the outstanding superiority of FeEDTA-OH in calcareous soils is due to its resistance to hydrolysis and subsequent formation of ferric hydroxide.

Studies with zinc-65 EDTA and carbon-14 ZnEDTA indicated that the zinc chelate was partially decomposed when passed through the cation exchange column. Eighty-five per cent of the zinc-65 was absorbed by the IR-120 resin and 39 per cent of the carbon-14. These exchange studies indicate that chelates may he affected both by cations and anions in the soil. Apparently, the primary adsorption in the soil is of the entire complex as an anion. Exchange of the metal in the chelate for other metals in the soil is an important factor in the less stable chelates. These studies also indicate that materials in the soil that normally fix iron and zinc sulfates may not react with the chelated forms that have an over-all negative charge. Apparently the change of the metals from cations to anions greatly increases their availability to plants.

ADSORPTION OF CHELATES BY CLAYS AND SOILS

Chelates probably react very readily with many components of soil. Adsorption studies were made with iron chelates in bentonite, kaolin, Florida red clay, Lakeland soil, and Parkwood soil. The Lakeland soil was fine sand collected from a citrus grove and had a pH of 6.2. The Parkwood soil was highly calcareous with a reaction of pH 8.0. Two hundred milliliters of a solution containing 10 ppm iron and an equivalent amount of EDTA were placed in a flask and one of the following then added: 100 gm of soil, 10 gm of kaolin, 50 gm of bentonite, or 100 gm of Florida red clay. In some of the flasks the FeEDTA was labeled with carbon-14 and in the others with iron-55 and -59. The flasks were shaken for two hours and the supernatant was filtered at intervals. Counts were made on two milliliters of the filtrate taken to dryness. Chemical analysis for iron using the *o*-phenanthroline procedure was made on another aliquot. The iron in solution was assumed to be chelated because its solubility was found to vary directly with the concentration of EDTA used in the extractant.

The iron chelates were found to be very active in the soil. Much more of the iron than the chelating agent was adsorbed, suggesting decomposition of the chelate (Fig. 1). However, the data from the chemical analyses (Table II) suggest that instead of a complete breakdown of the chelate there was exchange of iron in the soil for iron in the chelate. In addition, other metals apparently replaced some of the iron in the chelate. This is suggested by the data in Fig. 1 where in all instances more iron than chelating agent was adsorbed. Adsorption of either the chelate or chelating agent took place on all the clays and soils used. The bentonite adsorbed practically none of the EDTA whereas the Parkwood soil adsorbed the highest amount. The reason for the high adsorption of EDTA in the calcareous soil is unknown. When FeEDTA is shaken with a saturated solution of $CaCO_3$ all the iron precipitates but the EDTA remains in solution. Hence, in calcareous soils the way in which EDTA is fixed is probably by exchange of iron for calcium with the formation of iron hydroxide, and also by anion adsorption of the entire iron complex.

ABSORPTION OF CHELATES BY PLANTS

The entrance of chelated metals into plants has been explained in a number of ways, one being that exchange of the metals from the chelates takes place at the root surface. Another explanation is that the entire chelate is absorbed by the plant and the iron is removed from the chelate within the plant. There is some evidence that the latter explanation is the correct one. After growing plants in solutions containing carbon-14 FeEDTA, carbon-14 was found in the leaves of the plants.[7] Wallace *et al.*[8] have found that nitrogen-15 is taken up by plants when given nitrogen-15 FeEDTA. This could mean that the entire chelate is being absorbed or that the plant is taking up degradation products of the ligand. More recent work by Weinstein[9] and de Kock[2] using split root techniques suggest that the entire chelate is absorbed.

Comparisons were made of the date of uptake and the distribution of iron in plants when supplied as $FeSO_4$ and as FeEDTA. When these two forms of iron were tagged with radio iron and supplied to the roots of orange trees, autographs made three weeks after treatment disclosed a distinct difference in the distribution of iron in the leaves (Fig. 2). At the beginning of the treatment all plants were chlorotic and at the end of 11 days those that had received FeEDTA were green. Counts were made on different parts of the plants one, two, and eleven days following treatment.

There was much higher uptake of iron from the FeEDTA than from the $FeSO_4$. This is shown both by the autograph and by counts made on the plant ash (Table III). The highest amount of iron-55 and -59 was found in the new leaves, and the lowest amounts in the xylem and phloem. The isotope supplied as the chelate continued to increase in plants during the 11 days in which the studies were continued. On the other hand, the iron-55 and -59 concentration was not much greater in the plants 11 days following treatment with iron sulfate than in the samples taken 24 hours after the isotope was applied.

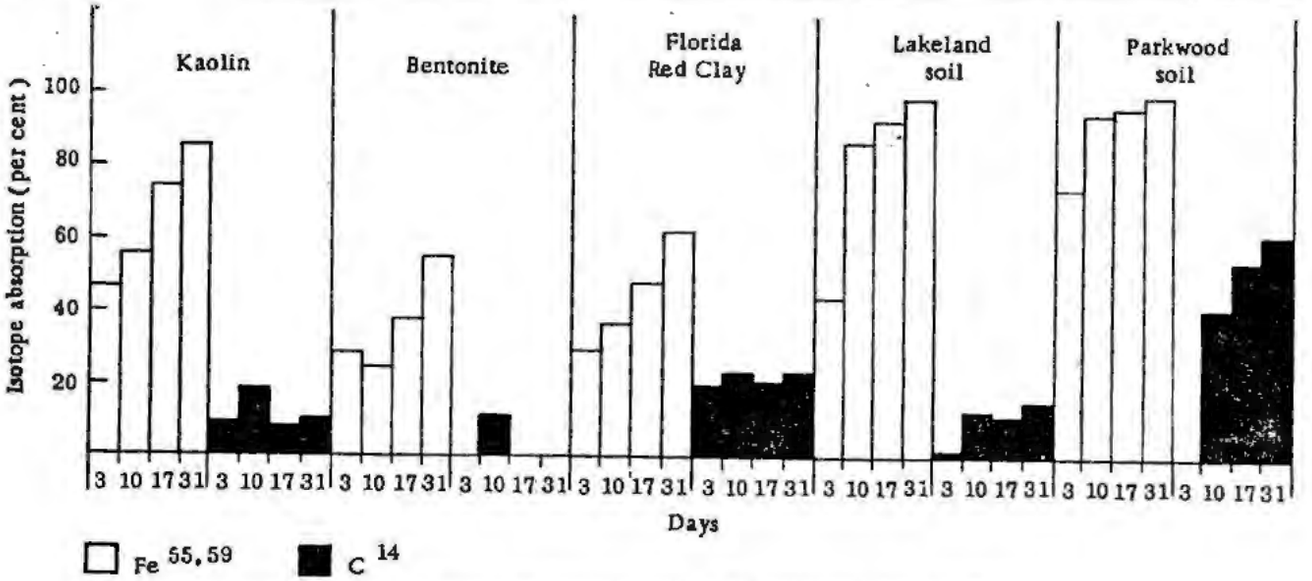

Figure 1. Isotope adsorption by clays and soils from $Fe^{55,59}$EDTA and from C^{14}FeEDTA

ABSORPTION OF ZINC-65 BY SEEDLINGS GROWING IN DIFFERENT SOILS

Zinc deficiency in citrus is common wherever this crop is grown. Soil applications of common zinc salts are usually not effective in alleviating the deficiency. Tests were made to determine whether chelated forms of zinc were more effective than inorganic forms. In a preliminary trial, ZnEDTA and $ZnSO_4$ tagged with zinc-65 were applied to citrus seedlings growing in four different types of soil. The soils used have a wide range of chemical and physical properties, but are typical of those found in the citrus growing areas of Florida where zinc deficiency is common. The St. Lucie soil is a fine white sand with an exchange capacity of one me or less per 100 grams. The Parkwood is a heavy calcareous soil that usually has an exchange capacity of 20 me or above. The Lakeland and Blanton soils are intermediate in their properties. The Lakeland fine sand has an exchange capacity varying from two to three me and the Blanton from three to six me. For this experiment, pots containing 4.5 pounds of soil were used. Grapefruit seedlings were transplanted to the pots and the zinc treatments were applied only after the plants had become established and the roots were well distributed in the soil. Zinc sulfate and ZnEDTA treatments were labeled with 20 microcuries of zinc-65 and applied in approximately 300 ml of solution at the rate of 40 pounds per acre of zinc. Plants were harvested 11 days after the treatment and counts were made on the ashed tissue.

Much larger amounts of zinc-65 were taken up from the ZnEDTA than from the $ZnSO_4$ in all the soils except the Parkwood (Table IV). The greatest difference in uptake between the two sources of zinc was in the St. Lucie fine sand, and the least difference

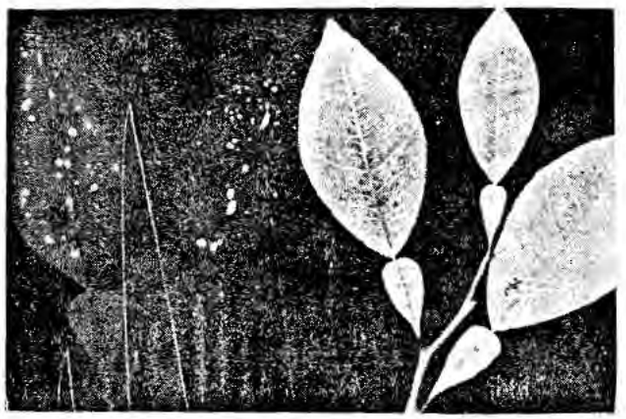

Figure 2. Radioautograph of seedling orange leaves made three weeks after treatment with radioactive iron. The leaves on the left were from a plant that received iron sulfate and the ones on the right were from a plant that received iron EDTA. Note the low amount and uneven distribution of iron in the plant that received iron sulfate

Table I. Adsorption of Chelates on Exchange Resin Columns

Chelate	*IR-120, %*	*IRA-400, %*
$Fe^{55,59}$EDTA	0	100
C^{14}FeEDTA	0	100
$Fe^{55,59}$EDTA-OH	76	100
Zn^{65}EDTA	85	100
C^{14}ZnEDTA	39	—

Table II. Iron Adsorbed from Added FeEDTA by Clays and Soils as Determined by Chemical Methods*

Adsorbing material	*Iron adsorbed, %*
Kaolin	46
Bentonite	44
Florida red clay	50
Lakeland soil	73
Parkwood soil	69

* The determination were made 17 days following the addition of the chelates to the clays and soils.

Table III. Uptake of Iron-55 and -59 by Orange Seedlings from $FeSO_4$ and FeEDTA

	24 hr cpm/gm		48 hr cpm/gm		11 days cpm/gm	
	$FeSO_4$	FeEDTA	$FeSO_4$	FeEDTA	$FeSO_4$	FeEDTA
New leaves	24	70	4	148	22	370
Intermediate leaves	14	54	18	64	16	264
Old leaves	12	44	10	76	10	328
New leaf wings	22	46	14	98	22	240
Intermediate leaf wings	6	42	12	48	12	158
Old leaf wings	6	22	14	50	10	182
New phloem	8	24	12	58	22	112
Intermediate phloem	8	32	14	48	12	140
Old phloem	6	14	2	80	18	120
New xylem	10	48	6	62	10	170
Intermediate xylem	—	36	2	50	2	104
Old xylem	6	30	4	48	6	98

Table IV. Uptake of Zinc-65 from $ZnSO_4$ and from ZnEDTA by Grapefruit Seedlings Growing in Four Different Soil Types

Soil	ZnEDTA cpm/gm	$ZnSO_4$ cpm/gm
St. Lucie	784	17
Lakeland	113	8
Blanton	77	11
Parkwood	36	28

was in the Parkwood soil. The uptake of zinc from ZnEDTA in these soils was inversely related to their exchange capacities.

MOVEMENT OF ZINC FROM ZnEDTA AND $ZnSO_4$ IN THE SOIL

Radioautographs made of plants treated with ZnEDTA and $ZnSO_4$ indicated that one of the explanations for a difference in uptake of zinc from these two sources was that $ZnSO_4$ failed to become well distributed in the soil (Fig. 3). Experiments were carried on with grapefruit seedlings growing in pots containing 22 pounds of soil. The two sources of zinc labeled with zinc-65 were applied to the soil in dilute solution and during the next six months the soil was leached several times with water. The distribution of the zinc in the soil was determined by taking three cores of soil from each pot. Counts were made on the soil samples with an end window tube that was enclosed in a small lead tube with a hole three millimeters in diameter in front of the window. The soil sampling tube contained a slit in the side. Counts showed that most of the zinc-65 applied as $ZnSO_4$ remained in the top one or two inches of soil. However, that applied as ZnEDTA was evenly distributed throughout the soil and considerable amounts leached through the pots. Obviously, one of the reasons for the greater availability of zinc in ZnEDTA is that it moves down in the soil to the root zone.

ABSORPTION OF ZINC BY PLANTS FROM SOIL AT DIFFERENT pH LEVELS

For the past three years, field applications have been made with ZnEDTA to zinc deficient citrus trees. This form of zinc has been much more effective in some places than in others even when the soil types were similar. One of the variables encountered in citrus groves is a difference in soil pH. Studies were made to determine the difference in uptake of zinc-65 by grapefruit seedlings growing in soil and adjusted to different pH levels and to which tagged $ZnSO_4$ and ZnEDTA had been applied. Twenty-two pounds of virgin Lakeland soil was put into pots and the pH's adjusted to 4, 5, 6, and 7. Grapefruit seedlings were transplanted to the pots and allowed to become well established before the treatments

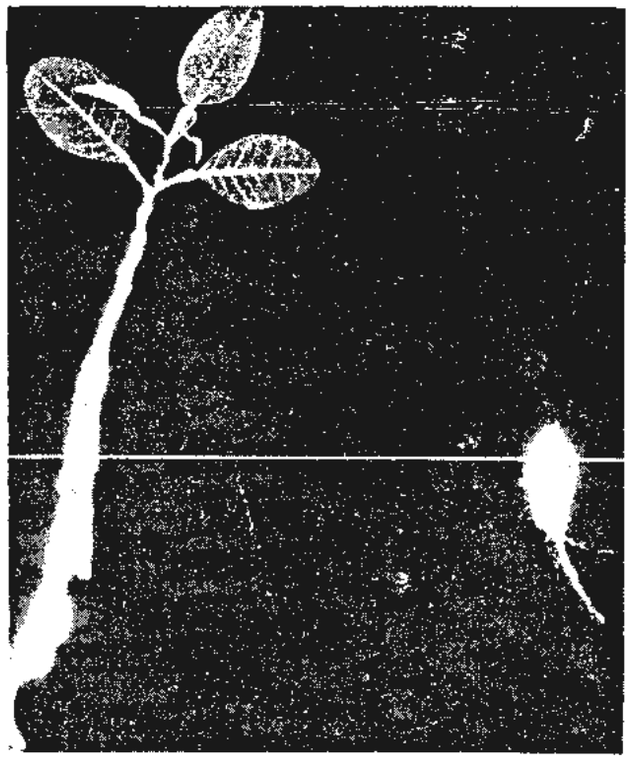

Figure 3. Radioautograph of grapefruit seedlings showing movement of zinc-65 after soil treatment with ZnEDTA (left) and $ZnSO_4$ (right). The horizontal line represents the approximate soil surface. There was uniform distribution of radioactive zinc supplied as ZnEDTA while that from $ZnSO_4$ was fixed near the soil surface. Note the absence of zinc-65 in the lower part of the root systems as well as in the top of the plant on the right

were applied. Zinc treatments labeled with approximately 50 microcuries of zinc-65 were applied to the soil in each pot at the rate of 20 pounds of zinc per acre. Each treatment was applied to 600 ml of water. Plants were harvested at intervals following the treatment and counts were made on the ash.

Three months after the treatments there was a much higher uptake of zinc-65 at all soil pH levels from ZnEDTA than from the $ZnSO_4$ (Table V). There was a tendency toward increased uptake of zinc-65 from $ZnSO_4$ with increasing acidity of the soil. This is in agreement with many observations made in the past; namely, that plants generally take up more zinc from the more acid soils than from those that have been limed. However, these studies indicated that when ZnEDTA was applied to the soil more of the zinc was taken up by plants from limed soil than from that which was highly acid.

RATE OF EXCHANGE OF ZINC IN ZnEDTA FOR IRON IN THE SOIL

The stability of the zinc chelate is much less than that of the iron chelate. Martell and Calvin[5] have reported that the stability constant for FeEDTA has a log value of 25 while that for ZnEDTA is 16. This means that in a mixture of iron and zinc, EDTA will chelate much more iron than zinc. It would be expected that when ZnEDTA is applied to the soil, exchange will take place between the iron present in the soil and the zinc in the chelate. The rate at which this exchange takes place determines the length of time that the zinc chelate is available to plants. One of the soil factors that is believed to affect the rate of exchange is the pH. Zinc is more effectively chelated by EDTA with increasing pH. Conversely FeEDTA tends to hydrolyze with increasing pH. Studies were made to determine the rate of exchange of zinc in ZnEDTA for iron in the soil. These studies were performed in the laboratory and a technique was used similar to that described for the adsorption work with iron chelates. Two hundred milliliters of ZnEDTA containing 20 ppm zinc labeled with zinc-65 was put into flasks containing 100 grams of Lakeland fine sand. The flasks were shaken for one-half hour. An aliquot was filtered for iron and zinc analysis and then the flask was shaken for an additional two hours, when more aliquots were taken. Additional iron and zinc determinations were made at intervals of two, five and thirteen days. The rate of exchange was determined by the rate at which iron came into solution and the zinc became fixed.

Table V. Uptake of Zinc-65 from ZnEDTA and $ZnSO_4$ by Citrus Seedlings Growing in Lakeland Soil Adjusted to Four pH Levels

Soil pH	*ZnEDTA cpm/gm*	*$ZnSO_4$ cpm/gm*
4	167	30
5	125	12
6	257	15
7	248	7

The initial rate of exchange was very fast. However, after two days the rate tended to be much slower (Fig. 4). The soil was approximately pH 5.3, which would be favorable for a rapid exchange, and, in addition, the presence of excess water also helped to speed up the exchange as compared with soil under field conditions. If complete exchange had taken place, there should have been approximately 2000 micrograms of iron in solution, whereas at the end of 13 days there was about 1000 micrograms.

ABSORPTION OF ZINC-65 BY PLANTS FROM VARIOUS SOURCES OF ZINC

Studies were made comparing various sources of zinc for citrus seedlings. Fourteen combinations of zinc, consisting of inorganic zinc, chelated zinc, wetting agents, and polymers were applied to the soil. Greenhouse studies were made using techniques similar to those reported previously for the zinc pH studies. Zinc sulfate was the only inorganic form of zinc used. The chelating agents were EDTA, EDTA-OH, N N^1-dihydroxyethylethylenediaminediacetic acid (DEDD) diethylenetriaminepentaacetic acid (DTP), *N*-hydroxypropyliminodiacetic acid (PDG) and acetylacetonate. The wetting agents were PR-51 (Atlantic Refining Co.), sodium alkylnaphthalene sulfonate, and lignosulfonate. Two polymers were tried: polyacrylic acid and polymeric acid anhydride. In preparing the zinc compounds, zinc-65 was first mixed with the carrier $ZnSO_4$ and an equivalent amount of chelating agent in the sodium salt was added to form a 1:1 chelate. Arbitrary amounts of the wetting agents and polymers were used: PR-51 0.1 gm, sodium alkylnaphthalene sul-

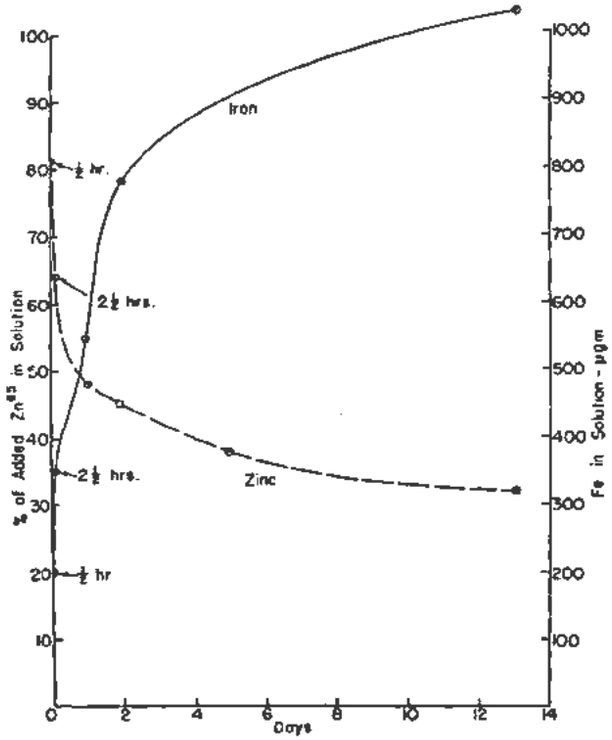

Figure 4. Rate of exchange of zinc-65 in ZnEDTA for iron in the soil

fonate 1 gm, lignosulfonate 1.4 gm, polymeric acid anhydride 0.5 gm and polyacrylic acid 2 grams in each pot.

Zinc EDTA was the most effective source of zinc tried (Table VI). However, all the chelated forms used with the exception of acetylacetonate were much more efficient than $ZnSO_4$. The wetting agents and polymers were not effective in greatly increasing the uptake of $ZnSO_4$ by the seedlings. PR-51 did not increase the availability of ZnEDTA. The soils used in these studies were highly acid, having a pH of approximately 5.3. This low soil pH is believed to have greatly increased the rate of exchange of the zinc in the chelate for iron in the soil. Some of the zinc chelates used would probably be even better sources of zinc in less acid soils than indicated.

Table VI. Uptake of Zinc-65 from Various Sources of Zinc by Citrus Seedlings

Source of zinc	*cpm/gm*
Zinc ethylenediaminetetraacetate (ZnEDTA)	125
Zinc N N^1-dihydroxyethylethylenediaminediacetate (ZnDEDD)	103
ZnEDTA + PR-51	87
Zinc isopropylenediaminetetraacetate (ZnIPDT)	70
Zinc *N*-hydroxyethylethylenediaminetriacetate (ZnEDTA-OH)	57
Zinc *N*-hydroxypropyliminodiacetate (ZnPDG)	47
Zinc diethylenetriaminepentaacetate (ZnDTP)	43
Zinc acetylacetonate	19
$ZnSO_4$ + polyacrilic acid	19
$ZnSO_4$ + polymeric acid anhydride	16
Zinc lignosulfonate	14
$ZnSO_4$	12
$ZnSO_4$ + PR-51 (wetting agent)	9
$ZnSO_4$ + sodium alkylnaphtalene sulfonate	6

SUMMARY

Studies have been made in the greenhouse, in the field and in the laboratory to determine some of the factors that affect the absorption of chelated iron and zinc by plants. These studies were made with chelates separately tagged $Fe^{55,59}$, Zn^{65}, C^{14}.

Solutions of FeEDTA, FeEDTA-OH, and ZnEDTA were passed through columns of cation and anion resins. All the chelates were adsorbed on the anion resin. FeEDTA showed no adsorption on the cation resin, but FeEDTA-OH and ZnEDTA were partially adsorbed.

Adsorption studies made with FeEDTA in clays and soils indicated that this chelate reacts very readily with these materials. When solutions of the chelate were in contact with the soils, exchange took place between iron in the chelate and iron in the soil. There was also exchange of other metals present in the soil for iron in the chelate. Also, either the entire complex or the chelating agent was adsorbed.

Absorption of iron-55 and -59 from $FeSO_4$ and from FeEDTA by plants indicated that the metal in the chelate is much more available than that in the inorganic form. Radioautographs of the leaves of citrus seedlings disclosed that iron supplied as FeEDTA was distributed uniformly throughout the leaf area, whereas that supplied as $FeSO_4$ was concentrated in small irregularly distributed spots.

Studies of the absorption of zinc-65 from ZnEDTA and $ZnSO_4$ by plants growing in four different soil types disclosed that the chelate was a much better source of this nutrient than $ZnSO_4$. There was considerable variation in uptake of zinc from the chelate by plants growing on the various soils and absorption was related inversely to the exchange capacities of the soils.

One of the reasons for the difference in availability of $ZnSO_4$ and ZnEDTA is believed to be the differences in penetration of these two compounds in the soil following their application. Most of the zinc sulfate was fixed in the top two inches of soil whereas ZnEDTA became well distributed.

Plants grown in soil at different pH levels took up more zinc from $ZnSO_4$ under very acid conditions than when the soil was limed to pH 7.0. However, when zinc was supplied as ZnEDTA much more of this nutrient was taken up from the soils limed to pH 6.0 or 7.0 than at pH 4.0 or 5.0.

The availability of chelated zinc to plants growing in soil is believed to be largely determined by the rate of exchange of the zinc in the chelate for iron in the soil. In the laboratory, the exchange rate was found to be very high during the first two days, but after 13 days under what was believed to be ideal conditions, complete exchange had not taken place.

Various zinc compounds and mixtures were compared as sources of zinc for plants growing in soil. Included were zinc sulfate, zinc chelates, wetting agents and polymers. With the exception of zinc acetylacetonate much more zinc was taken up by plants from chelates than from other sources tried.

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Analysis of the Movement of Substances in Plants by Means of Radioactive Isotopes

By A. L. Kursanov, USSR

The translocation of organic substances in plants is a very ancient problem of plant physiology, which preoccupied scientists as far back as the end of the eighteenth and the beginning of the nineteenth centuries. However, because of the intricacy of the problem and principally owing to the absence of any direct methods of studying the translocation phenomenon itself, this subject did not make much progress, so that by the forties of the twentieth century many problems related to it were in a very confused state.

And yet the great advances made in the study of other aspects of physiological activity of plants impel with ever increasing urgency a closer study of the nature and the regularities underlying the translocation of organic substances. It is, indeed, this process that ensures a correlation and unification of the activity of all plant organs. Hence, there is no doubt that the proper solution of the problem will at once unite as yet isolated chapters of plant physiology, and enable it to control with greater efficiency the development and nutrition of plants.

The concept of free diffusion of organic substances in the solution filling the sieve tubes has long since been abandoned because of the complete discrepancy between the rate of the diffusion process and the actual translocation rate of plastic substances in the plant. The situation has been changed but little by the assumption that diffusion is accelerated by streaming of the protoplasm filling the sieve tubes.

E. Münch's theory,[1] according to which the solution of organic substances flows through the sieve tubes under the influence of the difference in turgor pressure at the end-points of its path has likewise proved inconsistent and has now been given up. Moreover, the actual idea that in the conducting tissues of the phloem there occurs a flow of solutions of organic substances can hardly be regarded valid al all.

The extensive studies carried out hy T. Mason *et al.*[2] as well as those of H. Dixon,[3] W. Schumacher,[4] O. Curtis,[5] E. Raushal[6] and others have provided ample experimental evidence; but they have not solved the prohlem either of the mechanism of translocation or of the form of the substances involved. The problem as a whole remained vague arousing general scepticism.

The fundamental difficulty in the way of these investigations consisted in the fact that it was not possible to distinguish by means of the common chemical or cytological methods the translocated components from the remaining substances in the bast tissues. Another complication was due to the fact that in most cases the total amount of soluble substances (e.g., sugars) in the conducting paths of plants is maintained at a rather constant level, even in case of active translocation as judged indirectly by the disappearance of reserve substances in one part of the plant and their accumulation in another part.

All this precluded the possibility of ascertaining either the velocity of translocation or the concentration of the substances, or the composition of the mobile components. On such a scheme, the sieve cells seemed to take no part in translocation and it is only physiological experiments with ringing the bark that gave conclusive though indirect evidence of the important role of bast tissues in the translocation of organic substances in the plant. Within recent years this problem, owing to the use of labelled atoms, has entered a new stage of development.

It should be noted that hardly any other problem of plant physiology has overcome at once its inherent difficulties and received such an impetus for further advance as did the problem of translocation. Naturally enough, too little time has passed as yet to expect great achievements in this intricate problem. If, however, I take the liberty of discussing this "neglected" chapter of plant physiology, it is only for the sake of demonstrating how radioactive atoms, by removing the difficulties encountered by scientists of past days enable us now to follow up directly the "translocation of juices" in the body of the plant. These new opportunities are now being utilized in the Soviet Union, and the results so far obtained may contribute to some more general conclusions on this important aspect of physiological activity of the plants.

A leaf not severed from the plant and placed into a chamber containing carbon dioxide tagged with carbon-14, will form sugars and other photosynthetic organic compounds, which carry a radioactive label. By combining this method with partition paper chromatography we have found that sucrose is the first free sugar formed in the leaves of sugar beet

Original language: Russian.

and many other plants[7] as a result of photosynthesis.

Similar conclusions have been reached before by M. Calvin and A. Benson[8] in a study of the first products of photosynthesis in the unicellular alga, *Scenedesmus*.

Moreover, according to A. Nichiporovich and his associates,[9] who likewise employed isotopes, not only carbohydrates but also organic acids can be direct products of photosynthesis in the leaf, as well as amino acids which by themselves and as a result of secondary transformation yield the same mixture of diverse products of photosynthesis that serve for nutrition of the whole plant.

We removed fibro-vascular bundles from leaf petioles of sugar beet, cotton, *Plantago* and other plants. A test of their radioactivity (first as homogenates of plant material brought to constant weight, and afterwards as individual products isolated on paper by partition chromatography), showed that photosynthetic products penetrate very rapidly from mesophyll into the conducting system where they are conveyed to one or another part of the plant at a rate of 70 cm or more per hour[10,11,12] (Table I).

Some of these results are represented in Figs. 1 and 2. They demonstrate the beginning of the movement of assimilates from pumpkin leaves, and, as seen from the autoradiographs, the main stream of the assimilates is directed downwards from the leaves along the plant even in those cases when the leaves are situated near the apex or the zone of development of the fruit.

With a more prolonged exposure we are able to observe the radioactive assimilates reaching the roots. There they undergo various transformations owing to the absorbing function of the root system and its specific synthetic and biochemical activity, and then run upwards to the growing sprouts and fruits as a mixture of new products.[13] Thus, by means of radioactive carbon we are able to study visually the important function of the roots through which a considerable part of the photosynthetic products is passing and undergoing there the necessary transformations.

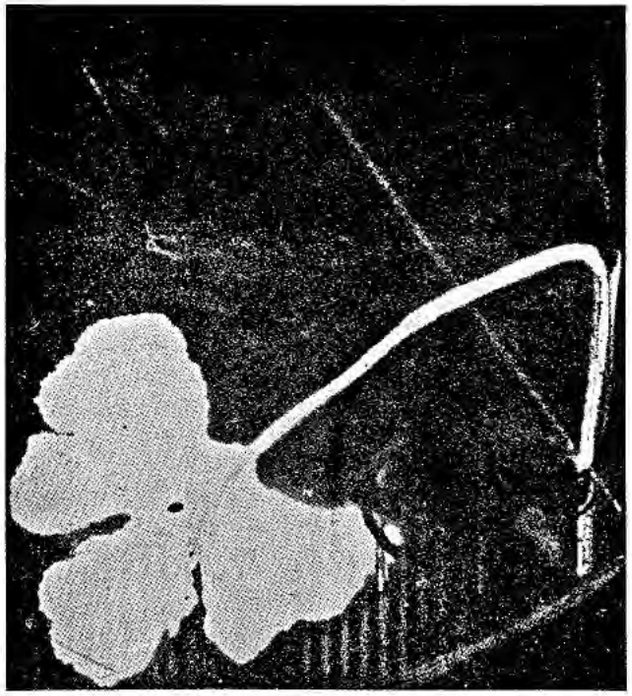

Figure 1. Translocation of assimilates in *Cucurbita pepo* from a leaf located close to the base of the plant. This autoradiograph shows the distribution of radioactive assimilates 40 minutes after treatment with $C^{14}O_2$

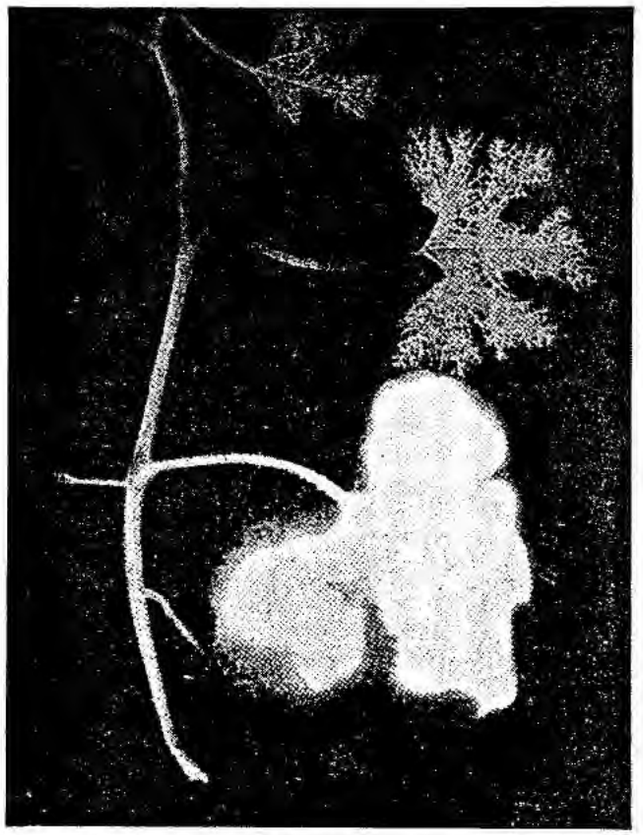

Figure 2. Translocation of assimilates in *Cucurbita pepo* from a leaf located nearer the top of the plant. This autoradiograph shows the distribution of radioactive assimilates 40 minutes after treatment with $C^{14}O_2$

It is apparent from same autoradiographs that some of the assimilates migrate directly into the growing sprouts and young fruits, without entering the roots. Of importance too, is the fact that part of the photosynthetic products passes from the assimilating leaf into other leaves of the plant, as shown by the above autoradiographs. Such an exchange of substances between the leaves of the plant, should undoubtedly contribute to the co-ordination of activity. The opportunity is thus

Table I. Translocation Rate of Assimilates along the Vascular Tissues of Some Plants

Name of plant	*Rate of migration, cm/hr*
Sugar beet	70–100
Haricot	60-80
Pumpkin	30–70
Cotton	35–40

provided of observing this phenomenon by means of radioactive isotopes for studying the correlations within the plant organism.

By using C^{14} we can readily follow up the changes in the direction and rate of translocation of assimilates in the plant in connection with the general course of its development. Such observations made by H. Pristupa in this laboratory on pumpkins showed that the translocation rate of the assimilates is greatest in young plants, aged 30–40 days (up to 72 cm/hr). In older plants it decreases to some 50 cm/hr towards the roots and not more than about 30 cm/hr from the leaves upwards to the growing points. Easily traced also are the changes in the stream of plastic substances associated with fertilization and the beginning of development of the fruit.

In the first few days the translocation rate of assimilates to the fertilized ovary of the pumpkin is but 12 cm/hr; by the tenth day of development it has already risen to 36 cm/hr and subsequently increases still more. Initially only the nearest leaf, whose axil contains the fertilized ovary, is the nutrient organ (Fig. 3); later on, with the increased requirement in nutritive substances, the sphere of influence of the growing fruit involves ever more distant leaves, whose assimilates are conveyed to the fruit either directly or via the root.

Interesting results have now been obtained with cotton,[11] in which the development of fibres occurs at the expense not only of the assimilates derived from the leaves, but also of the photosynthetic products formed in the green wall of the fruit. It has been found that the share of the walls in the formation of the fibres amounts to 15–18%. Such observations which became possible owing to radioactive isotopes may undoubtedly also contribute to a scientific solution of practical problems related, for example, to morphogenesis of grapes, pruning of fruit trees, abscission of tomatoes, etc., problems which hitherto have been taken up in a merely empirical way.

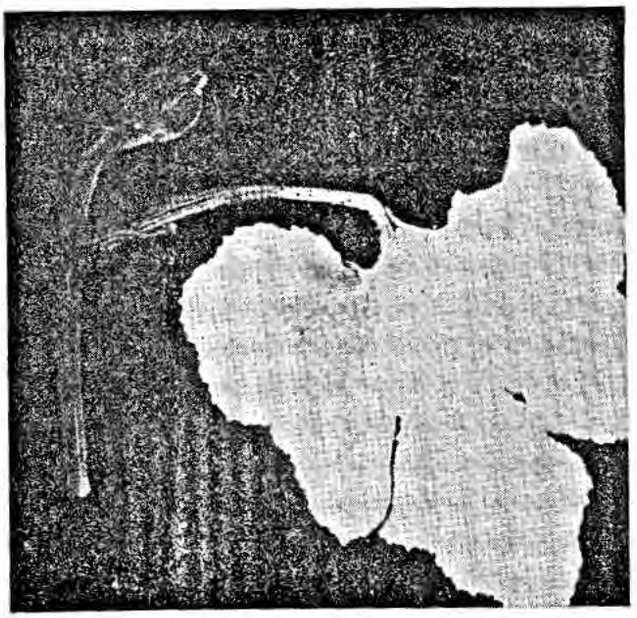

Figure 3. Autoradiograph showing inflow of radioactive assimilates into a 4-day old fruit of *Cucurbita pepo*

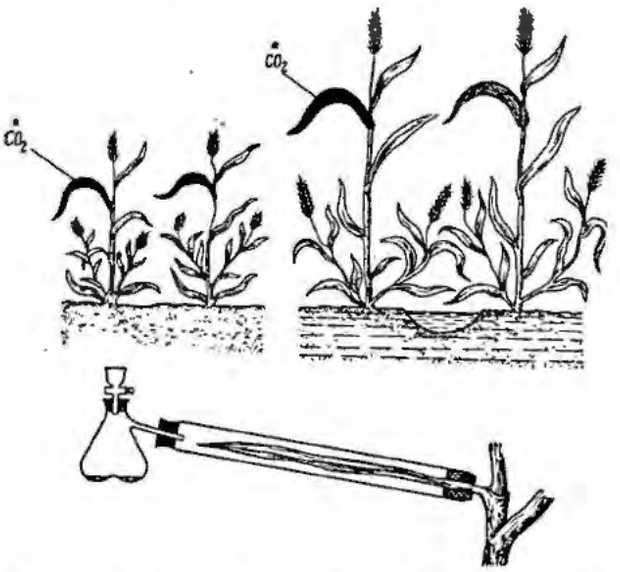

Figure 4. Scheme of translocation of assimilates in wheat from the leaves to the seeds as a function of irrigation. Below, apparatus for treating the leaf with $C^{14}O_2$ under field conditions

Simple methods, adapted for field tests, are helpful in following up, by means of radioactive carbon, the translocation of plastic substances under the actual conditions of the plant habitant. V. Zholkevich[14] has demonstrated in particular that in irrigated lands of Trans-Volga the assimilates formed in wheat leaves from labeled carbon dioxide are conveyed to the seeds of plants on irrigated areas much faster and more completely than in water-deficient plants (Fig. 4).

It has also been found by means of radioactive carbon that in potatoes of the far north the assimilation products of CO_2 are conveyed from the leaves chiefly to the stems and growing points which calls forth profuse growth of the leaves and a reduction in size and starch content of the tubers (Z. Zhurhitsky). At the present time agriculturists in the north are searching for ways and means of eliminating this undesirable phenomenon, using the same tracer technique to check the effectiveness of the measures applied.

This, however, is far from exhausting the scope of application of the tracer method to the study of the laws underlying the translocation of substances in plants. By combining radioactive elements with partition paper chromatography, we are well able to make out the composition of the translocated products as well, which is of paramount importance in many cases. Thus, at present we know that a mixture of sugars, organic acids and phosphoric esters among which sucrose and hexose-phosphates greatly predominate is translocated from the leaves of cotton to the developing fibres.[11] A similar mixture is also conveyed to the fibres from the walls of the fruit.

Sucrose greatly prevails also in the conducting system of sugar beet, *Plantago* and many other plants.[15]

In addition to non-nitrogen substances, amino acids are also translocated within the plants, the ratio between both kinds of substances varying in different plants, as well as with the direction of the stream. Thus, according to N. Pristupa, a mixture of sugar and amino acids (the latter making up as much as 30–40%) moves upwards and down along the stem of a pumpkin leaf treated with labelled carbon dioxide.

At the same time the composition of assimilates conveyed from the leaves to the fruits varies according to their age. Thus, during the first 4–5 days after fertilization in the pumpkin the ovary receives amino acids while on the 10th day of development the sugars become the chief source of nutrition (Table II).

Table II. Relative Composition of Organic Substances Conveyed to the Pumpkin Fruits at Various Periods of Development (Percentage of Total Radioactivity of the Fibro-vascular Bundles)

4 days		*10 days*	
Sugars	*Amino acids*	*Sugars*	*Amino acids*
45	55	85	15

It is a well known fact at the present that by changing the conditions of root nutrition illumination temperature, and water supply, it is possible not only to accelerate or retard the translocation of plastic substances, but also to change the very direction of the stream and, in addition, to influence the composition of the nutrient mixture conveyed from the leaves to one organ or another. It is apparent that the method of labelled atoms provides an approach to the problem of controlling the direction and composition of the flow of plastic substances in plants which is likely to open in the near future great hitherto unexplored possibilities for agriculture.

Finally, radioactive isotopes afford a more profound insight into the mechanism of transfer of organic substances in plants. According to the data available, this is by no means a passive flow of solutions but an active transfer of the molecules themselves due to a peculiar biochemical activity of the conducting cells. We are still far from understanding those complicated phenomena resulting in the transformation of energy of biochemical reactions into a form of energy which drives diverse organic substances rapidly in one direction or another. However, aided by labelled atoms we have secured now a starting point for investigations in this field as well.

As mentioned above, the translocation of substances, particularly of sugars in the fibro-vascular bundles, however intense, is as a rule accompanied by an almost complete invariability of the total sugar content in the conducting paths, and can therefore be detected only by means of labelled atoms[10] (Fig. 5).

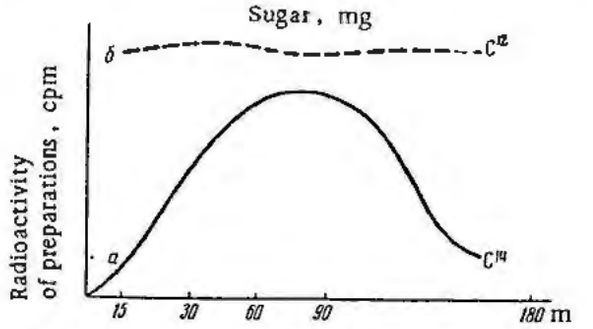

Figure 5. Translocation of labelled sugar from the leaf to the root along the fibro-vascular bundles in sugar beet. Curve *a*, sugars tagged with C^{14}; curve *b*, total sugar content

Hence, the sieve cells do not posses any reserve capacity to let through a wave of moving substances, and the act of translocation is based rather on the principle of push whereby the substance starts moving simultaneously throughout the length of the conducting path, without disturbing the continuity of the gradient, which is of considerable importance for the process at issue.

It is an established fact that the conducting cells of the phloem are distinguished by an extremely high rate of respiration—up to 5,000 μl O_2/hr/gm of active phloem. In such cells the act of translocation of sucrose is accompanied by a still more increased respiration. This leads to the assumption that a great amount of energy is expended for the transfer of molecules in the conducting cells[16] (Fig. 6).

In contradistinction to other plant tissues, the cytochromoxidase of the fibro-vascular bundles possesses a very high activity, which is still more enhanced upon passage of sucrose.[17] On the other hand, poisoning of cytochromoxidase of the leaf petiole (by enclosing part of it in a CO atmosphere) at once impedes the passage of the assimilates. (I. Dubinina). A close connection is thus suggested between the act of translocation of substances and the functioning of the cytochrome system (Fig. 7).

It has also been demonstrated by means of sugars tagged with radioactive carbon that translocation of substances in conducting cells is accompanied by their transformation. For example, according to O. Pavlinova of this laboratory, if the leaves of sugar beet are placed with the cut ends of their petioles into a fructose solution tagged with C^{14}, this sugar

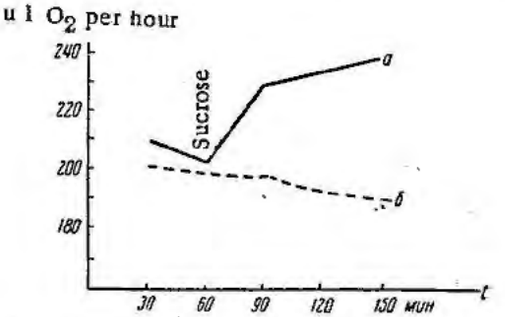

Figure 6. Respiration of fibro-vascular bundles (μl O_2/gm, fresh weight). Curve *a*, activity of sucrose; *b*, control

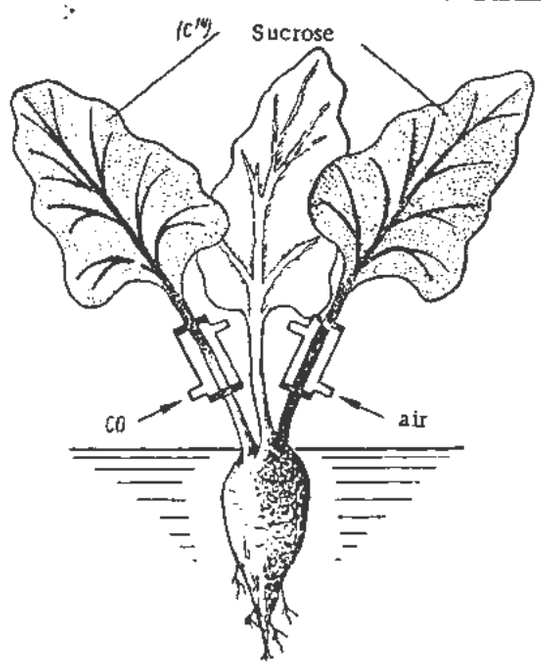

Figure 7. Diagram illustrating the influence of CO on translocation of sugars in sugar beet

is actively transformed in the fibro-vascular bundles into sucrose in which the major part of radioactivity is detected (Table III).

Table III. Formation of Sucrose from C^{14}-labelled Fructose in the Vascular Tissue of a Sugar Beet Leaf Petiole

No. of experiment	*Absorbed solution*	*Radioactivity of sugars in the conducting paths, cpm/gm of fresh tissue*		
		Glucose	*Fructose*	*Sucrose*
1	Fructose solution (2000 cpm/ml)	0	119	596 (glucose 212, fructose 333)
2	Same	0	68	458 (glucose 250, fructose 203)

It is also worth noting that sucrose thus formed, subjected to hydrolysis and separated on paper, appears to consist of glucose and fructose of approximately equal radioactivity. This finding suggests that, while passing along the fibro-vascular bundles, fructose undergoes an intense phosphorylation and isomerization. The glucose phosphate ester thus formed reacts with the phosphorylated fructose so completely that no radioactive glucose is freed but all of it is incorporated into sucrose.

More detailed investigations of the enzymes of fibro-vascular bundles of sugar beet and plantago, carried out in this laboratory by M. Turkina,[18] have shown that the conducting tissues of plants are capable not only of active oxidation-reduction reactions, but of many reactions of carbohydrate-phosphorus metabolism as well. It is likely that this ensures the mobilization of energy required for the transfer of molecules while the inert particles of sugar are converted into mobile ions either by coupling with phosphoric[19] or boracic acid (H. Gauch and W. Duggar).[20]

The various aspects of translocation of substances discussed in this paper are as yet fragmentary. A further elaborate study will be required before knowledge of the translocation of substances in plants reaches the level occupied by other well explored physiological processes. Still, the difficulties that had previously arisen are now being satisfactorily resolved with the aid of radioactive elements, so that there are no grounds for considering this problem more resistant to direct attack than any other problem of plant physiology.

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Use of Radioisotopes in Tracing Fungicidal Action

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Plant diseases, mostly caused by fungi, result in annual crop losses in the United States alone of almost three billion (10^9) dollars. Control measures which keep such losses from being much higher are aimed almost exclusively at the prevention of the germination and subsequent growth of fungus spores. These spores are very small; from 100,000 to 200,000,000, depending upon the species, are required to weigh one milligram. Before the ready availability of radioisotopes quantitative studies on interactions between fungus spores and toxicants could be carried out only with great difficulty. As a result almost no information was at hand on how much toxicant fungus spores took up when exposed to solutions or suspensions of fungicidal agents or how much was required on a weight basis to prevent germination. Radioisotopes have also been useful in determining possible penetration of applied fungicides into host plants. Here, too, very small quantities are involved, often of the same elements already present in plants.

In the work reported in this paper the toxicants used included C^{14}-labeled 2-heptadecyl-2-imidazoline and 2,3-dichloro-1,4-naphthoquinone, S^{35}-labeled ferric dimethyldithiocarbamate, elemental S^{35} and ions of Ag^{110}, Hg^{203}, and Ce^{144}. Rates of uptake by suspensions of spores from dilute solutions (1–10 ppm) were determined for most of the toxicants and spores of a number of species of fungi as well as the quantities in micrograms per gram of spore weight required to prevent germination of 50 per cent of the spores (ED50 values). In general the spores took up the toxicants very rapidly in large amounts and ED50 values ranged from 85 to over 10,000 micrograms per gram of spore weight. Sulfur was not accumulated by the spores but was reduced to hydrogen sulfide which was then released.[1]

In order to obtain information on possible common receptor sites, spores were exposed to more than one toxicant both simultaneously and consecutively and uptake determined. Results previously published[2] have shown that the three toxicants, 2-heptadecyl-2-imidazoline, silver, and cerium do not seem to have any receptor sites in common. Saturation of spores with one or two of these toxicants does not lessen uptake of the others. Extension of these studies to include many other ions has shown some unexpected competition between ions not closely related chemically and also brought out some instances in which previous exposure to or the simultaneous presence of one ion accelerates the rate of uptake of a second one. Thus silver increases the rate of uptake of mercury but does not influence the total quantity taken up. Pretreatment with fairly large amounts of mercury reduces subsequent silver uptake. Experiments with spores labeled with radioactive phosphorus have shown that silver has a marked effect on membrane permeability as measured by the release of phosphorus compounds into the ambient solution. Studies have also been made on the ease with which some of the toxicants taken up will exchange with non-radioactive toxicants added to the spore suspension. The effect of foreign ions on the release of ions previously taken up was also studied.

MATERIALS AND METHODS

The isotopes and labeled compounds, mentioned in the introduction, were obtained either from the Oak Ridge National Laboratory, Oak Ridge, Tennessee, or from commercial sources. All the materials were available at sufficiently high specific activity in relation to the quantities needed to inactivate the spores so that radioactivity determinations of the treating solutions could be made directly without any correction for self absorption even with the C^{14}-labeled compounds. Samples of spores, because of their small size, could also be taken in small enough quantities for direct counting without loss from self absorption.

Conidia of the following species of fungi have been included in these studies: *Neurospora sitophila* (Mont.) Shear and Dodge, *Monilinia fructicola* (Wint.) Honey, *Alternaria oleracea* Milbraith, *Aspergillus niger* van Tiegh, *Rhizopus nigricans* Ehr., *Cephalosporium acremonium* Corda, *Glomerella cingulata* (St.) Sp. and von S., *Myrothecium verrucaria* (Alb. and Schw.) Ditm. ex Fr., *Stemphylium sarcinaeforme* (Cav.) Wilts., *Venturia inaequalis* (Cke.) Wint. and *V. pyrina* Aderh. Cells of *Saccharomyces cerevisiae* Hansen were also used in some of the tests. Methods employed for culturing the fungi, harvesting the spores, determining the spore weights, and the nutrients added for germination tests are given in previous papers.[1,3]

In experiments on the interaction between the spores and various toxicants known weights of spores were suspended in solutions of the toxicant or toxicants in 15-ml centrifuge tubes with conical bottoms. The toxicants were in solution either in water or in 2 per cent acetone with organic compounds not readily soluble in water. A series of 12 tubes could be

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run at the same time. At appropriate intervals the suspensions of spores were centrifuged and aliquots of the supernatant and of the supernatant plus spores, when required, were taken for determination of the radioactivity. When placed in the centrifuge, the spores descended to the small area at the bottom of the tubes in a few seconds. In time studies the spores were considered out of contact with the solutions as soon as they aggregated at the bottom of the tubes.

Whenever desired, the remaining supernatant was removed from the tube and the spores resuspended in water or various solutions for studies on exchange or release of absorbed toxicant by various treatments. In many experiments the effect of a series of ions on the uptake or release of a radioactive ion was determined. The interfering ions which were not labeled as a rule were either used in pretreatments followed by exposure to the radioactive ion, or the spores were subjected to the ions simultaneously, or the ions were added after uptake of the radioactive ion and the subsequent release determined of the toxicant previously taken up.

Samples to be counted were transferred to one-inch cupped planchets, of either nickel plated steel, stainless steel, or glass, and dried under a heat lamp. With Hg^{203} and 2,3-dichloro-1,4-naphthoquinone special precautions had to be taken to prevent loss by volatilization. To prevent loss of mercury 0.5 to 1.0 mg of Na_2S was added to each planchet before drying and loss of the naphthoquinone was prevented by adding similar quantities of KOH. Radioactivity was determined by the use of conventional scaling equipment and end window type Geiger-Müller tubes with thin windows.

To determine effects of the toxicants on the outward movement of cell constituents, spores of *Aspergillus niger* were grown on potato dextrose agar in 100-ml tubes containing 25 ml of media each plus about 9 microcuries of P^{32} as phosphate. After seven to ten days the spores were harvested as usual and centrifuged with several changes of water. Spores grown in this way usually gave about 10,000 counts per minute per milligram of spores and released only about 1.5 per cent of the count to the ambient solution. The effect of added toxicant on the rate of release of phosphorus compounds to the suspending media compared to distilled water could then be determined.

The presence of small quantities of C^{14} in green plants from absorbed labeled fungicides was determined by the use of the methods of Van Slyke *et al.*[4] for wet combustion to CO_2 and the $C^{14}O_2$ was determined by proportional counting in Bernstein-Ballentine tubes.[5]

RATE OF UPTAKE OF TOXICANTS

The rate of uptake of toxicants as determined by the method described above was found to be exceedingly rapid. For practically all toxicants studied, depending somewhat on the quantities supplied in relation to amount required to inhibit germination and to saturate the spores, over 50 per cent of the quantity available was taken up by the time the first samples were taken, in one-half to five minutes. Typical curves showing cumulative uptake with time for 2-heptadecyl-2-imidazoline, cerium, mercury, 2,3-dichloro-1,4-naphthoquinone, and silver are shown in Fig. 1. The quantities available at the start of the test in terms of micrograms of toxicant per gram of spores were selected on the basis of information concurrently obtained on the quantities required to inhibit germination. Spores usually will take up two to three times the amount required to reduce germination by 50 per cent. This will become evident by comparing the amounts used in the experiments shown in Fig. 1 with the corresponding ED50 values shown in Table I. Mercury was taken up somewhat more slowly than the other toxicants studied. This is illustrated in the curve for the uptake of mercury by spores of *Monilinia fructicola*. The uptake of cerium also continues over a longer period of time as shown in the uptake curve for spores of *Alternaria oleracea*. As can be seen in Table I cerium is not toxic to most of the species studied.

QUANTITIES REQUIRED TO INHIBIT GERMINATION

By determining the quantity taken up by the spores and the effect on germination at several dose levels, a series of values are obtained which can be plotted to give a straight line on logarithmic-probability paper. For comparative purposes doses giving a 50 per cent inhibition of germination are usually used. ED50 values in micrograms per gram of spore weight for 2-heptadecyl-2-imidazoline, cerium, mercury, 2,3-dichloro-1,4-naphthoquinone, and silver and spores of a number of species of fungi are shown in Table I.

It is seen that the ED50 values range from 85 to 9300 micrograms per gram of spores. Values for sulfur are not included in the table since sulfur is not accumulated by the spores but is given off as hydrogen sulfide. If the quantity of hydrogen sulfide produced is considered as the dose then the ED50 value for *Neurospora sitophila* is about 11,500 micrograms per gram.[1] *Cephalosporium acremonium* was

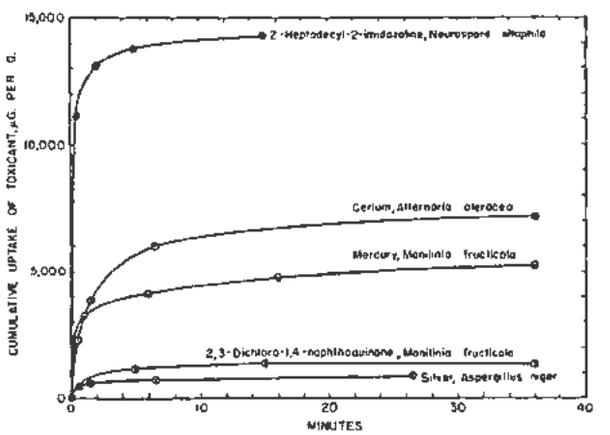

Figure 1. Typical curves showing rate of uptake of various toxicants from dilute solutions (2–10 ppm) by fungus spores

Table I. ED50 Values in Micrograms per Gram of Spore Weight for a Number of Toxicants far Spores of Representative Fungi

Toxicant	*Species*	*ED50 value*
2-Heptadecyl-2-imidazoline	*Neurospora sitophila*	5800
	Venturia pyrina	9300
Cerium	*Neurospora sitophila*	>970*
	Monilinia fructicola	4600
	Alternaria oleracea	>7100*
	Aspergillus niger	>8440*
Mercury	*Neurospora sitophila*	5030
	Monilinia fructicola	2830
2,3-Dichloro-1,4-naphtoquinone	*Neurospora sitophila*	560
	Monilinia fructicola	385
	Alternaria oleracea	400
	Rhizopus nigricans	680
	Myrothecium verrucaria	>1400*
Silver	*Neurospora sitophila*	165
	Monilinia fructicola	216
	Alternaria oleracea	360
	Aspergillus niger	540
	Venturia pyrina	85

* No effect on germination at these doses.

especially resistant to sulfur; 28 per cent of the spores still germinated after 53,600 micrograms of hydrogen sulfide had been given off per gram of spores.

Silver is the most toxic of the fungicides tested. 2,3-dichloro-1,4-naphthoquinone has an effect approaching that of silver and is the most toxic of the organic fungicides included in the studies. An examination of the table reveals some interesting differences between species. Although the naphthoquinone is quite toxic to spores of four species of fungi it has no effect on the germination of spores of *Myrothecium verrucaria* at the highest dose employed. Cerium is taken up in large amounts by spores of three species; it is toxic only to one of these three species. Although spores of *Neurospora sitophila* are active in taking up most of the toxicants studied, they took up comparatively small amounts of cerium. The ED50 values for 2-heptadecyl-2-imidazoline are the highest in the table at 5800 and 9300 for the two species listed. These values are reached rapidly, however, as shown in Fig. 1, from solutions containing only 2 ppm. This fungicide is used commercially for the control of apple scab (*Venturia inaequalis*) and pear scab (*V. pyrina*). The effectiveness of a given toxicant in preventing the germination of fungus spores depends upon the actual dose required to inactivate the spores and the ease with which this dose is obtained. It can thus follow that a toxicant inherently less effective on a weight basis may be more useful if it is taken up sufficiently more readily by the spores than a second innately more toxic.

The range of ED50 values of 85 to over 10,000 (if sulfur is included) shows that the innate toxicity of the fungicides studied is quite low. This is brought out clearly by examining the data given in Table II showing the LD50 values for a number of biocidal agents.

Table II. Approximate LD50 Values for a Number of Biocidal Agents

Toxicant	*Subject*	*Approx. LD50 μg/gm*
Atropine	Man	1.4
Botulinum toxin	Mouse	0.23×10^{-6}
O,O-diethyl *O*-*p*-nitrophenyl phosphorothionate	House fly	6.9
Diethyl *p*-nitrophenyl phosphate	House fly	0.5
2,4-Dichlorophenoxyacetic acid	Tomato	10
Penicillin	Staphylococci	2
Various fungicides	Spores	85–10,000

Some of the more effective toxicants listed, such as the phosphorus containing insecticides, 2,4-dichlorophenoxyacetic acid, and penicillin are of comparatively recent origin. There is no reason to expect that fungi are more difficult to inactivate than other organisms. The search for new fungicides would therefore be expected to disclose toxicants much more effective on a weight basis than those now known.

EXPOSURE OF SPORES TO MORE THAN ONE TOXICANT

When spores are exposed to various toxicants either singly, simultaneously, or successively the effects on uptake are determined by the nature of the toxicants and by the quantities used. If quantities insufficient to saturate receptor sites are presented, competition between toxicants for receptor sites may not be evident. In studies on the exchange of absorbed radioactive toxicant after the addition of more toxicant, the results may be confusing if much of the additional toxicant is taken up by the spores to saturate receptor sites and is therefore not available for exchange. Results previously published[2] have indicated that the toxicants 2-heptadecyl-2-imidazoline, silver, and cerium do not have receptor sites in common. When the experiments were extended to include mercury and silver, which would be expected to have some receptor sites in common, it was found, somewhat unexpectedly, that pretreatments or simultaneous treatment with silver increased the rate of uptake of mercury. Experiments with spores of *Neurospora sitophila* have shown an increase in the uptake of mercury as large as 75 per cent when silver was supplied simultaneously or previously.[6] The rate of uptake of silver was retarded when mercury was also present but after 20 minutes the amount of silver taken up was the same as if there had been no simultaneous exposure to mercury at the levels used in these tests.

These results have been verified in further experiments with spores of *Neurospora sitophila* and also other species. In Table III are shown results of pretreatments with silver or cerium on the subsequent uptake of mercury by spores of *Monilinia fructicola*. Spores treated with silver took up 105 micrograms per gram which reduced germination to 19 per cent.

Pretreatment with cerium did not reduce germination although 970 micrograms per gram of spores were taken up. On subsequent exposure to mercury the spores pretreated with silver took up about 70 per cent more mercury at the first sampling period at the highest dosage level than control spores or those pretreated with cerium. At the lowest dosage level any effect of silver was obscured by the fact that about 90 per cent of the available mercury was taken up by all three lots of spores at the time the first sample was taken.

Subsequent exposure to mercury following pretreatments with silver or cerium does not result in appreciable release of toxicant previously taken up.

In the experiment presented in Table III pretreatment with cerium had no effect on subsequent uptake of mercury. In other tests with *Neurospora sitophila*, spores which had taken up about 500 micrograms of cerium per gram took up as much mercury as the controls but spores which had received 980 micrograms per gram of cerium took up only about one-half as much. Further experiments have shown that there is competition for receptor sites between cerium and mercury.

The effect of silver in increasing the uptake of mercury is perhaps the result of an effect of some kind on cell permeability and does not necessarily indicate that there is no competition for the same receptor sites between these two ions. As a matter of fact in simultaneous treatments with silver and mercury in which the presence of the silver increases the rate of uptake of mercury, there is a lesser uptake of silver than when no mercury is present.

When copper and mercury are presented to spores at the same time there is some indication that copper, like silver, increases the uptake of mercury. This is more pronounced when spores have been pretreated with copper than when simultaneous treatments are used. If the amouuts of copper are large, uptake of mercury is reduced when copper is simultaneously present.

Mercury and cerium were found to show marked antagonism in uptake studies when fairly large quantities of interfering ion were used. This is true for spores of all species tested. Some common receptor sites for these two ions are apparently indicated.

EXCHANGE OF TOXICANT TAKEN UP

Studies on the rate and degree of exchange of fungicide previously taken up by the spores when an excess of non-radioactive toxicant is added to the ambient solution, have shown that of the materials under study silver is exchanged most rapidly and completely. The data indicate that the exchange is close to 100 per cent of the theoretical. Typical data for spores of *Neurospora sitophila* are shown in Table IV. Exchange was equally complete regardless of length of contact between silver and the spores. Some silver is also released when mercurous ion is added, no doubt because of replacement of the silver at some of the receptor sites.

Exchange with most of the other toxicants was usually less than 50 per cent. More radioactivity was released from spores previously treated with mercuric ion when an excess of mercurous rather than mercuric ions was added. This would indicate that the mercuric toxicant on contact with the spores has been reduced to the mercurous state.

Table IV. Effect of Length of Exposure of Spores of *Neurospora sitophila* to Silver on Subsequent Exchange with Non-radioactive Silver

Treatment with Ag^{110}		*Per cent exchanged on adding 1000 μg non-radioactive silver*	
Exposure time, hr	*Uptake, μg*	*in 5 min*	*in 65 min*
28.3	16.8	96	95
24.8	20.8	77	75
4.8	19.9	95	90
1.8	20.3	93	105
0.17	19.3	92	90
0.0014	6.0	90	90

2,3-dichloro-1,4-naphthoquinone was the only toxicant in which practically no exchange took place when non-radioactive toxicant was added subsequently. Some data are given in Table V. This indicates strongly that the toxicant has undergone chemical change and does not exist in the spores as the naphthoquinone. This is also shown by the fact that on the addition of spores to solutions of the naphtho-

Table III. Effect of Pretreatment with Silver or Cerium on Uptake of Mercury and Germination of Spores of *Monilinia fructicola*

Pretreatment			*Cumulative uptake of mercury after 10 and 85 minutes, μg/gm spore wt.*			*Germination after 10 and 85 minutes, %*	
	Uptake, μg/gm	*Germination, %*	*Quantity available*	*10 min*	*85 min*	*10 min*	*85 min*
Ag	105	19	10,000	7200	7375	3	3
			5000	4200	4400	9	3
			2500	2175	2340	11	5
Ce	970	100	10,000	4270	5730	29	7
			5000	3440	4185	41	49
			2500	2030	2345	92	92
None	—	100	10,000	4170	6030	47	9
			5000	3490	4305	65	46
			2500	2245	2410	74	72

Table V. Exchange on Addition of Non-radioactive 2,3-dichloro-1,4- naphthoquinone to Treated Spores

Species	Spore wt. mg	Treated spores	Radioactivity, cpm	
			Released on addn. of cold toxicant	Per cent released
Monilinia fructicola	10	1099	63	5.7
	30	1843	63	3.4
	90	2019	27	1.3
Myrothecium verrucaria	10	1080	36	3.3
	30	1720	36	2.1
	60	1940	54	2.8

quinone, uptake occurs for only a short time, usually not more than five minutes, after which the remaining toxicant will not even be taken up by a fresh lot of spores. If fresh toxicant is added uptake is resumed.

EFFECTS ON OUTWARD MOVEMENT OF CELL CONSTITUENTS

Effects of various toxicants on cell permeability were studied by using spores labeled with P^{32} and determining the degree of outward movement of phosphorus compounds subsequent to exposure. An experiment was carried out with silver, copper, mercury, zinc, cadmium and cobalt as indicated in Table VI. Spores of *Aspergillus niger* which gave 10,000 counts per minute per milligram were used. All the metal ions were tested at a dose of 1×10^{-5} equivalents per 9.8 mg of spores and in addition, silver, copper and mercury were also tested at lower doses. The particular dose chosen was based on the relative toxicity of the ions. Spores removed after 0.33 hour of exposure were unable to germinate at both doses of silver and at the highest mercury dose. The 1×10^{-6} equivalent dose of copper and zinc also seriously affected germination after 0.33 hour. Effects on release of phoshorus compounds from the cells were minor at this time but were noticeable with the spores treated with silver and copper.

Further samples were taken after 17.0, 66.5, and 170 hours. After 17 hours effects on germination were greater and the release of cell contents as a result of treatment with silver has become very pronounced. It is clear that copper, mercury, and zinc have brought about inability to germinate without any marked effect on release of cell contents. Examination at later time intervals up to 170 hours shows that a release of cell contents equivalent to 35 to 45 per cent of the phosphorus compounds was brought about by silver and the highest dose of copper. Killing the spores by heat released less than 30 per cent of the cell contents. Although after 170 hours the spores were unable to germinate in all the other lots except the control and that treated with cobalt, there was little outward movement of phosphorus compounds. Spores suspended in water lost only 2.2 per cent of their cell contents and those exposed to 1×10^{-5} equivalent of mercury, zinc, cadmium, and cobalt only up to 8.3 per cent.

The effect of silver in releasing cell contents and therefore altering permeability of the membrane is of interest in view of the finding that pretreatment with silver increases subsequent uptake of mercury. It would appear that the effect on permeability is not merely the result of killing of the spores since the spores were also killed by mercury, zinc, and cadmium without a comparable effect on cell permeability.

At both doses of silver used in the tests represented in Table VI germination was completely inhibited at the first sampling period. To study the effect of silver over a wider dose range, an experiment was set up in which the quantity of silver was varied from 200 to 25,600 micrograms per gram of spores (9×10^{-7} to 2.4×10^{-4} equivalents per gram) and the effect on the germination and release of cell contents studied over a 116-hour period. The results on release of phosphorus compounds up to 67 hours are plotted in Fig. 2. The numbers on the curves represent doses of silver expressed as micrograms per gram times 1×10^{-3}. At the highest doses of silver 3200 to 25,600 micrograms per gram the final effects on release of cell contents were the

Table VI. Effect of Various Metal Ions on the Per Cent Germination and the Outward Movement of Phosphorus Compounds from Spores of *Aspergillus niger*

Treatment		Germination and cumulative percentage of P content released by spores after various time intervals in hr						
		0.33		17.0		66.5	170	
Metal ion	Quantity in equivalents per 9.8 mg spores	Germination	P	Germination	P	P	Germination	P
Ag+	2×10^{-7}	0	1.5	0	26.9	39.3	0	35.3
	1×10^{-5}	0	1.7	0	34.3	40.1	0	36.1
Cu++	3×10^{-6}	100	1.7	15	1.3	5.6	0	9.0
	1×10^{-5}	6	2.2	0	2.8	14.6	0	45.3
Hg++	1×10^{-6}	3	0.8	0	1.0	1.8	0	1.7
	1×10^{-5}	0	0.4	0	1.9	4.5	0	8.3
Zn++	1×10^{-5}	6	0.3	0	1.5	3.4	0	2.3
Cd++	1×10^{-5}	100	0.9	12	1.7	5.6	0	4.9
Co++	1×10^{-5}	100	0.4	100	0.4	4.7	42	2.3
Distilled water		100	−0.2	100	0.2	1.3	39	1.2
Spores killed by heat		0	26.7	0	27.6	26.8	0	21.9

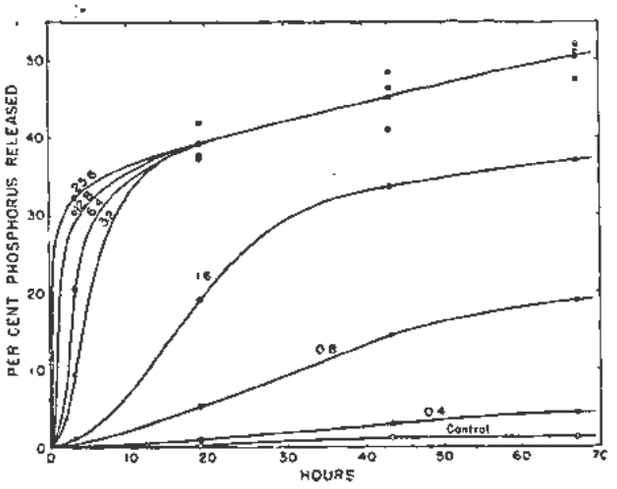

Figure 2. Effect of various doses of silver on the permeability of the spore membrane determined by the outward movement of phosphorus compounds. Numbers on curves represent μg/gm of spores × 10⁻³

same but there were differences in the speed with which the release set in. At all these doses germination was completely inhibited at the first sampling period. At 1600, 800, and 400 micrograms per gram (the values for 200 micrograms were not plotted since they were essentially the same as the control for the first 67 hours) some germination response persisted even up to 67 hours even though cell permeability was affected to the extent that from 4.4 to 37.0 per cent of the phosphorus compounds had leached out. The results, compared with those in Table VI suggest that silver has more of an effect on cell permeability than some other toxicants quite apart from its greater toxicity. This follows since cell contents are released at doses giving less than 100 per cent kill while, with cadmium, zinc, mercury, and low doses of copper there is very little release of cell contents even though all spores are unable to germinate.

UPTAKE OF TOXICANTS BY HOST PLANTS

The absorption of fungicides by host plants has been investigated in a preliminary way. Experiments with S^{35}-labeled ferric dimethyldithiocarbamate have shown that a very small amount of S^{35} can be found in leaves of plants near treated leaves.[7] This has been found to be the result of absorption of CS^{35}_2, a decomposition product of the dithiocarbamate. Whether any other portion of the molecule is absorbed has not been determined since C^{14}-labeled toxicants have not been available. Some uptake through the root system of small tomato and bean plants occurs when 2-heptadecyl-2-imidazoline or 2,3-dichloro-1,4-naphthoquinone is added to the medium. The amounts are small, however, as shown in Table VII for bean plants and the naphthoquinone. In view of the known instability of this toxicant it is very unlikely that the absurbed material has remained unaltered chemically. Fungicides that are active systemically will no doubt be found in the future and the use of isotopes should aid in discovering them and in the process of studying their action.

Table VII. Uptake of 2,3-dichloro-1,4-naphthoquinone through Roots of Bean Plants

Time after addn. of quinone, hr	*Content of tops, μg/gm fresh wt.*
2.5	0.4
23.0	2.2
43.0	8.2
74.0	15.4

SUMMARY

Rates of uptake and the quantities required to reduce germination by 50 per cent have been determined for spores of representative species of fungi with the use of S^{35}, S^{35}-labeled ferric dimethyldithiocarbamate, C^{14}-labeled 2-heptadecyl-2-imidazoline and 2,3-dichloro-1,4-naphthoquinone, and Ag^{110}, Ce^{144}, and Hg^{203}. Germination was inhibited 50 per cent after 85 to 10,000 micrograms per gram of spore weight had been taken up. Sulfur is an exception in that it is not accumulated by spores but is reduced to hydrogen sulfide and released. Lethal doses were removed from dilute solutions (1–10 ppm) within one-half to five minutes. The fungicides studied are much less toxic on a weight basis than many animal poisons, insecticides, herbicides, and bactericides. The results suggest that further search for better fungicides should be rewarding since there is no reason to believe that fungi are more difficult to kill than other organisms.

Studies on interferences among various toxicants on uptake and subsequent release by fungus spores have yielded information on competition or lack of competition for receptor sites. Certain toxicants, such as 2-heptadecyl-2-imidazoline, cerium, and silver, seem to have no receptor sites in common and saturation of spores with one of these toxicants does not interfere with subsequent uptake of the others. Toxicants competing for the same receptor sites interfere with each other and rates of uptake are reduced when they are used simultaneously. The presence of a second toxicant may increase the rate of uptake. When silver and mercury are used together the rate of uptake of mercury is increased and that of silver decreased. The results indicate that silver has an effect in increasing permeability of the cell membrane. Studies on effects of various substances in decreasing or increasing uptake should lead to more efficient practical use of fungicides and thereby lessen present losses caused by fungus diseases.

Effects on the permeability of cell membranes were studied by determining the release of phosphorus compounds into the ambient solutions when spores labeled with P^{32} were exposed to various toxicants. Silver was the most active in releasing cell contents even at doses which did not completely inhibit germination while other ions such as mercuric, cadmium, zinc, and copper could produce 100 per cent inhibition of germination with comparatively little effect on cell permeability.

Silver which had been taken up by spores exchanged rapidly and practically completely with

silver subsequently added to the medium. The other toxicants did not exchange so readily; usually the degree of exchange was less than 50 per cent. With spores treated with mercuric ion more exchange occurred on adding mercurous ion than when more mercuric ion was supplied. Practically no exchange occurred with spores treated with 2,3-dichloro-1,4-naphthoquinone. This indicates that the naphthoquinone has undergone chemical change on contact with the spores.

Preliminary experiments have indicated that there is little uptake of toxicants by host plants in tests with ferric dimethyldithiocarbamate, 2-heptadecyl-2-imidazoline, and 2,3-dichloro-1,4-naphthoquinone. Radioactive tracers are especially useful in studying uptake by host plants and possible activity of toxicants as systemic fungicides.

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Uses of Radioisotopes by the Hawaiian Sugar Plantations

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Since 1946 radioisotopes have been in continuous use here for several purposes. These may be classified as (1) essential elements as tracers of absorption, translocation, metabolism and photosynthesis (examples: C^{14}, P^{32}, Rb^{86}, Ca^{45}, Mo^{99}); (2) tracers of leaching and distribution of irrigation water (examples: P^{32} and Rb^{86}); (3) sources of radiation for instruments weighing streams of bagasse and sugar, measuring light and temperature, measuring soil moisture, measuring crop growth (examples: Sr^{90}, Co^{60}, Cs^{137}); (4) biological effects of radiation (Co^{60}).

The following examples have been selected for this paper because of their direct application to the growing and manufacture of sugar on the plantations.

TRANSLOCATION OF PHOTOSYNTHATE

Tagged elements are ideal tools for measuring translocation. A number of practical problems in sugar cane culture are associated more or less directly with the translocation and storage of soluble carbohydrates. Hence, factors affecting rates of movement as distinct from photosynthetic manufacture have been investigated in some detail here.

Material and Methods

For the more exact work plants in culture solution have been used. A glass tube is slipped over a leaf, sealed with a split stopper and plastic, and $C^{14}O_2$ introduced. After a short period of photosynthesis in sunlight, the tube is removed and the plant subjected to the measurements planned.

Results

In the first experiment a large, single stalk plant was fed through blade 5. Eighteen hours later the alcohol soluble fraction of every part of the plant was strongly tagged. The fed leaf and the growing tip were particularly rich.

For the second experiment a large stool of cane with 16 stalks in all stages of development was fed $C^{14}O_2$ through blade 3 of stalk 9. The plant was harvested 44 hours after feeding. Figure 1 shows the relative specific activity of the spindle of each stalk. Table I gives the percentage distribution of the photosynthate among the parts of the stool.

In experiment 3 the roots of a single tall stalk of sugar cane were enclosed so that the respired CO_2 could be absorbed from an air stream bubbling through the culture solution. The $BaCO_3$ was collected at short intervals and its relative specific activity determined. The first radioactive $BaCO_3$ appeared 6 hours after feeding blade 5 with $C^{14}O_2$ in sunlight. Total distance from fed blade to the top of the roots was 258 cm. Hence, the minimum rate of translocation from leaf to root is 43 cm/hr or 0.7 cm/min.

Maximum specific activity of CO_2 from roots was reached during the first night following the morning feeding (9:00 A.M.). Following this there is a decline until at 8 days a relatively constant level has been reached (Fig. 2).

Harvested after 8 days the plant was found to have a general distribution of C^{14}. Even the oldest leaf, about ready to drop, received some C^{14} from leaf 5.

Discussion

The experiments described above demonstrate the high speed of movement of photosynthate in a general circulatory system. The wide range of specific activities shown by the same tissue of different stalks of a stool may be interpreted in terms of relative stalk vitality, i.e., the ability to take from a common pool more or less than its share. From a practical standpoint, this idea has been helpful in the study of the incidence of dead cane due to competition in a crowded, high-yielding field.

FEEDING SUGAR CANE THROUGH LEAVES

Introduction

It has been known for some years that some minor (trace) elements can be successfully fed to plants by leaf spray. It is only in recent years that a serious

* Hawaiian Sugar Planters' Association.

Table I. Per Cent of Total Counts Found in Parts of the Stool

Stalk no.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*Roots*	*Stubble*
% of counts	7.2	0.2	1.0	0.7	1.4	0.1	0.5	0.003	68.5 (Fed)	0.05	0.006	0.08	0.003	0.3	0.1	1.2	17.2	1.3

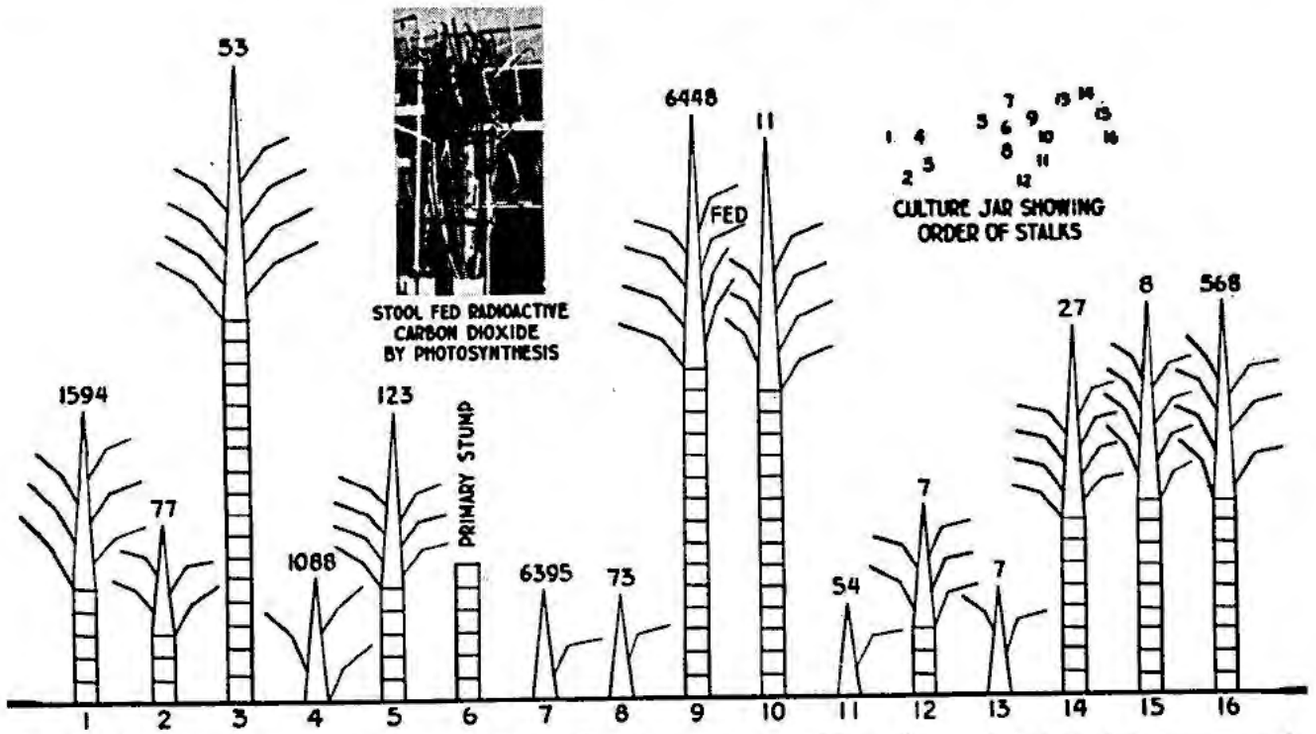

Figure 1. Translocation of photosynthate in experiment 2. Radioactive sugars move rapidly to all parts of stool. Stool of sugar cane fed through leaf 3 of stalk 9, 2:30–3:30 March 9. Numbers show radioactivity of spindle after 44 hours

effort has been made to supply some of the major elements in that way. Detailed studies have been made here of the uptake and distribution of urea tagged with N^{15}, P^{32}, Rb^{86}, radioactive sucrose and others. All materials thus far tested are absorbed by the leaf and distributed more or less throughout the plant. In general the absorption curves are logarithmic with half-times ranging from 1 day for sucrose to about 15 days for P^{32} and Rb^{86}. Absolute values depend upon the quantities applied and the environmental conditions.

Experimental Results

Figure 3 shows results of a single experiment. From a practical standpoint in the Hawaiian sugar industry phosphate application via the leaf is of greatest interest. Many of the soils fix phosphate so quickly and completely that fertilizer applications lose their effectiveness. Hence, a detailed study of utilization of leaf application was made. Figure 4 shows the efficiency of absorption with different concentrations.

From these data it can be readily calculated that the total required phosphate may be supplied in this manner.

The absorption of sugar, urea and other organic compounds is also of great importance because a number of hormone-like substances are being used in control of weeds, control of sugar cane flowering, etc.

Figure 5 illustrates the striking differences in uptake and translocation of radioactive 2,4-D in

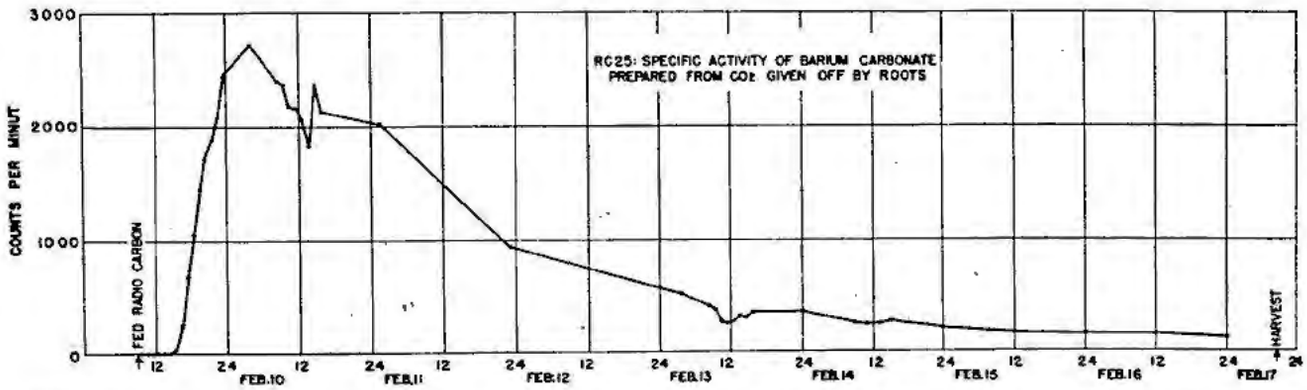

Figure 2. Root respiration of photosynthate in experiment 3. RC 25: specific activity of barium carbonate prepared from CO_2 given off by roots

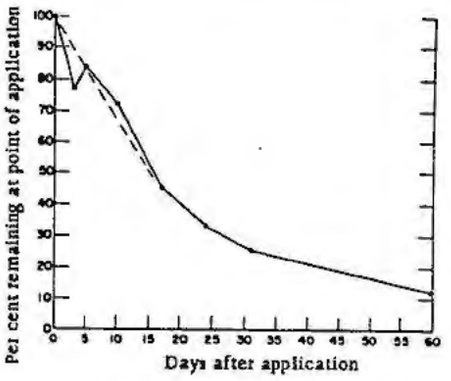

Figure 3. Absorption of P^{32} applied in solution to the sugar cane leaf

sugar cane and beans. The very low concentrations of 2,4-D reaching the vital growing parts of sugar cane may explain its high resistance to this chemical as contrasted with the bean plant. The point of application is shown by a star. A much larger proportion of the 2,4-D remained in the treated leaf of sugar cane.

RUBIDIUM-86 AS A SUBSTITUTE TRACER FOR POTASSIUM

Introduction

The available radinactive isotope of potassium (K^{42}) has such a short half-life that it is not useful for much of the work with sugar cane. This is unfortunate since this element is a major fertilizer constituent and is used in large tonnages here. Therefore, a study of rubidium-86 was made to determine whether it would serve as a tracer for potassium. This element has a strong beta and gamma ray and convenient half-life of 19.5 days.

Methods and Material

Healthy sugar cane plants 4½ months of age were fed a dose of Rb^{86} via the culture solution. After one week, when most of the rubidium had entered the plant, the plant was returned to the normal, rubidium-free, culture solution. Two months later the plants were harvested, divided into many

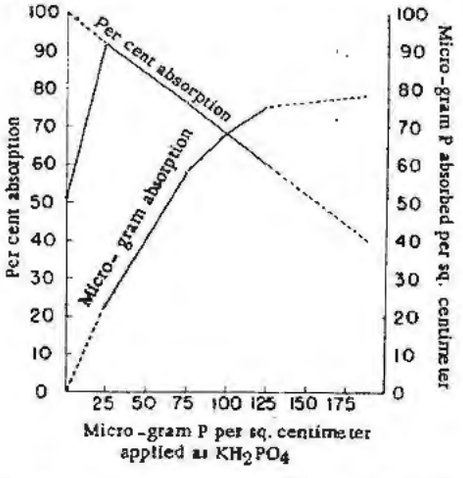

Figure 4. Efficiency of absorption of P^{32} by the sugar cane leaf

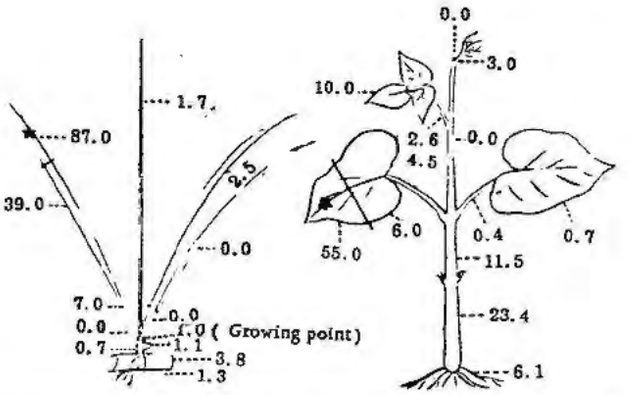

Figure 5. 2,4-D is absorbed and translocated much more rapidly by the bean plant (right) than by sugar cane. Solution applied at star

anatomical parts, and analyzed for potassium and rubidium. Potassium was determined with a flame photometer and Rb^{86} by Geiger counting.

Results

Figure 6 summarizes the results, expressed as per cent distribution of the two elements. Two conclusions are evident:

1. Two months is sufficient time for rubidium to reach its final distribution in the growing sugar cane plants; and
2. Rubidium is distributed within the plant in the same proportion as potassium.

Hence, Rb^{86} may be safely used as an index of potassium distribution. This relationship between the two elements is of great interest. For some time it has been suspected that rubidium may partially substitute for potassium as an essential element. Apparently the large difference in their atomic weights does not materially alter their "affinities" for different tissues. The forces which determine their relative concentration in different tissues apply

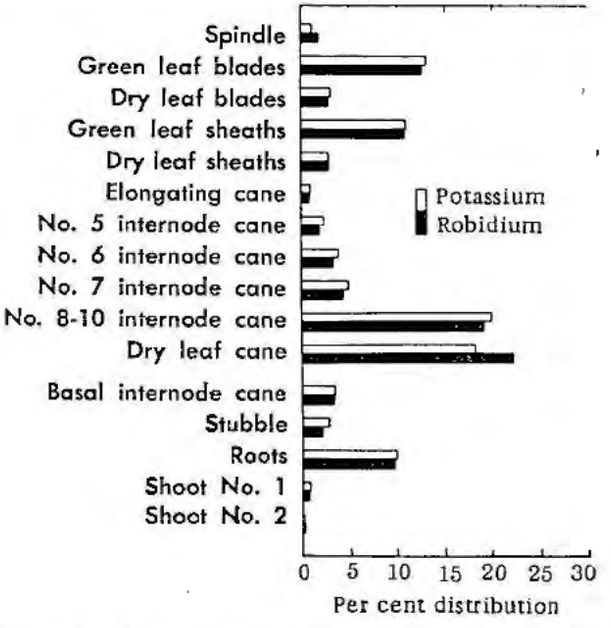

Figure 6. Distribution of potassium and rubidium in the cane plant

alike to the two elements. This is all the more remarkable in this case where total potassium present was many times the total rubidium.

MEASURING SUGAR CANE GROWTH WITH Co^{60}

Introduction

The usual method of measuring growth of sugar cane is by tabulating elongation of the stalk above a fixed base mark. This has the disadvantages inherent in small selected samples as well as the obvious error of substituting length for mass. It is also difficult to reach stalks in the center of the plots.

The gamma-ray weighing method described here is very satisfactory for standard sugar cane plots and may well be useful for many other crops. Without disturbing the plants in any way, the entire vegetative mass is weighed, every stalk, large and small being included.

Materials and Methods

Figure 7 is a close view of the equipment shown in Fig. 8 in place for shooting through a sugar cane plot consisting of 6 lines 16 feet long. The Co^{60} slugs of 100 mc each are mounted in the pockets of the two upright poles. The track and cart carrying the portable scaler or ion chamber are on the opposite side of the plot. The cart is rolled forward and backward at a uniform speed. This gives an integrated value which completely eliminates errors due to irregularities of stand in the lines. Sources and receiver are raised in steps of 1-foot. Each measurement with a scaler is run to a total of 10,000 counts. At a rate of 3000 cpm the time at each level, including resetting the sources, is about 5 minutes. When the cane is 9 feet tall, total time per plot is 45 minutes.

Results

The results given in Fig. 9 are expressed as tons per acre-foot. The two varieties of sugar cane were planted in the spring and fall. Measurements at six months compare the winter and summer growth rates. Totals for the entire height of sugar cane show that summer growth rate is about twice that for winter.

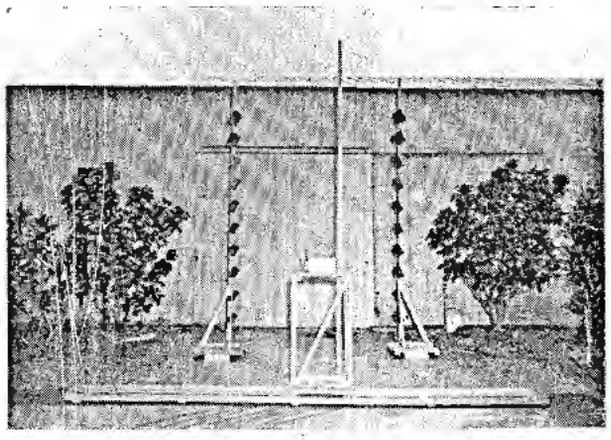

Figure 7. Equipment for weighing sugar cane in the field

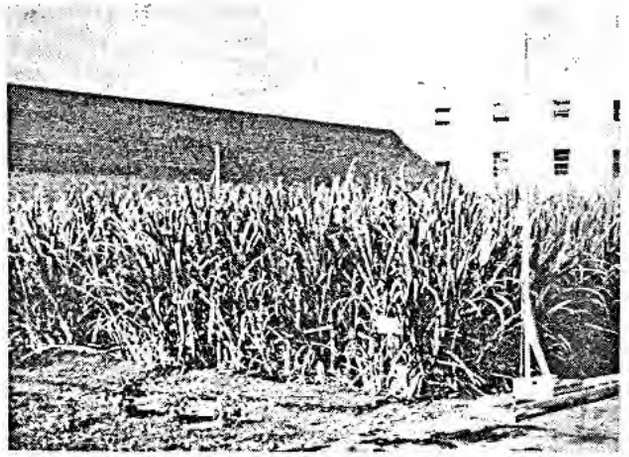

Figure 8. A plot of sugar cane being weighed with Co^{60} and an ion chamber

The method has been applied to plots of sugar cane which were hand-harvested and weighed the following day. The two methods agree very well and the gamma-ray scale leaves the cane undisturbed for future measurements.

TRACING IRRIGATION WATER TAGGED WITH RADIOISOTOPES

Introduction

In Hawaii over 50 per cent of the million ton crop of sugar is grown on irrigated plantations. Uniform distribution of water in long irrigation lines has been of especial interest during times of water shortage. It has become of even greater importance in recent years as the practice of adding soluble

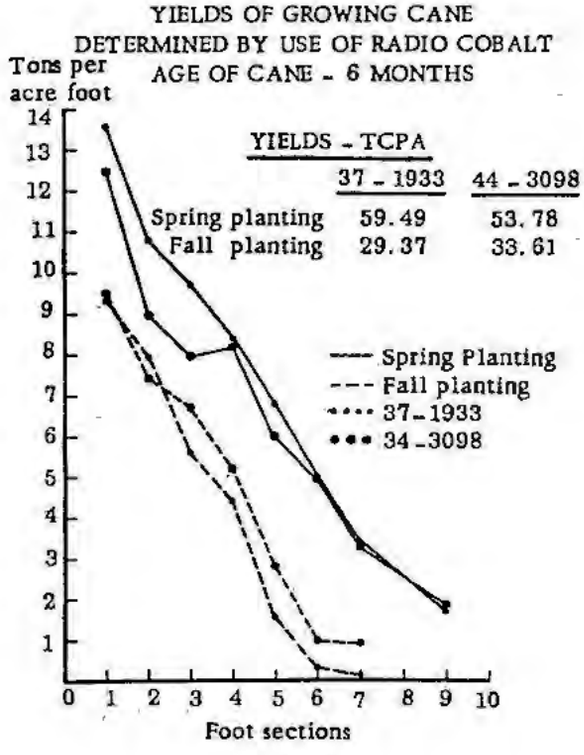

Figure 9

fertilizer to the water has grown. In order to simplify the measurement of the amount of water percolating into the soil at points along the cane line a method has been devised in which the water is tagged with a radioactive element which is later measured at any chosen points. From the data thus obtained it is hoped that field layouts may be redesigned to give the best possible results on the various soil types.

The Method

The method is based on the fact that some elements are so strongly adsorbed by most soils that they will be completely retained on the surface while the water percolates downward. The first element considered was radioactive phosphorus (P^{32}), which had the advantage of ready availability and cheapness, in addition to the fact that it has a convenient half-life and emits only a strong beta ray making shielding easy. It works well on many soils. But the discovery that it does leach readily through some soils led to the search for an element of more universal applicability. Rubidium-86 seems to fill the bill. It also emits a strong beta ray and has a convenient half-life (19.5 days). However, it emits some gamma rays which increases the cost of shipment and necessitates more care in handling in the laboratory. These problems are not too serious and rubidium has been adopted for routine use in studying water distribution on Hawaiian sugar plantations.

The test lines are isolated for a hundred days until decay has reduced the radioactivity to a negligible quantity.

Procedures

Approximately 1 mc of Rb^{86} per 100 ft of irrigation line is diluted to a convenient volume. A capillary tube is inserted in the outlet of an aspirator bottle which will give a constant rate of flow for a period in excess of the estimated irrigation time. The irrigation weir is adjusted to maintain a constant flow of water into the line throughout the entire period. The Rb^{86} and the irrigation water are fed into a mixing box which gives a thorough mix before the water starts down the line. Any desired number of sampling stations are set up where both water and soil samples are to be taken. The repeated water samples are required because Rb is so strongly adsorbed that it is picked up by the surface soil, thus reducing the activity of the water as it goes down the line. Each station is covered by four layers of cheesecloth about 15 inches wide extending across the line. This does not hinder percolation into the soil but does prevent surface pickup of Rb from passing water. Thus a pickup correction is eliminated since all of the residual radioactivity in the soil must come from water which has gone down at that point.

Water flowing over each station is sampled continuously to give a true integrated mean activity.

From the activity of the water at zero station, a few feet below the mixing box, the rate of water flow into the line may be calculated.

For assay of Rb^{86} content 50 ml of water in moisture cans 7 cm in diameter are counted at infinite thickness with a mica end window counter.

The interval between time of arrival of irrigation water at each station and the time of stopping irrigation is recorded for each test. Record is also kept of the flow of water down the cane line for each 10 foot section.

When the last of the irrigation water has disappeared, the cheesecloth is removed and soil samples are taken with a cylindrical soil sampler 1 in. in diameter, which penetrates to a 1 inch depth. It has been found that sampling to this depth recovers all the Rb^{86}. Twelve samples are taken: two at the water lines which are marked during irrigation and the other ten equally spaced across the line. The total area sampled is 9.4 square inches. The soil sample is dried, weighed, and thoroughly mixed. Fifty grams of the sample is weighed into 7 cm moisture cans and leveled to an even layer for counting. From the activity of this aliquot, the total count in the whole sample is calculated.

A reference standard is prepared by adding to 100 grams of dry inactive soil the amount of Rb^{86} present in 9.4 cubic inches of an approximately average water sample. This standard is dried, thoroughly mixed and a 50 gram aliquot counted. The calculated total activity is equivalent to 1 linear inch of water of known activity.

Thus from the total activity of the soil sample and the activity of the integrated water sample of a station in a cane line, the amount of water which has gone down at that station is:

Linear inches of H_2O,

$$I = \frac{\text{Total activity of soil sample}}{\text{Total activity of standard soil}} \times \frac{\text{Activity of standard water}}{\text{Activity of water sample}}$$

Acre inches of water absorbed at any station are:

$$\text{Acre inches} = I \times \frac{\text{width of wetted line}}{\text{width of cane lines}}$$

For example, if the wetted line is 2 ft wide and the cane lines are 5 ft, acre inches $= 0.4\ I$.

Experimental Results

Over a hundred tests, covering practically all the irrigated cane areas of the Islands have been run to determine the efficiency of the various irrigation practices. The results of three of these tests are recorded in Table II. These lines were 200 feet in length and five sampling stations were set up at 20, 60, 100, 150 and 190 feet (A, B, C, D, E) below the head of the line.

The very large differences in inches of water absorbed along the lines is due to line slope and rate of application of water. In order to get enough water into the soil at the points of lowest absorption, it is

Table II. Distribution of Irrigation Water Tagged with Rb^{86}

Test no.	Line slope %		Sampling stations A	B	C	D	E
0–16	0.75	Water activity	16.3	15.8	15.4	14.7	14.3
		Soil activity	5084	4782	4267	2745	2300
		Acre inch irrigation	4.43	3.26	3.40	2.46	2.60
0–19	1.5	Water activity	17.0	15.1	14.9	11.5	8.0
		Soil activity	3726	4254	3546	4136	2698
		Acre inch irrigation	2.58	3.04	2.56	5.11	6.61
0–13	2.7	Water activity	21.3	17.8	14.3	9.7	6.3
		Soil activity	3575	3984	5760	5412	2125
		Acre inch irrigation	1.23	1.43	4.74	7.93	4.46

Figure 10. Soil moisture will be measured by dropping ion chamber to level of Co^{60} in adjacent pipe

necessary to flood other sections of the line with 3 or 4 times the needed amount. This represents a great waste of water. The effects become even more disturbing when irrigation water is the medium for fertilizer application, a practice in wide use in Hawaii.

MEASURING SOIL MOISTURE WITH GAMMA RAYS

Some years ago it was found here that the gamma rays from Co^{60} could be used to measure soil moisture changes with sufficient accuracy for most needs. The method is now being used continuously.

Materials and Methods

Figure 10 shows a field installation in which the Co^{60} source is lowered in one pipe and the ion-chamber is just ready to be lowered. Figure 11 is a cross section of an 18-inch pot, showing plastic pipes, Cs^{137} source (A) and G-M tube receiver (B). With the source and tube each 4 inches long the measurement is an integrated value for a rectangle of soil. Any depth can be chosen.

Results

Figure 12 shows the calibration curve for the pot. The width of the dots represent the error of about 1% moisture. The absolute moisture value is read in 5 minutes without disturbing the soil or roots.

GAMMA-RAY SCALE FOR STREAMS OF BAGASSE AND SUGAR

Numerous uses may be made of radioisotopes within the sugar mill. The first continuous use to be made here is in the gamma-ray scale for streams of bagasse and sugar. This gives a continuous record of milling and production performance as well as the integrated weight for any period of time.

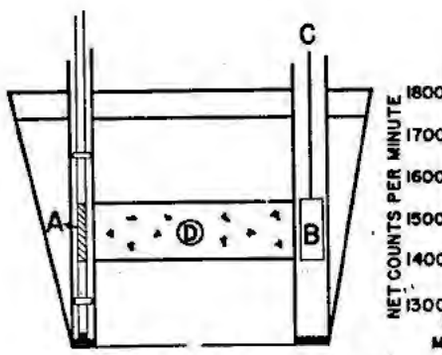

Figure 11. Diagram of a pot of sugar cane with pipes for measuring soil moisture with G-M tube and Cs^{137}

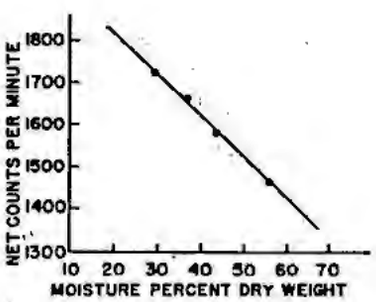

Figure 12. Soil moisture calibration curve for pot shown in Fig. 11

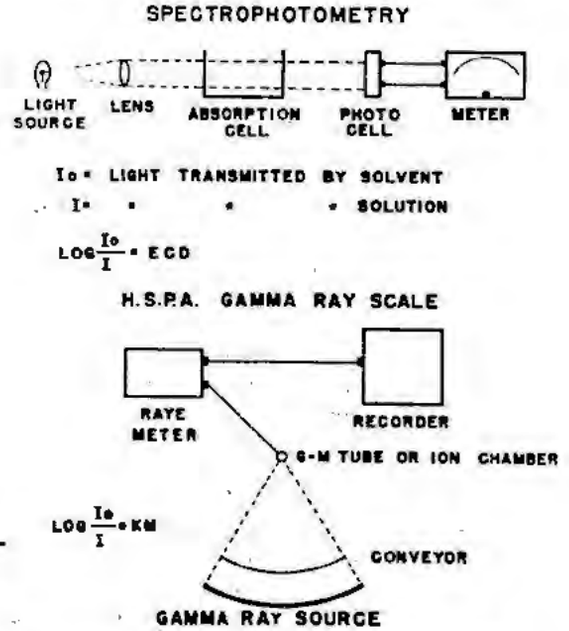

Figure 13. Schematic drawing of gamma ray scale

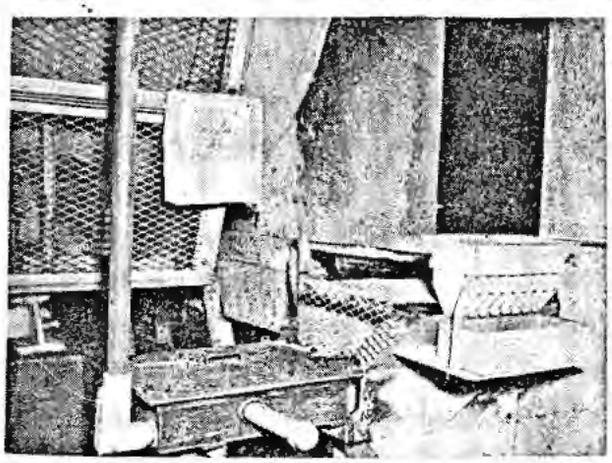

Figure 14. Sugar stream falling between Cs^{137} source and ion chambers in box at left

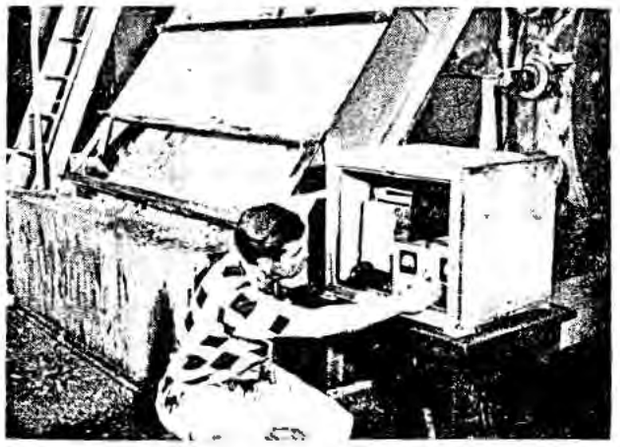

Figure 15. Bagasse stream falling between Cs^{137} source and receiver with recorder at right

Materials and Methods

The principle of the measurement is illustrated in Fig. 13. Although the system lacks the theoretical simplicity of a parallel beam of light passing through a homogeneous medium, in practice an entirely empirical calibration yields constants which are highly reproducible from day to day. Figures 14 and 15 show a sugar stream and a bagasse stream, respectively, falling through the gamma-ray beam. The recorder is equipped with a precision integrator which totals the weight in each batch.

The source is a tube of cesium-137 which is ideal for this work since it emits a soft γ-ray and has a half life of about 37 years. The receiver is a pair of ion chambers which are in balance when no sugar is flowing. A 10% absorption of gamma rays is spread across about two thirds of the chart. This is the normal rate of flow and there is sufficient chart space to take care of the highest loads encountered.

Results and Discussion

Calibrations against weighed loads of sugar indicate reproducibility under continuous operation within $\pm 1\%$ of the true value. Reliability over very long periods is now being tested.

The records from these machines give very valuable pictures of mill operation. The uniformity of load at the crushers and the efficiency of output from the batch crystallizers are recorded. There are a number of operations within the mill where such records and integration may be well worth while. With minor modifications the same equipment is applicable to other operations.

The Importance of Isotopes in Agriculture

By S. Mitsui,* Japan

The induction of mutations by radioactive isotopes promises to lead to the production of higher-yielding crops which tolerate heavy applications of fertilizer and are resistant to damage by insects and disease. Little work has been done in Japan on this subject and much remains to be done in the future.

Radioisotopes as tracers, however, have been used in various aspects of agriculture in Japan. For instance, the harmful effect of hydrogen sulphide on nutrient uptake by rice plants has been widely studied with the use of labelled sulphur. Hydrogen sulphide is considered to be one of the principal causes of unproductive paddy soils in Japan. The transfer of labelled calcium and phosphorus from the feed to eggs in the hen's body has also been clarified. In addition studies with labelled phosphorus have shown that foliar application of phosphorus to mulberry trees helps to promote its availability particularly to younger leaves.

As illustrated above, agriculture in Japan is different from that of other countries where the uses of isotopes have already been extensively advanced. Knowledge of the use of radioisotopes already accumulated in these countries might be helpful, but often needs modification when applied to our country. For instance, the improved method of fertilizer placement for upland crops by the use of radioisotopes can hardly be applied without modification to the fertilizer placement for lowland rice which is the principal crop in Japan. Therefore, so far as the use of radioisotopes in agriculture is concerned, location of the country is important.

Cultivated crops in Japan are receiving the highest doses of chemical fertilizers in the world. Therefore, much more efficient use of fertilizers is required. Much wider use of isotopes, particularly the labelling of various phosphate fertilizers with P^{32} in large-scale field experiments is urgently required.

Soil erosion control is now attracting attention as one of Japan's most vital problems. For this purpose, investigations of soil movement due to rain-fall are required. However, no case has been reported on the application of the isotope technique for such studies. Co^{60} would be tightly fixed by soil colloid and the energy and half-life appear to be quite suitable for surveying eroded soils. Also the activation of a quantity of soil in an atomic pile might be helpful.

Lowland rice is sometimes seriously attacked by plant hoppers, but the life cycle of the insect during the winter season remains unknown. Labelling the insect with a suitable isotope would be quite helpful in elucidating the cycle and in ensuring a rational protection of lowland rice against insect damage. Finally, a large-scale γ-field trial would probably give rise to much more efficient varieties of lowland rice, which is the main crop not only of Japan but also of other vast Asian countries. Needless to say, the average yields of lowland rice in these countries are surprisingly low and much improvement will be required in the future.

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Research by Means of Radioactive Isotopes Concerning Penetration and Residues of Phospho-Organic Insecticides in Plants

By K. A. Gar and R. Y. Kipiani, USSR

The phosphorus-organic insecticides are of extremely great value in the struggle against agricultural pests and have become widely used in farming. A sound place in agriculture has already been won by such effective compounds as diethyl-4-nitrophenylthiophosphate (thiophos, parathion, NIUIF*-100), dimethyl-4-nitrophenylthiophosphate (metaphos, vofatox). Also widely spread are such compounds as S-1 2-dicarboethoxiethyl-0, 0-dimethyldithiophosphate (carbophos, malathione) and their analogues which are somewhat less toxic for insects, much less so for warm-blooded animals, and hence they may be used on a much wider scale than thiophos or metaphos.

An intense study has also been made of such compounds as octamethyltetramide of pyrophosphoric acid and a number of other compounds capable of penetrating and accumulating in plant tissues thereby making them insect-proof for a rather long period of time. The wide use of these compounds in agriculture will allow sharp reduction in the number of treatments against sucking pests (mites, aphids) and thereby an increase in the economic effect of insecticide treatments, that is, will make them cheaper.

The phosphorus-organic insecticides, however, are quite selective in their action and have a number of peculiarities which require a most thorough study. Most of the phosphorus-organic insecticides are very toxic for warm-blooded animals, hence the importance of the question as to what proportions of their residues are permissible and how long are they preserved on the surface of the treated agricultural plants. No less important are the possibility of penetrations of phosphorus-organic insecticides into plants and the duration of time in which their toxicity is preserved as well as the ways by which these insecticides penetrate into the body of warm-blooded animals and the danger of and precautions against their poisoning. The knowledge of the behaviour and metabolism of phosphorus-organic insecticides within the insects provides a more intelligent and expedient approach to the search for new insecticides of greater effectiveness.

But the solution of these problems encounters serious difficulties because of complicated and laborious and in some cases insufficiently specific methods of chemical analyses of minute quantities of phosphorus-organic insecticides and of their decomposition products in plant and animal tissues. Analysis of these products for the content of phosphorus, sulphur, chlorine and other elements cannot provide information on the insecticide admixtures since all these elements are abundant in plant and animal tissues as well. Exceptionally great promise in this respect is opened by the use of the methods of labeled atoms, P^{32}, S^{35}, C^{14} and other isotopes which allow one to follow up the circulation and transformation of insecticides within the bodies of animals and plants.

The task of the present investigation is first of all a practical one, viz. elucidation of the conditions of application of phosphorus-organic insecticides. This should provide the most expedient use of these compounds as well as the greatest safety of labour. The study was therefore carried out with compounds already used or prepared for use in agriculture (Table I). Synthesis of labeled compounds by an original method was carried out at the J. V. Samoilov Scientific Institute of Fertilizers and Insectofungicides by Y. A. Mandelbaum, K. D. Shvetsova-Shilovskaya, I. L. Vladimirova, V. I. Lomakina and Z. M. Bakanova under the guidance of Professor N. N. Melnikov,[1,2] and at the Institute of Plant Protection of the Academy of Sciences of Georgian SSR the synthesis of the preparations was accomplied by R. Y. Kipiani and G. V. Gegenava. Biological investigations at the Samoilov Institute were carried out by K. A. Gar, Y. N. Fadeev, V. I. Chernetsova and Z. Z. Golubeva; and at the Institute of Plant Protection of the Academy of Sciences of Georgian SSR by R. Y. Kipiani and G. V. Gegenava.

The compounds prepared were tested for their relative radioactivity. The absolute radioactivity of the compounds was of no importance for the present purposes, but, if necessary, it could be determined by means of an uranium radioactivity standard. The preparations studied had a specific activity up to 4 mc/gm which, with our device, produced up to 1300 cpm/min/μg.

Original language: Russian.

* Name derived from Russian initial letters of the J. V. Samoilov Scientific Institute of Fertilizers and Insectofungicides.

Table I. Chemical Characteristics of the Compounds

Formula, name	d_4^{20}	n_d^{20}	*Melting point, °C*	*Boiling point, °C*	*Label*
$(C_2H_5O)_2PSOC_6H_4NO_2$ (NIUIF-100, thiophos)	1.2704	1.5374	+6	115–117 (0.03 mm Hg)	P^{32}, S^{35}
$(CH_3O)_2PSOC_6H_4NO_2$ (metaphos, vofatox)	1.3518	1.5600	+36	120 (0.8 mm Hg)	P^{32}, S^{35}
$C_2H_5OPS(OC_6H_4NO_2)_2$ (NIUIF-101)	—	—	to 125	—	P^{32}, S^{35}
$(CH_3O)_2PSSCHCOOC_2H_5$ $CH_2COOC_2H_5$ (carbophos I, malathione)	1.2076	1.4960	—	160–170 (3.5 mm Hg)	P^{32},
$(C_2H_5O)_2PSSCHCOOCH_3$ CH_2COOCH_3 (carbophos IV)	1.2237	1.4979	—	116–120 (0.025 mm Hg)	P^{32},

STABILITY OF RESIDUES OF PHOSPHORUS-ORGANIC INSECTICIDES

These investigations[4] were initially carried out by dispersing 1–3% dust preparations on small glass cups at a rate of about 10 kg/hectare (the minimum expenditure rate of preparations used in field conditions) with subsequent determination of total loss of P^{32}. The results of estimations of phosphorus loss from dusts of diethyl-4-nitrophenylthiophosphate and ethyl-4,4′-dinitrodiphenylthiophosphate exposed under various conditions in a finely dispersed state are summarized in Tables II and III. It will be seen that the most important disintegration factor of the residues of these two phosphorus-organic compounds is the ultra-violet part of the solar spectrum. The loss of the residues of diethyl-4-nitrophenylthiophosphate under direct sunlight is completed within a few hours while in the diffused sunlight the loss is several times slower. Still more slow is the loss of the residues of diethyl-4-nitrophenylthiophosphate under the same temperature conditions, but in absolute darkness.

These data clearly point to the photochemical character of decomposition of the diethyl-4-nitrophenylthiophosphate residues which are transformed under the influence of sunlight into another more volatile compound evaporating from the surface. Ethyl-4,-4′-dinitrodiphenylthiophosphate which is a crystalline product is decomposed more slowly than diethyl-4-nitrophenylthiophosphate.

The residues of the preparations were also tested for the degree of hydrolysis. This was estimated by their solubility in organic solvents (dichlorethane, benzol) in which, in contradistinction to the products of complete hydrolysis, they readily dissolve.

Extraction of the dusts exposed to direct sunlight, in a shaded place (in small porcelain vessels) and on leaves showed that after a 24-hour exposure the residue of diethyl-4-nitrophenylthiophosphate dissolves in dichlorethane (up to 76–85%). After a 72-hour exposure in the shade, 83% dissolves in dichlorethane, after light exposure 73%, and on the leaves, independently of the illumination conditions it dissolves in amounts varying from 62% to 64%. The residue of ethyl-4,4′-dinitrodiphenylthiophosphate, after 72-hour exposure, dissolves in dichlorethane (82% to 87%) independently of the exposure conditions.

In the experiments at the Georgian Institute of Plant Protection the preservation time of diethyl-4-nitrophenylthiophosphate was estimated by placing a small amount of the insecticide (its alcohol solution) on drawing paper. This was subsequently exposed to different conditions and the residue subjected to chromatographic analysis.

Chromatographic analysis was carried out with the aid of a solvent consisting of 30% acetone, 30% methyl alcohol and 40% water (with this solvent, diethyl-4-nitrophenilthiophosphate had an R_f of 0.62). In this way the fact was confirmed that decomposition of the residues of diethyl-4-nitrophenylthiophosphate from the solutions proceeds under the influence of temperature at approximately the same rate as from the dusts. The residue of the preparation on the paper exposed to light contained

Table II. Loss of Phosphorus from Finely Dispersed Dusts of Diethyl-4-nitrophenylthiophosphate and Ethyl-4-,4′-dinitrodiphenylthiophosphate

Compound	*Exposure conditions*	*Phosphorus residue, %* *					
		1 * / 1	5 / 5	24 / 5	27 / 8	72 / 8	96 / 8
1% dust of diethyl-4-nitrophenylthiophosphate	In open place	59.0	43.5	12.3	—	5.7	0.0
	In shady place	—	88.5	64.0	37.8	—	16.4
3% dust of ethyl-4,′-dinitrodiphenylthiophosphate	In open place	100	92.7	75.1	66.0	55.0	33.5
	In shady place	100	97.0	93.2	91.3	—	86.0

* The numerator indicates total number of exposure hours; the denominator, the number of exposure hours in the sunlight.

Table III. Effect of Temperature on Loss of Phosphorus from Finely Dispersed Dusts of Diethyl-4-nitrophenylthiophosphate and Ethyl-4,4'-dinitrodiphenylthiophosphate

Time of exposure, hr	Residue of phosphorus from 1% dust of diethyl-4-nitrophenylthiophosphate after exposure at temperature of			Residue of phosphorus from 1% dust of ethyl-4,4'-dinitrodiphenylthiophosphate after exposure at temperature of	
	15°C	22°C	45°C	22°C	45°C
50	99	95	72	95	83
100	96	72	50	86	79
200	77	60	38	80	72
300	65	52	30	77	65
600	57	37	—	63	53

diethyl-4-nitrophenylthiophosphate and its hydrolysis products.

The content of the hydrolysis products after a 6-day exposure (including 12 hours under direct sunlight) amounted to about 40%, while in the sample kept in diffuse light it was about 20%, and in darkness, about 15% These products when separated on paper had an R_f of about 0.56 and 0.97 (Fig. 1). The product characterized by an R_f of 0.56 was found, in the main, on chromatograms of drops exposed to light. The product characterized by an R_f of 0.97 should be referred to the products of complete hydrolysis of diethyl-4-nitrophenylthiophosphate for it was always formed as a result of alkaline hydrolysis of diethyl-4-nitrophenylthiophosphate. Thus, the phosphorus-containing residues of diethyl-4-nitrophenylthiophosphate on a treated surface do not consist entirely of the insecticide, but partially of its hydrolysis products.

The study of diethyl-4-nitrophenylthiophosphate residues on treated surfaces showed that its use may be permitted for treating cucumbers in hothouses for two days before the next harvesting. The solution of this problem by the usual sanitary-hygienic methods of estimating the quality of the crop on laboratory animals would require almost one year of laborious investigations, while with the aid of labeled atoms similar results were obtained literally within several days.

STABILITY OF CARBOPHOSES

A similar study was carried out with two esters of dithiophosphoric acid:

S-1, 2-dicarboethoxiethyl-0, 0-dimethyldithiophosphate (carbophos I), and

S-1, 2-dicarbomethoxiethyl-0, 0-diethyldithiophosphate (carbophos IV).

Since the estimation of stability of dusts exposed to direct sunlight was found inconvenient and the results obtained difficult to compare with each other, irradiation by a quartz lamp PRK-4 was used at a 50-cm distance from the burner. In other respects the method did not differ from that described above. The experiment was carried out with 3% dusts and 0.1% emulsion of a 30% concentrate. (The emulsion drops were placed on the bottom of small cups and exposed after being dried in the air.) The remainder of the preparation was tested for total phosphorus as well as for the dichlorethane soluble fraction. Table IV presents data concerning total phosphorus residue, phosphorus content of the dichlorethane fraction obtained by extracting the residue with water and dichlorethane (content of intact preparation in the residue) and total residue of the intact preparation with due account to the losses caused by evaporation and hydrolysis.

It will appear from the data of Table IV that total loss of phosphorus as well as hydrolysis proceeds more rapidly in the dust residues than in the emulsion residue. Carbophos IV proved somewhat more stable than carbophos I; this appeared more distinct when the stability of emulsions was estimated. A comparison of the results obtained with those on thiophos stability clearly indicates that decomposition and loss of carbophos from the surface is due to two factors—hydrolysis and evaporation while with thiophos the principal part is played by evaporation.

An estimate was also made of decomposition of carbophos I and carbophos IV and of loss of phosphorus residues from finely dispersed dusts of these two compounds exposed to heat in a dark thermostat. It will be seen from Table V, that loss of phosphorus from the dust of carbophos I as caused by heat proceeds at approximately the same rate as phosphorus loss from the dust of thiophos. By its heat stability carbophos IV approximates ethyl-4, 4'-dinitrodiphenylthiophosphate.

In conclusion, mention should be made of the stability of carbophos I and carbophos IV emulsions to hydrolysis. When 0.1% aqueous emulsions (on distilled water) of these preparations were kept in summer time in glass flasks both in the light and darkness under natural temperature of the air (which varied within the limits of 15–30°C), carbophos I was hydrolyzed in 75 days by 25% in darkness

Figure 1. Radiochromatograph of partition of diethyl-4-nitrophenylthiophosphate by means of the solvent mixture: acetone, ethyl alcohol, water: (a) point of application; (b) product with $R_f = 0.57$; (c) diethyl-4-nitrophenylthiophosphate, $R_f = 0.62$; and (d) product of complete hydrolysis, $R_f = 0.97$

Table IV. Resistance of Carbophos I and Carbophos IV Preparations to Ultra-violet Rays

Preparation		Time of exposure under quartz lamp, hr					
		1	3	6	10	20	40
Carbophos I, 3% dust	Total P^{32} residue, %	98	90	86	84	82	80
	P^{32} content in dichlorethane fraction, %	70	54	41	35	31	29
	Residue of non-decomposed preparation, %	68.5	48.6	35.2	29.4	25.4	23.2
Carbophos I, 0.1% emulsion	Total P^{32} residue, %	99	95	93	92	91	90
	P^{32} content in dichlorethane fraction, %	74	57	46	40	38	36
	Residue of non-decomposed preparation, %	73.2	54.0	42.7	36.8	34.6	32.4
Carbophos IV, 3% dust	Total P^{32} residue, %	97	95	87	84	77	74
	P^{32} content in dichlorethane fraction, %	75	62	48	41	35	30
	Residue of non-decomposed preparation, %	72.5	59.0	41.8	34.4	27	22.2
Carbophos IV, 0.1% emulsion	Total P^{32} residue, %	99	99	98	97	95	91
	P^{32} content in dichlorethane fraction, %	80	71	60	58	56	55
	Residue of non-decomposed preparation, %	79.0	70.2	58.7	56.2	53.2	50.0

Table V. Loss of P^{32} from the Dusts of Carbophos I and Carbophos IV at Different Temperatures

Temperature, °C	Time of exposure, hr				
	0	1	3	20	44
	Carbophos I, P^{32} residue, %				
0	100	94.8	97.2	94.4	89.3
25	100	99.1	98.1	95.7	95.9
45	100	94.4	90.0	79.6	71.4
	Carbophos IV, P^{32} residue, %				
0	100	100.0	100.0	95.2	94.3
25	100	100.0	98.2	98.4	94.0
45	100	100.0	92.4	78.1	82.2

and, approximately, by 40% in the light; within the same time period carbophos IV was hydrolyzed in the light by 50%, and in darkness by 40%.

STABILITY OF RESIDUES OF DIMETHYL-4-NITROPHENYLTHIOPHOSPHATE (METAPHOS)

The preservation time of metaphos residues placed on a surface as 2% dust was determined only by evaluating P^{32} residues. The method applied did not differ from that described above. The loss of phosphorus from a 2% dust is illustrated by Fig. 2. The rate of phosphorus loss from the same dust as influenced by ultra-violet rays is:

Time of exposure, hr.	0	1	3	7	11	15	25
P^{32} residue, %	100	88	87	77	77	60	46

It will appear from these data that dimethyl-4-nitrophenylthiophosphate is less resistant to heat. As to its resistance to the ultra-violet part of the spectrum it lags behind carbophos I and IV as far as phosphorus residue is concerned. It will be noted that hydrolysis of carbophos I and IV is greatly accelerated by ultra-violet raws while metaphos remains more resistant to hydrolysis. A total of only 2% of the preparation was hydrolyzed upon exposure of 0.05% metaphos emulsion in distilled water for 15 days to the temperature of about 20°C.

PENETRATION OF PHOSPHORUS-ORGANIC INSECTICIDES INTO PLANTS

The phosphorus-organic preparations differ in their capacity to penetrate into plants. Moreover, the differences in their light resistance and hydrolyzing capacity increase the variability of the storage time of phosphorus-organic preparations in plants. Hence, only some of the phosphorus-organic insecticides can be used as systemic-intraplant preparations.

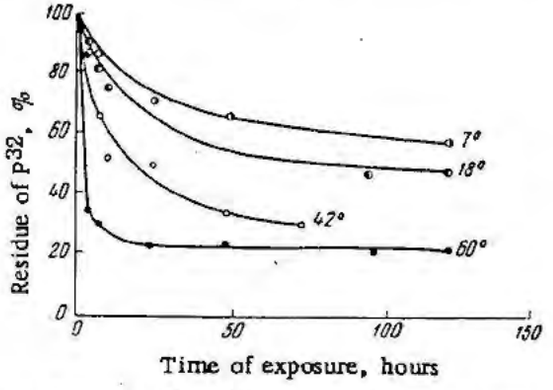


Figure 2. Preservation time of the residues of dimethyl-4-nitrophenylthiophosphate upon dispersing 2% dust in a thin layer in darkness

The properties of the intraplant insecticides are eventually attributed to diethyl-4-nitrophenylthiophosphate and dimethyl-4-nitrophenylthiophosphate and to some other compounds used as contact insecticides. A study of this question is not only of theoretical, but of practical significance as well, since it contributes to a clear-cut determination of the possible ways of utilization of these preparations.

PENETRATION OF DIETHYL-4-NITROPHENYLTHIOPHOSPHATE INTO PLANTS

Diethyl-4-nitrophenylthiophosphate (thiophos) was examined both under natural and conservatory conditions.[5] The emulsions of diethyl-4-nitrophenylthiophosphate concentrate were placed on the leaves of plants or used for spraying the soil under the plants. The amount of insecticide penetrating into the plants was determined by estimating the radioactivity of the aqueous and dichlorethane leaf extracts as well as by direct determination of radioactivity of the leaves.

Water and dichlorethane extraction were used because, according to N. N. Melnikov and other scientists,[6,7] the solubility of diethyl-4-nitrophenylthiophosphate in water amounts at 25°C only to 0.002%; in organic solvents (benzene, dichlorethane) the solubility of diethyl-4-nitrophenylthiophosphate is high and at 25°C exceeds 10%. On the other hand, the products of hydrolytic decomposition of diethyl-4-nitrophenylthiophosphate which, according to Melnikov, follows the scheme:

1. $C_2H_5O_2PSOC_6H_4NO_2 + H_2O \rightarrow (C_2H_5O)_2PSOH + NO_2C_6H_4OH$
2. $(C_2H_5O)_2PSOH + H_2O \rightarrow C_2H_5OPS(OH)_2 + C_2H_5OH$
3. $(C_2H_5O)PS(OH)_2 + H_2O \rightarrow C_2H_5OH + H_3PSO_3$
4. $H_3PSO_3 + H_2O \rightarrow H_3PO_4 + H_2S$
5. $H_2S + O \rightarrow H_2O + S$

are soluble in water. Thus, by extracting plants simultaneously with equal volumes of water and dichlorethane and by separating then the extracts and determining their P^{32} content, one can judge the decomposition of diethyl-4-nitrophenylthiophosphate or of other similar compounds. Studies were also carried out on penetration of diethyl-4-nitrophenylthiophosphate into plants after spraying the soil with an emulsion and on treating the leaves with the emulsions and dusts of this insecticide.

It was thus found that when the roots of various plant species (chrysanthemums, hydrangeas, cinerarias, beet, potato, cotton, cabbage, etc.) are immersed into an emulsion of diethyl-4-nitrophenylthiophosphate for various time periods or when the soil under the plants is sprayed with emulsions containing 0.025–0.25% of the active principle, considerable amounts of radioactive phosphorus can be detected in the plants. For example, when cabbages were sprayed with a 0.2% emulsion, the content of P^{32} in the plants amounted to 290 mg (computed in diethyl-4-nitrophenylthiophosphate units) per kg fresh weight. In chrysanthemums up to 60 mg of the preparation were found, and in potato leaves much more. However, when the plants were infested with aphids, the latter were not completely eradicated. An analysis of the poisoned green aphids (*Aulacorthum pelargonii* Kalt) from chrysanthemums that contained more than 50 mg/kg of diethyl-4-nitrophenylthiophosphate showed up to 22 mg of the preparation per kg live-weight of the insects, and still the insects survived.

The analysis of gray cabbage aphids (*Brevicorine brassicae* L.) that lived on cabbage sprayed with a diethyl-4-nitrophenylthiophosphate emulsion and containing about 100 mg of the preparation per kg fresh weight showed 49–80 mg/kg of phosphorus (computed in diethyl-4-nitrophenylthiophosphate units) in the bodies of the aphids, but in spite of this the insects were still alive.

Periodic observations of the rate of diethyl-4-nitrophenylthiophosphate penetration and decomposition in plants sprayed with an emulsion of the preparation showed that its hydrolysis proceeds at a high rate. In plants sprayed with less concentrated emulsions, hydrolysis of the preparation was completed within 30 days, while with more concentrated emulsions it required a somewhat longer period of time. It was also found that the rate of hydrolysis of the preparation in plants depends on the illumination and temperature conditions.

Special experiments were undertaken to obtain more detailed information regarding the influence of illumination on the velocity of diethyl-4-nitrophenylthiophosphate hydrolysis in plants treated by spraying the soil (this was done with a 0.05% emulsion at a rate of 50 ml per bowl of 0.8 l capacity). For this purpose the chrysanthemums and hydrangeas used were kept in the conservatory in sunlight, in shady places and on the ground. The experiments were carried out in summer (in the first days daily temperature varied from 17°C to 20°C, and in the following days it fell to 10–14°C). The results of one of the experiments are illustrated by Fig. 3 and the data of Table VI.

It thus appears that the preparation penetrates into the plants rapidly. The largest amount of radiophosphorus was found in plants on the fifth to the fourteenth day after showering. Decomposition of diethyl-4-nitrophenylthiophosphate in plants begins almost as soon as it gets there. The preparation penetrated more actively into plants exposed to light than into those shaded either in the conservatory or on open ground. At an earlier stage the total quantity of non-decomposed preparation in plants exposed to light was also higher than in shaded ones, while later on the preparation content in the former did not exceed that in the latter. This is due to the fact that decomposition of diethyl-4-nitrophenylthiophosphate in plants exposed to light proceeds somewhat more rapidly. Decomposition of diethyl-4-nitrophenylthiophosphate in hydrangeas was slower than in

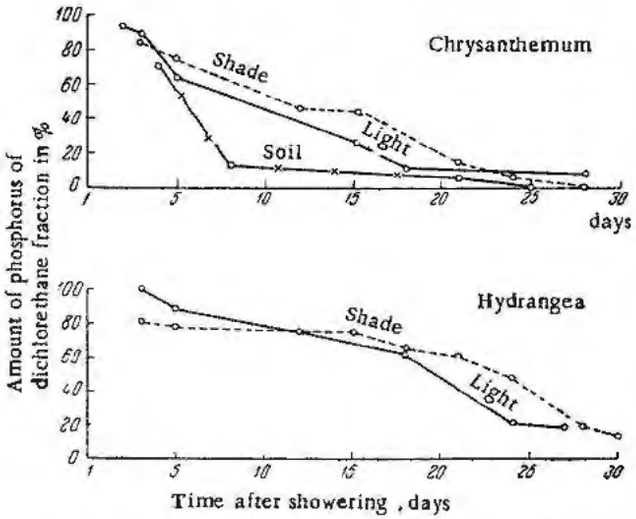


Figure 3. Phosphorus content of the dichlorethane fraction upon extraction of the leaves of plants sprayed with P^{32}-tagged diethyl-4-nitrophenylthiophosphate

chrysanthemums. From the plants in the open ground diethyl-4-nitrophenylthiophosphate was lost more rapidly than from those in the conservatory. An analysis of leaves from various nodes of the plants did not show any substantial difference in the distribution of phosphorus.

In summarizing we can state that the character of penetration and decomposition of diethyl-4-nitrophenylthiophosphate in plants was very similar in different experiments. The amount of the preparation in the plants sprayed with a 0.05% emulsion of diethyl-4-nitrophenylthiophosphate did not, in the main, exceed 80–100 mg/kg; of this, even during the first days of the experiment, a considerable part of phosphorus was extracted with water. Decomposition of diethyl-4-nitrophenylthiophosphate was, in the main, completed within 30 days after spraying.

It should be noted that eradication of aphids living on sprayed plants was incomplete, becoming most pronounced on the 4–5th day after spraying, when the thiophos content in the plants amounted to about 50 mg/kg. As a rule, diethyl-4-nitrophenylthiophosphate lowered the vitality of the plants.

The above evidence seems to show conclusively that the so-called "systemic" action of diethyl-4-nitrophenylthiophosphate (penetrating into the plants through the roots) upon aphids living on the plants is weak, within the range of doses tolerable for the plants (up to 100 mg per kg of fresh weight). Obviously, it cannot be of any practical importance.

PENETRATION OF DIETHYL-4-NITROPHENYLTHIOPHOSPHATE INTO PLANTS VIA THE LEAVES

Similar experiments were carried out to elucidate the penetration of diethyl-4-nitrophenylthiophosphate into the leaves of plants treated with emulsions and dusts of the preparations.

When haricot was treated with 1–3% dusts of diethyl-4-nitrophenylthiophosphate and ethyl-4-4'-dinitrodiphenylthiophosphate labeled with P^{32}, the expenditure rate being 40 kg/ hectare in conservatory conditions in cold and cloudy autumn days, the penetration of diethyl-4-nitrophenylthiophosphate amounted to 13 mg/kg fresh weight, 60% of the

Table VI. Penetration and Distribution of Phosphorus (in Terms of Diethyl-4-nitrophenylthiophosphate) into Plants Sprayed with Emulsion of Diethyl-4-nitrophenylthiophosphate

Time after spraying, days	Plants in conservatory: Shade: Total amount of preparation mg/kg	Plants in conservatory: Shade: Amount of preparation dissolved in dichlorethane, mg/kg	Plants in conservatory: Light: Total amount of preparation mg/kg	Plants in conservatory: Light: Amount of preparation dissolved in dichlorethane, mg/kg	Plants in soil: Total amount of preparation mg/kg	Plants in soil: Amount of preparation dissolved in dichlorethane, mg/kg
			Chrysanthemum			
2	—	—	83	77	45	—
3	43	36	62	56	—	—
4	—	—	—	—	59	42
5	47	35	114	73	—	—
8	—	—	—	—	46	6
12	—	—	—	—	—	—
15	37	16	34	9	—	—
18	—	—	88	10	—	—
21	28	4	—	—	70	5
24	32	2	—	—	—	—
28	25	0	57	4	—	—
30	—	—	52	0	—	—
			Hydrangea			
3	22	18	18	18	—	—
5	66	52	94	83	—	—
15	59	44	—	—	—	—
18	69	44	33	20	—	—
21	48	29	20	5	—	—
24	23	—	30	7	—	—
28	25	5	24	5	—	—
39	37	5	—	—	—	—

preparation remaining intact. The ethyl-4-4′-dinitrodiphenylthiophosphate penetrated in lesser quantities, but under these conditions it underwent practically no hydrolysis.

Quite different results were obtained when treating plants exposed to direct sunlight. In this case it appeared that the haricot leaves sprinkled with 1% dust of diethyl-4-nitrophenylthiophosphate at a rate of 40 kg/hectare contained on the second day up to 8.8 mg of phosphorus per kg of leaves, and on the 9th day up to 13 mg (estimated in diethyl-4-nitrophenylthiophosphate units). It should be mentioned that the preparation wholly dissolved in water and did not dissolve in dichlorethane.

In wheat leaves treated with similar amounts of 1% dust of diethyl-4-nitrophenylthiophosphate, up to 27 mg of preparation per kg of leaves were found on the second day, but on subsequent days no preparation could be detected. In a parallel experiment the plants were treated with 3% dust of ethyl-4-4′-dinitrodiphenylthiophosphate at the same rate: on the second day there were found in haricot leaves 0.6 mg of this compound and on the ninth day up to 3 mg per kg of leaves. The preparation dissolved in dichlorethane and did not dissolve in water. In wheat leaves, 1.3 mg/kg of this compound was found on the second day; and on the 17th day, 15 mg/kg. On the second day 100% dissolved in dichlorethane, and on the seventeenth day only 22%.

These data point to the conclusion that in sprayed plants protected from direct sun rays, the solubility of diethyl-4-nitrophenylthiophosphate and ethyl-4-4′-dinitrodiphenylthiophosphate penetrating into the leaves in dichlorethane is preserved for a rather long period of time (up to 15–20 days). If the plants are illuminated by direct sun rays, diethyl-4-nitrophenylthiophosphate can penetrate into the leaves in somewhat greater amounts, but it rapidly loses its solubility in dichlorethane. This suggests that it is decomposed. Ethyl-4-4′-dinitrodiphenylthiophosphate is, apparently, preserved in the leaves for a longer period of time.

Diethyl-4-nitrophenylthiophosphate penetrates into the plant in amounts considerably less than those necessary for the manifestation of the insecticidal effect on the aphids through plants.

At the Institute of Plant Protection of the Academy of Sciences of the Georgian SSR the penetration and decomposition of diethyl-4-nitrophenylthiophosphate labeled with phosphorus-32 and sulphur-35 was studied on lemon trees and vines. Spraying the soil under the plants was used as well as either dipping the leaves into or sprinkling them by an emulsion of the preparation. The penetration was followed up both by estimating the content of the preparation in samples cut out of the leaves and by radioautography.

The results obtained show that in grape-vine saplings sprayed with a 0.3% emulsion the preparation keeps penetrating into the leaves for not less than 7 days and its concentration in the leaves gradually increases. However this amount is not great: in the above experiment it constituted not more than 0.2 mg/cm² of leaf surface (disregarding hydrolysis). When the plants were dipped into a 0.5% emulsion the maximum quantity of preparation in the leaves was found to be 0.07 mg/cm² of the leaf surface. This amount remained constant for 7 days. No transfer of the preparation from the sprayed to non-sprayed parts was found when individual parts of the plant were treated (Fig. 4).

Special experiments were undertaken to determine the rate of diethyl-4-nitrophenylthiophosphate hydrolysis in the leaves of the plants. In these experiments, the soil under one-year-old grape-vines was sprayed with a 0.5% emulsion of the preparation. After 24 hours the plants were taken from the soil, thoroughly rinsed with running water and placed into uncultivated soil. The leaves from the upper part of the plants were periodically cut and tested for total P^{32} and phosphorus unextractable with water. The percentage of phosphorus left in the leaves rinsed with water, related to the initial quantity of phosphorus prior to washing, was used as an index of the residue of the non-decomposed product.

It will be seen from the data of Table VII that although the penetration of the preparation through the roots was cut off, its content in the leaves continued growing up to the 7th day. This points to its translocation from the roots and stem. The hydrolysis of diethyl-4-nitrophenylthiophosphate in plants began from the first days on and by the 10th day about 90% of the preparation was hydrolyzed.

In the next experiment the rate of decomposition of diethyl-4-nitrophenylthiophosphate in grape-leaves was tested under various conditions of illumination.

Figure 4. Radioautograph of a leaf-blade of grape: left side immersed in an emulsion of diethyl-4-nitrophenylthiophosphate; right side not immersed

Table VII. Penetration and Decomposition of Diethyl-4-nitrophenylthiophosphate in Grape Leaves

Duration of experiment, days	Content of diethyl-4-nithophenyl-thiophosphate in mg/cm² of leaf surface	Percentage of decomposition of diethyl-4-nitrophenylthiophosphate
2	0.014	—
3	0.019	48
4	0.027	65
5	0.031	77
6	0.035	81
7	0.037	83
8	0.028	85
9	0.021	87
10	0.016	91

For this purpose, young grape-vines were sprayed with a 0.3% emulsion of diethyl-4-nitrophenylthiophosphate. Some of the plants were placed in darkness, some in diffused light and some under a quartz lamp at a distance of 60 cm from the source. As shown by the above data, hydrolysis of diethyl-4-nitrophenylthiophosphate in the leaves exposed to quartz lamp illumination proceeds considerably faster than in diffused light, and nnder the conditions of diffused light faster than in darkness.

Thus, the data obtained at the Institute of Plant Protection of the Academy of Sciences of the Georgian SSR on diethyl-4-nitrophenylthiophosphate penetration into the leaves of the vine in general confirm those obtained in this Institute with different plant species and show that diethyl-4-nitrophenylthiophosphate, although penetrating into the plants, does not remain there for a long time due to its being decomposed and transformed into non-toxic compounds.

PENETRATION OF DIMETHYL-4-NITROPHENYLTHIOPHOSPHATE INTO PLANTS

These studies undertaken in the Scientific Research Institute on Fertilizers and Insectofungicides pursued the task of not only evaluating the penetration of dimethyl-4-nitrophenylthiophosphate into the leaves, but of finding out how much the penetration of the preparation used in the form of a 2% dust differs from that of a water emulsion containing 0.025% of the preparation.

In the experiments conservatory hydrangeas and chrysanthemums were used. Some of the plants were placed in the sunny side of the greenhouse conservatory and others in the shady side, being additionally protected by sheets of paper so that no sunlight could reach the leaves. Some of the leaves from the experimental plants were removed at intervals, sprayed with water and tested by the usual method for the P^{32} content and for its solubility in dichlorethane. The results of the test are shown in Figs. 5 and 6.

Graphs *B*, *E* and *G* show the presence of dimethyl-4-nitrophenylthiophosphate residues (fraction soluble in dichlorethane) in the leaves in mg/kg of fresh leaves of dusted and sprayed plants. Graphs *A*, *C*, *D*,

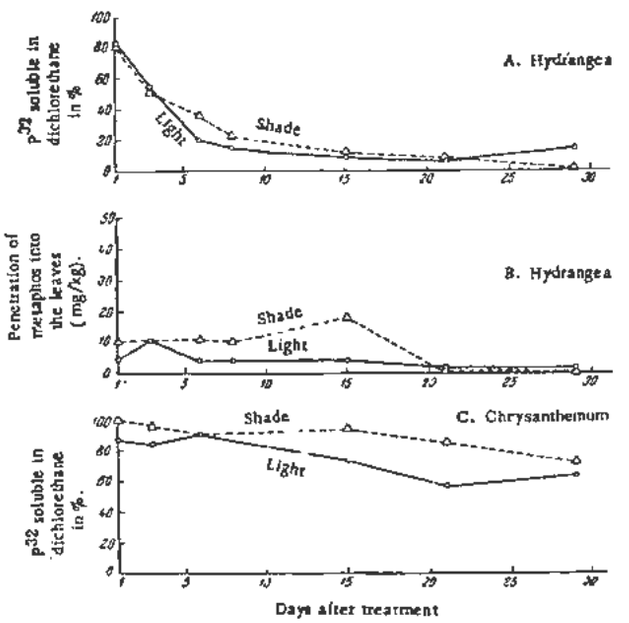


Figure 5. Penetration and decomposition rate of dimethyl-4-nitrophenylthiophosphate in hydrangea and chrysanthemum leaves dusted with 2% metaphos. Percentage of nondecomposed preparation in hydrangea leaves, A; in chrysanthemum leaves, C. Absolute amounts of nondecomposed preparation in the hydrangea leaves, B

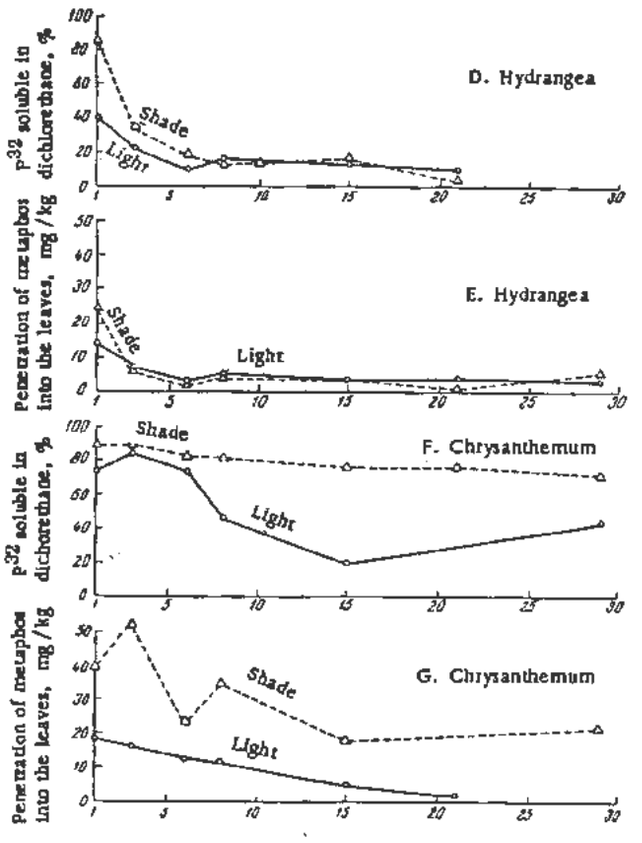


Figure 6. Penetration and decomposition rate of dimethyl-4-nitrophenylthiophosphate in hydrangea and chrysanthemum leaves sprayed with 0.1% metaphos emulsion. Percentage of nondecomposed preparation in hydrangea leaves, D; in chrysanthemum leaves, F. Absolute amounts of nondecomposed preparation in hydrangea leaves, E; in chrysanthemum leaves, G

and *F* show the degree and rate of decomposition of dimethyl-4-nitrophenylthiophosphate penetrated into the leaves of the hydrangeas and chrysanthemums treated in various ways. From the data of graphs *A*, *C*, *D* and *F* one can see that the preparation, independently of the plant species, is being decomposed at a somewhat higher rate upon spraying than upon dusting the plants. Light likewise accelerates the decomposition of dimethyl-4-nitrophenylthiophosphate both after spraying and after dusting.

However, the difference in the decomposition rate of dimethyl-4-nitrophenylthiophosphate becomes most pronounced when a comparison is made of the corresponding data from different plant species — chrysanthemum and hydrangea. With both spraying (Graphs *D* and *F*) and dusting (Graphs *A* and *C*) decomposition of the preparation proceeds considerably faster in hydrangea than in chrysanthemum. With spraying, dichlorethane-soluble P^{32} constituted 15% in the leaves of the hydrangea in the shade on the fifteenth day, while in the leaves of chrysanthemum, under similar conditions, about 80% of P^{32} passed into dichlorethane. After dusting, the percentage of P^{32} in hydrangea constitued, on the fifteenth day, 6% in dichlorethane, and in chrysanthemum 94%.

The fact that dimethyl-4-nitrophenylthiophosphate is decomposed at a different rate in different plant families is in itself very interesting. This phenomenon is obviously due to the difference in the enzyme activity of the plants and primarily of phosphatases which hydrolyze phosphoric acid esters. Certainly the influence of the micro-anatomic structure of the leaves cannot be disregarded. At any rate, when studying the velocity of loss of toxic residues of phosphorus-organic insecticides in the leaves of the plants a differential approach is necessary. A mechanical transfer of the results obtained in a study of one plant species to others, cannot, obviously, be justified.

A less intense decomposition of the preparation by the plant favours the accumulation in the leaves of greater amounts of non-decomposed dimethyl-4-nitrophenylthiophosphate or of its metabolites soluble in dichlorethane. In complete agreement with the data characterizing decomposition of the preparation both in case of dusting and spraying, very large absolute amounts of dimethyl-4-nitrophenylthiophosphate were found in chrysanthemums. The table and diagrams show that these quantities are several times greater than those of dimethyl-4-nitrophenylthiophosphate in the leaves of hydrangea where hydrolysis proceeds considerably faster. It should be noted that the difference in the rate of hydrolysis and in the absolute content of the preparation in the leaves of chrysanthemum in the shade and in light is more pronounced than in the leaves of hydrangea. Decomposition of the preparation in the leaves of chrysanthemum in the light proceeds much faster than in the shade, and, therefore, in the light the absolute amounts of dimethyl-4-nitrophenylthiophosphate soluble in dichlorethane are considerably less than in the shade. In hydrangea this difference is only slightly expressed.

Table VIII. Hydrolysis of Diethyl-4-nitrophenylthiophosphate in Grape Leaves

Exposure conditions	*Amount of non-decomposed diethyl-4-nitrophenylthiophosphate, %*		
	in 3 days	*in 6 days*	*in 9 days*
Vines in darkness	86	69.5	70.5
Vines in diffused light	81.5	61.5	44
Vines under quartz lamp	74.1	52	39

The figures concerning the penetration of phosphorus (as estimated in dimethyl-4-nitrophenylthiophosphate units) into the leaves of dusted chrysanthemums are not included in the diagram, for the amount of phosphorus penetrating into the leaves varies considerably due to the fact that they are usually unevenly covered with dust. However, the quantity of insecticide penetrating into the leaves of the plant placed in shade reached 60 mg/kg.

The above data show that dimethyl-4-nitrophenylthiophosphate differs considerably from diethyl-4-nitrophenylthiophosphate in its capacity to penetrate into the leaves. This becomes especially distinct when shaded plants are treated. The maximum amounts of diethyl-4-nitrophenylthiophosphate penetrating into the leaves of dusted chrysanthemums in shade constituted only 10 mg/kg while up to 25 mg/kg dimethyl-4-nitrophenylthiophosphate penetrate even into the plants located in sunlight.

Special experiments on seventeen plant species using the P^{32} tagged preparation were undertaken to examine the character of hydrolysis of dimehtyl-4-nitrophenylthiophosphate in various plants. For the administration of greater quantities of the preparation, the method of vacuum infiltration was used. The plants were placed in a vacuum desiccator containing an emulsion of labeled dimethyl-4-nitrophenylthiophosphate. When the air was slowly let in, the pressure in the desiccator increased and the leaves filled up with the emulsion. After that, the leaves were rinsed and placed into small glasses filled with water. The leaves were periodically analyzed. The degree of decomposition of the preparation was determined by extracting the leaves with equal volumes of water and dichlorethane.

It will be seen from Table IX that within 5 days 90–95% decomposition of metaphos occurred in the following plant species: cherry, currant, tomato, grape, hydrangea, cotton, and wheat. In eight plant species (cabbage, maple, apple, beet, haricot, raspberry, cucumber, tea) disintegration reached 80% in only two days. Only in two plant species, lemon and trifoliate, disintegration, even on the seventh day, did not exceed 31%. These data show that in spite of substantial specific differences decomposition of metaphos proceeds in plants at a very high rate.

The penetration of metaphos into apple leaves was also followed up under natural conditions. Examination was made of leaves of different ages. The trees were sprayed with a 0.15% emulsion of a 25% pre-

Table IX. Rate of Metaphos Decomposition in the Leaves (Percentage of P^{32} in Water Fraction)

Plants	Days after infiltration						
	1	2	3	4	5	6	7
Cabbage	59.0	88.7	91.8	—	95.0	—	97.2
Maple	70.0	94.5	95.0	—	93.0	—	97.4
Apple (young leaves)	56.3	81.4	94.5	—	96.5	—	96.6
Beet	69.0	95.0	96.3	—	96.8	—	98.2
Lemon	20.6	38.0	44.0	—	35.0	—	31.0
Haricot	39.4	74.8	94.7	—	96.0	—	95.0
Cherry	14.1	44.0	73.0	—	90.0	—	92.0
Raspberry	49.5	80.0	88.0	—	92.0	—	92.3
Currant	12.5	30.0	51.0	—	70.0	—	83.0
Tomato	24.8	77.0	90.0	—	93.0	—	88.0
Cucumber	22.4	81.0	86.0	—	96.0	—	99.0
Trifoliate	13.7	13.7	21.8	—	13.0	—	27.0
Cotton	25.6	51.0	71.0	—	63.0	—	78.0
Wheat	12.8	48.0	63.5	—	89.0	—	97.0
Grape	24.0	60.0	75.0	—	99.0	—	94.6
Tea	71.0	93.0	98.0	—	99.0	—	98.0
Hydrangea	51.0	76.8	88.7	—	93.0	—	94.2
Chrysanthemum	9.4	10.3	23.8	—	26.0		perished
Chrysanthemum*	29.0	40.0	—	59.0	—	84.0	77.0

* Infiltrated in mild conditions.

Table X. Decomposition of S^{35}-labeled Metaphos in Apple Leaves Sprayed with a 0.15% Aqueous Emulsion (Percentage of S^{35} in Aqueous Fraction)

Age of leaves	Treatment of leaves	Time of taking samples, days				
		0	1	2	3	6
Young	Washed	3.8	40.6	60.0	58.0	100.0
	Unwashed	7.7	44.0	40.0	40.0	77.0
Medium	Washed	4.6	24.0	49.0	60.0	85.0
	Unwashed	5.6	39.0	26.0	40.0	77.0
Old	Washed	3.7	28.0	35.0	58.0	75.0
	Unwashed	9.6	38.0	34.0	29.0	43.0

paration. Each leaf was cut into two parts along the vein, one part being analyzed without rinsing, and the other after thorough spraying with water. The results of the analysis are presented in Table X. It follows from the data that decomposition of the preparation in leaves after administration by means of vacuum infiltration and by treating the leaves on trees under natural conditions proceeds approximately in the same way. However, it is faster in younger leaves and in the inside of the leaves than on the surface. This clearly indicates the enzymatic nature of metaphos hydrolysis.

A study was also made of hydrolysis of P^{32} labeled dimethyl-4-nitrophenylthiophosphate in soil and in sunflowers while the preparation penetrated into the plants via the roots. When the sunflowers reached the phase of two true leaves they were sprayed with a 0.1% emulsion of 25% concentrate metaphos at a rate of 50 ml per bowl of 300–400 cm^3 capacity. At various intervals after spraying, one plant was taken for analysis and simultaneously about 1 gm of soil from the middle of the bowl in which the plant grew. The plants were washed and then the leaves, stem and roots dissected separately and analyzed. In the leaves the content of dimethyl-4-nitrophenylthiophosphate per 1 kg fresh weight and the distribution of P between water and dichlorethane were determined. In the stem, roots and soil, determination was made only of the degree of dimethyl-4-nitrophenylthiophosphate decomposition without estimating its quantity.

Table XI shows that P^{32} of dimethyl-4-nitrophenylthiophosphate used for spraying the soil penetrates into the plants rather actively but is not accumulated there because of very rapid disintegration of the preparation. In all samples the maximum decomposition was found in the leaves, a lesser one in the stem, and the least in the soil.

This pronounced tendency of the decomposition degree to increase in the direction of soil-root, stem-leaves can obviously be explained by the enzymatic character of dimethyl-4-nitrophenylthiophosphate hydrolysis which begins in the soil, probably under the influence of soil microflora, then proceeds intensely in the root system and at the highest rate in the stem and leaves. Hydrolysis in the leaves is probably accelerated by photochemical processes. However, one should bear in mind that the percentage ratio of

Table XI. Penetration and Decomposition of Dimethyl-4-nitrophenylthiophosphate in Sunflower Tissues and in the Soil Sprayed with 0.1% Metaphos Emulsion

Time of taking sample after spraying, days	Leaves: Amount of phosphorus evaluated in metaphos units, mg/kg	Leaves: Fraction soluble in dichlorethane, %	Leaves: Fraction soluble in dichlorethane, mg/kg	Fraction soluble in dichlorethane, %: Stem	Root	Soil
1	3.8	51.4	1.95	89.3	—	95.3
4	147.0	8.4	12.3	18.3	28.0	47.0
6	177.0	5.6	9.9	—	29.7	20.7
8	288.0	1.6	4.6	12.6	18.0	25.0
15	—	1.2	—	2.6	10.9	—

the dichlorethane soluble fraction in plants is undoubtedly influenced by the difference in the rate of absorption by the roots of dimethyl-4-nitrophenylthiophosphate and of its disintegration products. It is certain that the more mobile molecules of phosphoric acid, which is one of the decomposition products of dimethyl-4-nitrophenylthiophosphate, should be absorbed at a higher rate than the non-decomposed preparation. This can greatly influence the increase in the relative content of the water soluble fraction of the roots of the aerial parts of the plant. The application of the above vacuum-infiltration method excludes such a mistake for the preparation is introduced once so that no additional supply of the preparation and of its decomposition products is possible from without in the course of the experiment.

The rapid disintegration of dimethyl-4-nitrophenylthiophosphate both in the soil and plant tissues apparently precludes the possibility of its being used as a systemic insecticide.

The next question, of theoretical as well as of practical importance, is that of the fate of the preparation penetrating into the plant, of the mechanism of its detoxication or, on the contrary, activation. The ever growing use of phosphorus-organic insecticides, particularly of the so-called systemic insecticides which penetrate into and are translocated within the plant, make it urgent that a most thorough study of the behaviour of such compounds in the plant be made.

For warm-blooded animals there is a direct relationship between anticholinesterase activity of phosphorus-organic compounds and their toxicity for these animals. Estimations of the inhibition of cholinesterase of animals and of certain insect species show that outside the body there remains a complete correlation with the toxicity of these compounds. It was noted, however, that for certain compounds this correlation does not hold, viz. small doses of the preparation when administered to animals cause a considerable inhibition of cholinesterase and simultaneous poisoning of the animals, but to obtain the same degree of cholinesterase inhibition outside the body much greater doses of the preparation are required. This is the case in particular with pure octamethyl preparations.

Gardiner and Kilby[8] showed with the aid of labeled octamethyl that inhibition of the cholinesterase activity inside the body requires a 790–60,000 times lesser quantity of the preparation than is necessary to achieve the same degree of cholinesterase inhibition in the experiments outside the body. This led to the conclusion that within the animal octamethyl is transformed into some new many times more active compounds. This activation was found to take place in liver.[9,10] Aldridge and Barnes[11] found in a similar test of some more compounds that in the body of animals thiophos is activated approximately 10 times and metaphos 12 times. According to our data, in the rabbit a 24–25-fold increase in metaphos activity is accompanied by a 50% inhibition of cholinesterase activity, and a 73–260-fold increase corresponds to an 80% inhibition of same.

What happens then to phosphorus-organic insecticides in plants? Does an activation of phosphorus-organic insecticides occur and if so in what form?

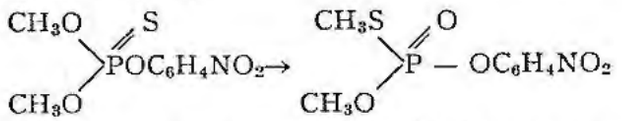
As already mentioned much work in this direction was done by a number of foreign students who showed, for example, that one of the very interesting systemic phosphorus-organic poisons, octamethyltetramidpyrophosphoric acid (octamethyl) undergoes an oxidative breakdown in plants and is transformed into a physiologically more active form of oxide.[12] As to the derivatives of thiophosphoric acid, there is no distinct evidence indicating their activation within the plants. However, there are many data on the activation of these compounds within the animal body. As already mentioned, according to one of the current theories, activation of thiophosphoric compounds is due to their oxidation to oxygen analogues which, in a general way, may be represented as follows:

$$\begin{matrix} R_1 \\ \\ R_2 \end{matrix}\!\!>\!\overset{\displaystyle S}{\overset{\|}{P}} - X + \frac{2O_2}{HOH} \rightarrow \begin{matrix} R_1 \\ \\ R_2 \end{matrix}\!\!>\!\overset{\displaystyle O}{\overset{\|}{P}} - X + H_2SO_4$$

The oxygen analogues are much more toxic for warm-blooded animals than thio-derivatives and more dangerous.[13, 14, 15]

According to another theory, activation of thiophosphoric compounds is accomplished through isomerization.[16,17] The S-alkyl-isomers formed as a result of this reaction are likewise physiologically more active than thiophosphates. For dimethyl-4-

nitrophenylthiophosphate this can be represented in the following way:



An attempt has been made in this laboratory to check the possibility of oxidative activation of dimethyl-4-nitrophenylthiophosphate in plant tissues. For this purpose recourse was taken to the separation of the dimethyl-4-nitrophenylthiophosphate decomposition products by paper chromatography.

An inspection of the hydrolysis scheme of dimethyl-4-nitrophenylthiophosphate which is analogous to that of diethyl-4-nitrophenylthiophosphate will show that almost all the individual intermediate breakdown products of dimethyl-4-nitrophenylthiophosphate, except the last phase (the ultimate breakdown product), contain in their molecule simultaneously one phosphorus and one sulphur atom. Should oxidation of dimethyl-4-nitrophenylthiophosphate to dimethyl-4-nitrophenylphosphate take place, there would be among the hydrolysis products some compounds containing only phosphorus but no sulphur, and vice versa. It seemed worthwhile therefore to separate these products in order to ascertain whether or not oxidation took place. Preparations of dimethyl-4-nitrophenylthiophosphate labeled with P^{32} and S^{35} were used.

Preliminary experiments were carried out to ascertain how the alkaline hydrolysis products of metaphos can be separated. To carry out chromatographic partition, a glass device was constructed securing the possibility of obtaining descending chromatograms.

The chromatograms were analyzed either by cutting them into 1-cm strips and estimating their radioactivity directly under the counter or by obtaining radioautographs by exposing the chromatograms with roentgen films.

For chromatographing purposes the common Whatman drawing paper, washed first with hydrochloric acid, water, alcohol and then again with water, was used. In the first experiments the chromatographic solvent had the following composition: isoamyl alcohol, 100 ml; formic acid, 14.6 ml; and water, 5.6 ml.

ALKALINE HYDROLYSIS OF DIMETHYL-4-NITROPHENYLTHIOPHOSPHATE

Preliminary chromatograms of the dimethyl-4-nitrophenylthiophosphate emulsions labeled with P^{32} have been obtained. The first emulsion was a fresh one, the other one was preliminarily subjected to alkaline hydrolysis and mostly contained water soluble products of metaphos hydrolysis. The chromatograms of these two samples (in the form of diagrams) and of an arbitrary mixture of the two are presented in Fig. 7, a, b, c.

The non-decomposed emulsion gives one principal peak with $R_f = 0.85$ which corresponds to dimethyl-4-nitrophenylthiophosphate; the second peak with $R_f = 0.045$ probably corresponds to the ultimate decomposition product of dimethyl-4-nitrophenylthiophosphate, i.e., to phosphoric or thiophosphoric acid; and the third maximum somewhat obliterated with $R_f = 0.45$ corresponds to an unknown decomposition product. The hydrolysis emulsion gives the highest peak with $R_f = 0.05$, which undoubtedly correspond to phosphoric or thiophosphoric acid, that is, to the ultimate alkaline hydrolysis product of metaphos; the two other small peaks ($R_f = 0.9$ and $R_f = 0.47$) coincide with those which were obtained with the nonhydrolyzed emulsion. The chromatogram of the mixture repeats these results.

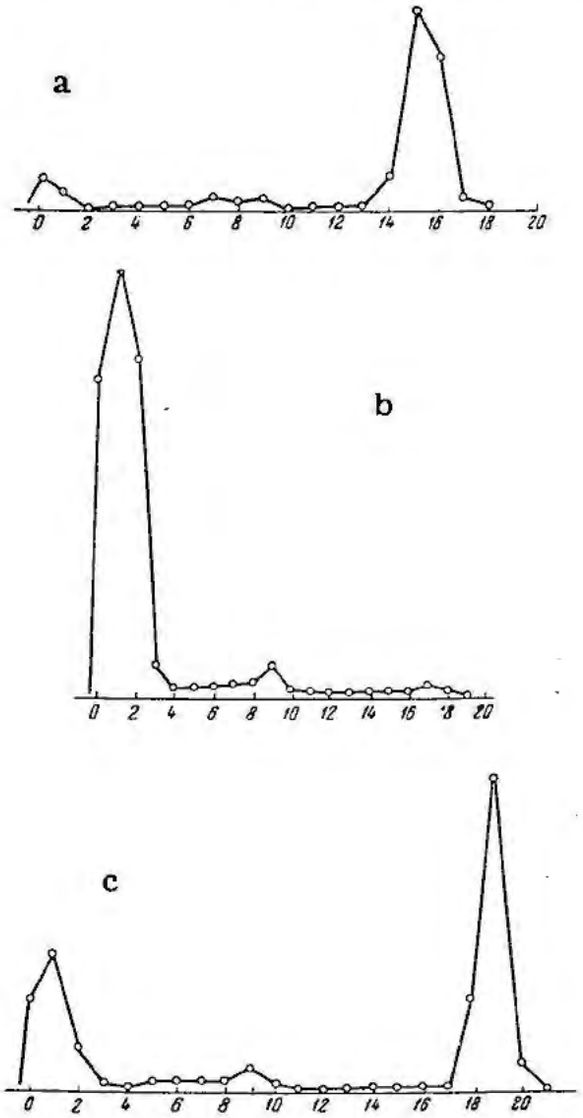


Figure 7. Graphic representation of distribution of the peaks of phosphorus-containing compounds on paper chromatograms of metaphos emulsions with different degrees of hydrolysis. Horizontal axis, distance from the application point of the drop, cm; vertical axis, radioactivity: (a) slightly decomposed metaphos emulsion; (b) decomposed emulsion; and (c) mixture of decomposed and nondecomposed metaphos emulsion

Thus, alkaline hydrolysis of dimethyl-4-nitrophenylthiophosphate yields, at least, three products

one of which ($R_f = 0.9$) is a non-decomposed compound, the second ($R_f = 0.05$) is phosphoric or thiophosphoric acid, and the third ($R_f = 0.45$) is a small amount of an unknown product. As far as its position on the chromatogram is concerned, this product is considerably more soluble in water than dimethyl-4-nitrophenylthiophosphate.

A STUDY OF HYDROLYSIS OF DIMETHYL-4-NITROPHENYLTHIOPHOSPHATE IN HYDRANGEA LEAVES

After the preliminary chromatographic analysis of the alkaline hydrolysis products of dimethyl-4-nitrophenylthiophosphate, its breakdown in the leaves of plants was followed up. As already mentioned, in the leaves of plants there occurs an extremely rapid decomposition of dimethyl-4-nitrophenylthiophosphate leading to the formation of water-soluble sulphur and phosphorus containing products. Yet, the decomposition of dimethyl-4-nitrophenylthiophosphate in plants is of a distinctly enzymatic character as here the conditions are milder than in alkaline hydrolysis. It seemed probable therefore that in plants the hydrolysis products may be somewhat different, at least, in the quantitative respect. It was to elucidate these questions that special experiments were undertaken.

Hydrangea leaves were infiltrated with a 0.05% P^{32} labeled metaphos emulsion. Periodically two leaves were taken for analysis, extraction with water and dichlorethane. In addition, the juice of the leaves and their alcohol extracts were also analyzed. Analysis of the chromatograms was done by the radiometric method.

Analysis of chromatograms shows that with the plant juice three maxima are obtained with R_f coinciding with those for hydrolyzed emulsion. This testified to the identity of these products. As to alcohol extracts (Fig. 8), the number and position of the peaks do not change, but the ratio between the radioactive products in the first two peaks is greatly altered. While in the case of juice the first peak is higher than the second one, in the case of alcohol extracts this ratio changes. Moreover, the ratio of the sum of counts of the first two peaks to the third one remains constant. This can presumably point only to the fact that, in case of juice chromatograms, part of the product related to the second maximum remains combined and cannot be washed out from the application point, probably due to the presence in the juice of a considerable quantity of colloidal proteins which, are denatured by alcohol treatment and are no longer an obstacle to the more complete separation of the mixture.

The estimation of the content of individual peaks and the graphic representation of the course of metaphos decomposition (in per cent, Fig. 9) are therefore based on the alcohol extract chromatograms. It will be seen that the content of non-decomposed metaphos in hydrangea leaves continuously decreases, reaching by the third day nil per cent, while the phosphoric (or thiophosphoric) acid of the product forming the second peak increases.

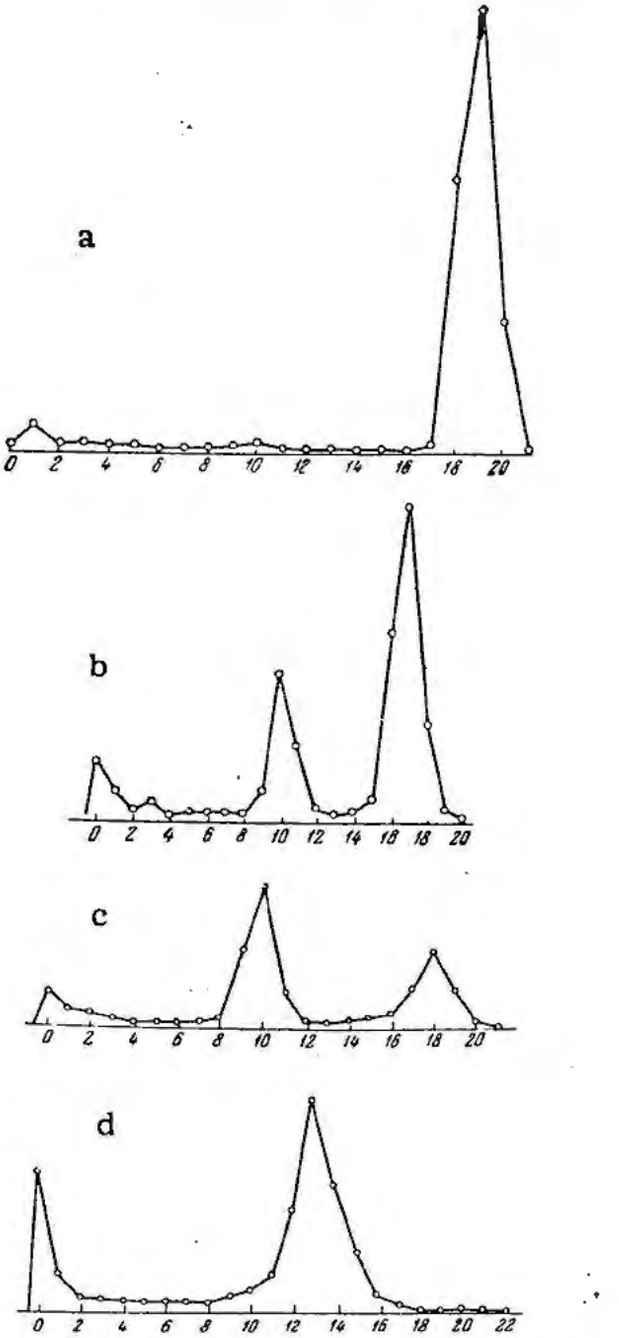


Figure 8. Graphic representation of distribution of the peaks of phosphorus-containing compounds on paper chromatographs of alcohol extracts from hydrangea leaves infiltrated with metaphos, at different times after infiltration. Alcohol extract from hydrangea leaf: (a) in 1 hour; (b) 24 hr; (c) 48 hr; (d) 72 hr. Horizontal axis, distance from the drop point, cm; vertical axis, radioactivity

In another experiment the process of decomposition was somewhat slower, and on the seventh day it amounted to 94%. It is worth noting that the ratio between the quantity of the second peak product and phosphoric acid remained practically the same, at least for 5–7 days, amounting in one experiment to 3 : 1, and in another to 2 : 1.

Further breakdown of the hydrolytic products could not be followed up owing to loss of radiophosphorus activity. It should be assumed that, later on,

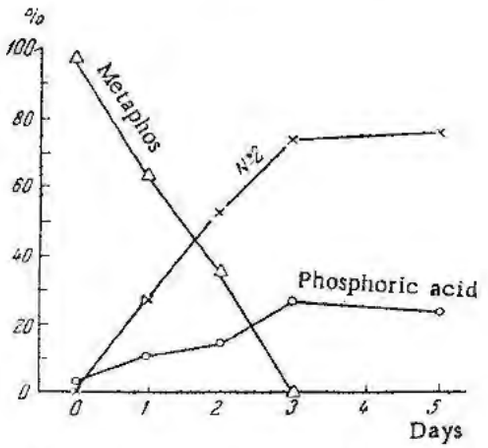


Figure 9. Course of metaphos decomposition in hydrangea leaves

the correlation between the activity of the products of the first and second peaks becomes different, for the second product disintegrates to phosphoric acid.

In the Institute of Plant Protection of the Academy of Sciences of Georgian SSR a study was made of hydrolysis of 0.3% emulsion of diethyl-4-nitrophenylthiophosphate in the juice of leaves of grape and lemon outside the plants with subsequent chromatographic partition of the hydrolytic products (in the solvent mixture, acetone, ethyl, alcohol, water). It will be noted that an accumulation of a phosphorus containing product was observed with $R_f = 0.97$; i.e. that of complete hydrolysis of diethyl-4-nitrophenylthiophosphate (Fig. 10).

It is a characteristic fact that hydrolysis of diethyl-4-nitrophenylthiophosphate in the juice of these plants (in experiments outside the plants) proceeded at the same rate as the hydrolysis of diethyl-4-nitrophenylphosphate under the same conditions in distilled water. There is no doubt therefore that the rate and character of diethyl-4-nitrophenylthiophosphate hydrolysis inside the plants are closely associated with enzymatic activity.

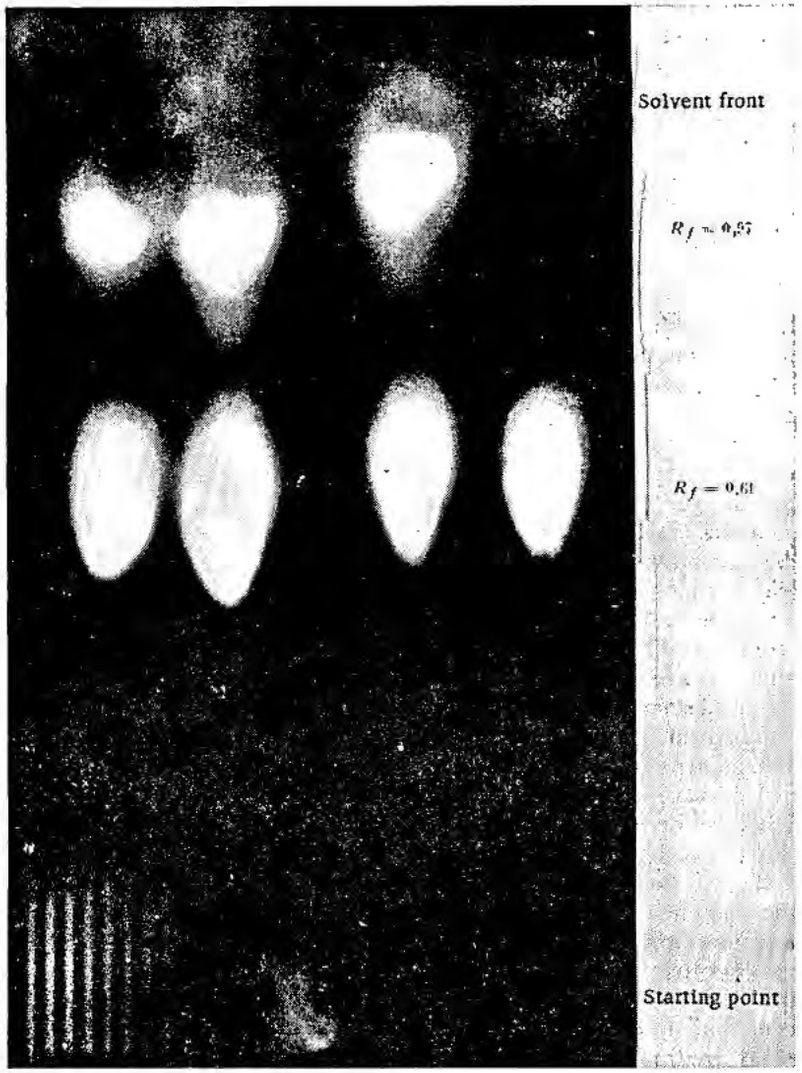


Figure 10. Radiochromatogram of partition of diethyl-4-nitrophenylthiophosphate and its hydrolysis products upon exposure of the preparation to plant juices and to water: (a) diethyl-4-nitrophenylthiophosphate; (b) same in water; (c) same in juice of lemon leaves; and (d) same in juice of grape leaves

THE POSSIBILITY OF OXIDATIVE DECOMPOSITION OF DIMETHYL-4-NITROPHENYLTHIOPHOSPHATE IN PLANTS

According to the theory already mentioned, thiophosphoric compounds can be activated by oxidation to the respective phosphates, that is to say, in the course of the oxidation process the sulphur atom leaves the thiophosphate molecule. We made an attempt to study this possibility with respect to plants, and for this purpose P^{32} and S^{35} labeled compounds were used. Some of the hydrangea leaves were infiltrated with P^{32} labeled dimethyl-4-nitrophenylthiophosphate and others with the same compound tagged with S^{35}. Three days after infiltration alcohol extracts were prepared from the leaves and then chromatographed radioautographs were obtained by exposing the chromatogram with roentgen films.

The chromatograms of the mixture of alcohol extracts containing both P^{32} and S^{35} did not differ from individual chromatograms of P^{32} and S^{35} extracts. Thus, at least with this chromatographic mixture, it proved impossible to note any difference in the breakdown products P^{32} and S^{35} tagged dimethyl-4-nitrophenylthiophosphate in plants. All the recorded maxima contain both phosphorus and sulphur and perhaps in the plant tissue there occurs no oxidation of dimethyl-4-nitrophenylthiophosphate to methyl-4-nitrophenylphosphate. It is possible that ultimately thiophosphoric acid disintegrates to phosphoric acid with sulphur being split off. Phosphoric acid formed as a result of decomposition of thiophosphoric acid, as well as hydrogen sulphide acid or its salts probably do not separate in this chromatographic solvent, since they are absolutely insoluble in it and remain at the point of application. At any rate there were found no intermediate decomposition products of dimethyl-4-nitrophenylthiophosphate which would contain phosphorus and be devoid of sulphur.

SUMMARY

1. The method of labeled atoms was used to study the preservation-time of residue of certain phosphorus-organic insecticides on treated surfaces as well as the penetration of these insecticides into plants treated in various ways.

2. To carry out these investigations synthesis was made of P^{32} or S^{35} tagged diethyl-4-nitrophenylthiophosphate; dimethyl-4-nitrophenylthiophosphate; S-1, 2-dicarboethoxiethyl-0, 0-dimethyldithiophosphate; S-1-2-dicarbomethoxiethyl-0, 0-diethyldithiophosphate; ethyl-4, 4-dinitrodiphenylthiophosphate and of some other compounds.

3. The estimation of the preservation-time of the residues of phosphorus-organic insecticides on treated surfaces with the use of labeled atoms is extremely simplified and accelerated, so that the results can be obtained within a few days.

4. A study of the preservation-time of residues of diethyl-4-nitrophenylthiophosphate, dimethyl-4-nitrophenylthiophosphate and of other compounds showed that they are preserved on treated surfaces for a very short time. Decomposition of residues of the phosphorus-organic insecticides is greatly favoured by the ultra-violet part of solar radiation.

5. Evidence was obtained concerning the penetration of diethyl-4-nitrophenylthiophosphate and dimethyl-4-nitrophenylthiophosphate into plant leaves upon spraying the soil under the plants with the respective emulsions and upon treating the leaves with dusts and emulsions of same compounds. The data obtained make it probable that both compounds do not possess properties of intraplant-systemic insecticides, for their hydrolysis inside the plants proceeds very rapidly and hence the concentration of the preparations within the plants is insufficient for poisoning insects.

6. By combining the methods of paper chromatography and of labeled compounds it became possible to show that hydrolysis of dimethyl-4-nitrophenylthiophosphate in plants results in the formation of the same products as in alkaline hydrolysis of dimethyl-4-nitrophenylthiophosphate. No evidence was obtained to show that in the leaves of plants there is oxidation of dimethyl-4-nitrophenylthiophosphate.

7. The study of the preservation time of the preparation residues on plant surfaces and of its penetration and preservation within the plants made it possible to use a number of these preparations for treatment of agricultural plants shortly before harvesting.

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Some Notes on the Pollination of Alfalfa (*Medicago Sativa*)

By Y. Demarly,* France

MATERIAL

In most plants of the genus *Medicago,* pollination is carried out mostly by Hymenoptera of such genera as *Bombus, Apis* and *Megachile.* It is very easy, particularly in cultivated alfalfa, to distinguish between flowers which have and have not been visited by insects. Before pollination, the filaments of the stamens are held inside the keel by two fleshy ridges at the base of the wings. When an insect, in search of nectar, inserts its proboscis toward the nectaries, it causes the components of the flower to be separated: the filament of the stamen, on being liberated, strikes hard against the standard after having swept the hairy ventral aspect of the insect, soiled with various pollens. Thus, on the pollinated flowers, the filaments of the stamens are in plain sight (Fig. 1). On the surface of the stigma, there are now found pollen grains produced by the stamens of the plant itself, which make up the autopollen, and pollen grains brought from other plants by the insect, which make up the allopollen.

METHODS

Various authors have studied the dispersion of pollen and the rate of self-fertilization in alfalfa by the use of genetic methods: Waldron[1] used purple-flowered plants, which were cross-bred with yellow-flowered plants. Burkart[2] used a similar method.

The work of Tysdal, Kiesselbach and Westover,[4] in Nebraska, also is based on the cross-breeding of *Medicago falcata,* bearing yellow flowers, and *Medicago sativa,* bearing purple flowers.

Even though these methods gave estimates which, on the whole, were correct, some inaccuracies still are to be feared.

1. The "flower-color" characteristic, which is frequently used, may be one of the effects of a pleiotropic gene; indeed, this generally is the case for the "white flower" characteristic, related to less vigor, a slower rate of growth, and less early flowering. Natural selection which occurs at the seedling stage, can thus very well eliminate, from the offspring, some of the white flowered individuals, and falsify the results.

2. The work carried out in Germany by Rudorf[3] showed that there was a phenotypic homogamy, in alfalfa, for which the pollinizing insects are responsible: the bumble bees which gather nectar in the flowers of a given color preferentially visit all plants bearing flowers of the same color. The results achieved by most authors, starting from studies bearing on cross-breeding between plants which are differentiated by the color of their flowers, thus probably allow for a cross-fertilization rate which is smaller than it actually is.

3. The use of a genetic character, whichever it be, is rather difficult in most autotetraploid plants; since alfalfa has a genome which is very close to that type, it takes a very long time before a pure line, homozygous for a given characteristic, is developed. When the "purple flower" characteristic is used, it is necessary to test, by self-fertilizing, whether the line is really fixed for this character.

UTILIZATION OF PHOSPHORUS-32

It is of considerable interest, therefore, to use some means of tagging the pollen physically, in order to study its distribution and, subsequently, its rate of natural self-fertilization.

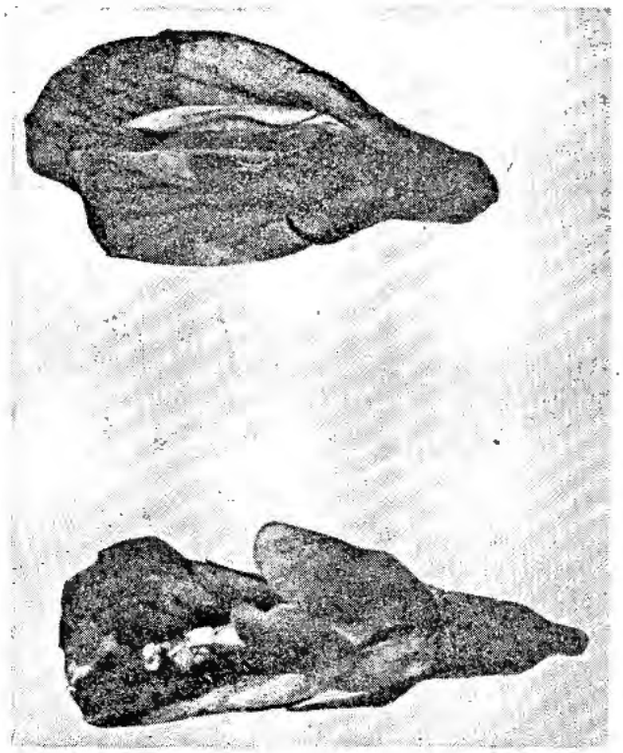

Figure 1. Alfalfa flower: above, not-pollinated; below, pollinated

Original language: French.

* Chargé de Recherches à l'Institut National de la Recherche Agronomique.

The small volume of the pollen grain could be considered a source of problems: this pollen grain, indeed, consists essentially of a nucleus which is rich in thymo- and ribonucleic acids. It would thus seem that phosphoric acid would be suitable for tagging purposes. The half-life of P^{32}, in fact, appeared to be suitable for such an application.

By means of tubes containing labeled phosphoric acid, which were attached to the main roots of the plant, it became possible to achieve a rapid absorption and homogeneous distribution of the material in the various stalks at the end of a few hours. Throughout the duration of the experiment, despite the use of doses going up to one millicurie, the plant showed no sign of weakening. *In vitro*, the pollen germinated normally.

From the very beginning of absorption, there was significant activity in the anthers of a plant so treated. However, the activity curve for pollen reached its maximum only nine days from the start of the experiment. This, as a matter of fact, may be readily explained: it is likely that the parent cells of the pollen grain show an increase in metabolism at the moment of meiosis; the exchanges of phosphorus with the cells of the nourishing layer then are very active. During the maturation period which follows, the pollen grains isolated by the extine, in the process of formation, show a considerable drop in their metabolic exchanges.

With a dose of one millicurie, a mass of ten grains of pollen can readily be detected nine days after the beginning of the treatment.

STATISTICAL TESTS USED

The work was carried out for two consecutive years, on approximately 12,000 flowers. Since the radioactivity of the pollen was rather weak, it was indispensable that the measurements be repeated as often as possible.

We compared the average of these values with the use of the "t" test, of Student-Fisher. This enabled us, allowing for the activity read on the counter in the controls, to determine the smallest significant difference at the probability threshold of 5 per cent.

RESULTS

The duration of receptivity of alfalfa stigmata varies with the climate: it is approximately 48 hours under average conditions. Thus, the experiment gives maximal accuracy through the 48 hours which follow the 9th day.

At that time, by gathering the pollinated flowers located on concentric circles of different radii around the marked plant, it is possible to find the marked pollen again on the stigmata of the various plants.

Two precautions were taken:

1. The analysis was carried out only on plants the flowers of which were essentially of the same color as those of the labeled plants, which rules out any phenotypic homogamy.

2. The diffusion of labeled phosphorus through the soil was avoided, or at least, controlled. In fact, the work of Barbier and Husson[5] demonstrated that corn roots excreted a small fraction of their own phosphorus into the ground.

Thus, it was to be expected, even though the principal roots had been put in sealed tubes, that there would be some measure of contamination through the soil. This was not the case, since we gathered and tested with a counter the non-pollinated flowers of plants located one meter from the tagged plant. These flowers were significantly inactive.

A statistical interpretation of the results achieved showed that the dissemination of pollen was limited to a small circle, which did not exceed nine to ten meters in diameter. In that zone, we were able to note the following distribution gradients. by setting up an index of 100 for all of the pollen disseminated from one plant:

Distance, m	*1*	*1–2*	*2–3*	*3–4*	*4–5*	*5–8*	*10*
Proportion of disseminated pollen	33	23	13	10	10	10	0

Thus, we are far away from a haphazard distribution of pollen, since 30 per cent of that produced by a given plant is deposited on the pistils of its immediate neighbors. As a matter of fact, this mode of distribution admits of a biological explanation: *Bombus terrestris*, which is the most active pollinating agent, has very short flights, from one plant to the next.

Furthermore, the flowers collected in a circle 10 meters in diameter, with the treated plant as the center, showed no significant activity (Fig. 2). In view of the proportion of the flowers examined, the probability that pollen transport to that distance

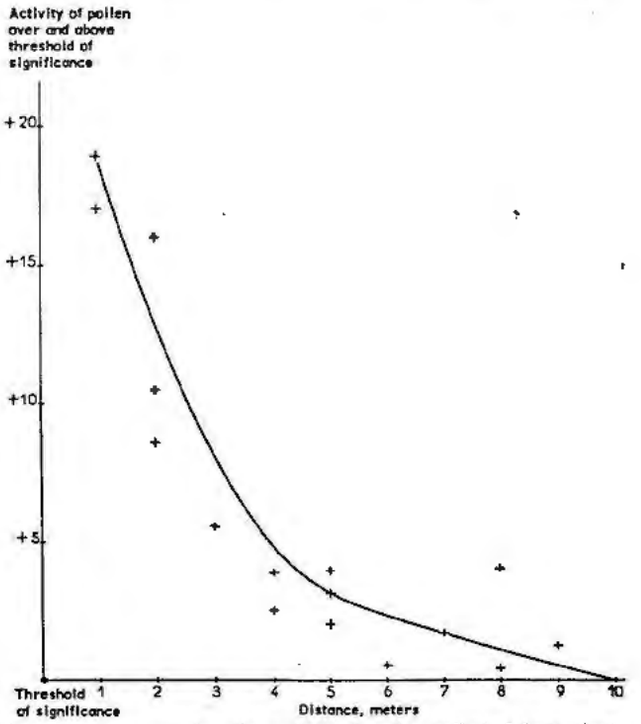

Figure 2. Curve showing the activity of pollen collected from plants pollinated at various distances from a tagged plant

would be missed by analysis is about 1/25,000. Thus, the precision of this test is entirely acceptable, and it is likely that it cannot be achieved by genetic analysis.

Another study had to do with the selection of the pollen tubes by the style.

It is easy, using a standard microscopic counting technique, to determine the number of pollen grains present on a stigma. The activity of this pollen mixture can be determined and, from this, the proportion of auto- and allopollen deposited on the stigma.

From these measurements, which entail some inaccuracies, because of difficulties in counting and collecting of the pollen grains from the stigmata, it can be estimated that the number of grains deposited on a stigma at the time of pollination reaches approximately 1500. Of these, approximately 1200 come from the plant itself. The proportion of allopollen on the stigma thus is about $\frac{1}{5}$.

This small amount of allopollen, compared with the high rate of cross-fertilization, implies a high measure of selectivity against pollen tubes derived from autopollen. A pollen tube derived from the pollen of a foreign plant is 40 times more likely to fertilize an ovule than a tube formed by autopollen.

This selection is expressed by a slowing down of the elongation of the pollen tube of the autopollen, conditioned no doubt by the critical influence of the concentration of growth substances in the female tissues.

The use of tagged phosphorus thus makes it possible to clarify some aspects of floral biology in all cases where genetic observations are difficult and lengthy.

Part of this work finds practical application in the determination of the isolating distances which are indispensable for the production of seeds of a given variety.

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Absorption of Radioactive Sulfur by the Fruit System in Comparison to the Roots of Peanuts

By Henry C. Harris,* USA

The peanut plant flowers above ground, and after fertilization the flower forms a gynophore (peg) which grows downward into the soil to a depth of about two inches. The gynophore grows by elongating back of the ovaries and by that means the ovaries are transferred from an aerial to a subterranean position. Thus both the root and fruit system of the peanut develop in the soil.

It has long been suspected that the fruit system absorbs nutrients and water[8] directly from the soil. More recently it has been demonstrated that a supply of calcium around the developing fruit is necessary for normal yields.[2,3,4] Bledsoe *et al.*[1] devised a technique for growing peanuts in sand culture with the root system completely isolated from the fruit system. Using that technique, they conducted tracer studies with Ca^{45}. The necessity of an external supply of calcium in the fruiting area for normal fructification was shown to be the result of an inadequate movement of calcium from the plant to the developing fruit. These and other results[10] cause one to speculate on the possibility of the fruit playing a part in the absorption of other nutrients.

In the southeastern part of the United States it is common practice to dust peanuts with sulfur and to apply calcium sulfate to the foliage in the early blooming stage. These treatments probably have more effect on harvested yield of quality peanuts than any others used. The assumptions are that the sulfur dust decreases diseases and that calcium sulfate falls on the surface of the soil, supplying calcium to the developing fruit.[7] However, these assumptions may not be the complete explanation of the results. It is known that sulfur dusting has an effect on the color of growing peanuts. This suggests that the sulfur in these treatments may have a nutritional effect on the peanut, especially on soils deficient in sulfur, and many of the soils in the state of Florida are deficient.

The sulfur applied to the plant as dust or gypsum could enter the plant through the foliage, or fall on the soil and enter through the fruit or root system. Leaf absorption has been studied only in a preliminary way. The primary purpose of the experiments reported in this paper is to compare the absorption of radioactive sulfur as sulfate by the fruit system and roots of peanuts.

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EXPERIMENTAL WORK

Two sand culture experiments were conducted in which the root system of the peanuts was completely separated from the fruit system (Fig. 1). The first experiment was with the Dixie Runner variety in 1952, and the second one was with the Early Runner variety in 1954. These peanuts are the runner type with the branches reclining, but they tend to have one central upright part with few or no fruiting organs.

The first experiment was conducted as follows: 48 lb. of washed coarse builder's sand were put in a 4 gallon glazed pot, filling the pot to about ½ inch of the top. The bottoms were taken out of quart size milk bottles, and the outside of each bottle was painted with aluminum paint. Two of the bottles were set in each pot. Some of the same kind of sand was used to fill the milk bottles, thus the columns of sand in the bottomless bottles extented upward about 6 inches above the rim of the pot. One peanut was planted in each milk bottle, and in that manner the peanuts grew in an elevated position. For the fruiting zone, metallic pans, the inside of which were coated with asphaltum and the outside painted with aluminum paint, were filled to about one inch of the top with 60 pounds of the same kind of sand. Permegard wire (1 × 1 inch mesh), coated with aluminum paint, was put over the top of each pan. This was to prevent the foliage from contacting the treatment in the fruiting zone. One pan was slipped under each

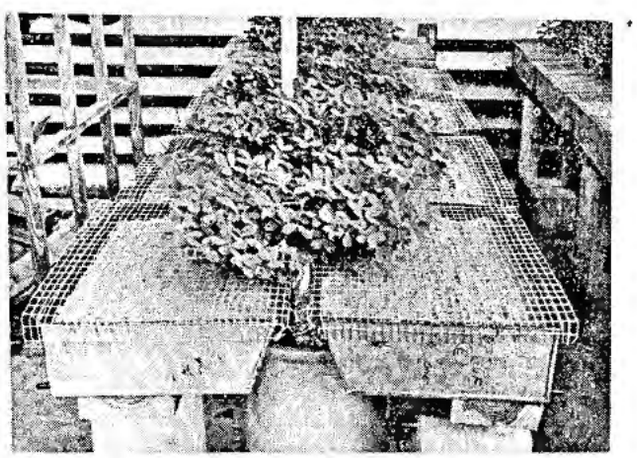

Figure 1. Young peanuts growing with root and fruit systems in separate media

side of the young peanuts as shown in Fig. 1. Thus the two peanuts in a pot with a pan on each side was one culture. The branches were trained so they did not lie in the small opening between the pans.

A modification of the Shive-Stahl method[9] was used for applying the nutrient solution to the root area. The pan treatment was carefully poured in the pan through a funnel and attached small hose. This prevented splashes on the foliage.

All peanuts were grown on Hoagland's No. 2 complete nutrient solution[5] from March 1 to May 23, 1952. At the end of this period pegs were beginning to form freely and only distilled water had been applied in the fruiting zone. On the latter date the root zone was thoroughly flushed with water and differentiation in root or fruit treatment for sulfur begun and continued until the end of the experiment. The sulfur deficient nutrient solution was prepared by using magnesium nitrate in place of magnesium sulfate of the No. 2 solution.

The treatments (listed in Table I) were replicated and randomized in blocks.

June 5, 1952, carrier free S^{35}, 21.6 microcuries as sulfate, was applied to each of the pots indicated in Table I, or 10.8 microcuries to each of the two pans of a culture, as the case might be.

Four weeks after the activity was applied, the various parts of the plants were sampled according to physiological age, the material dried, and saved for assay.

The assay was made in the following manner: 0.5 gram of the dry material was put into a small calibrated test tube. Five ml of concentrated nitric acid was added and the material slowly refluxed digested for about two days. It was then made up to 5 ml with water, 1 ml aliquot put in a stainless steel planchet, two drops of 10 per cent sodium hydroxide added and evaporated to dryness under a lamp. Counts were then made on the sample with a Nuclear Measurements Corporation proportional counter. A pre-set time of four minutes, after flushing with P-10 gas, was used to evaluate each sample. Background was determined in a ten minute period. Counts by this method would include a few from K^{40} because potassium was not removed from the sample.

In the second experiment with Early Runner peanuts in 1954 each pot contained three milk bottles with one plant each. Thus a culture unit was three plants. The treatments (listed in Table III) were in randomized blocks and replicated three times. Otherwise the experiment was essentially the same as the previous one.

The peanuts, inoculated with the legume organism, were planted April 8, 1954 and grown on the No. 2 complete nutrient solution until June 17, 1954, when the plants were freely blooming and pegs were forming. Up to that time only water had been applied to the fruiting area and a complete nutrient solution to the root zone. On that date the roots were flushed with water and differentiation in root or fruit treatment begun and continued to the experiment's end.

Carrier free S^{35}, 48 microcuries as sulfate, was applied June 23, 1954 to each pot requiring it or 24 microcuries to each of the two pans of a culture, as indicated under treatments in Table III.

July 1, 1954, one entire side of each culture was harvested and July 12, 1954, nineteen days after activity was applied, the other side of each culture

Table I. Square Root of Counts per Minute for 0.1 Gram of Peanut Plant as Affected by Position of S^{35} Application Four Weeks before Harvest and by Nutrient Treatment (1952)

Treatment[a]		Pot no.	Leaves			Stems			Pegs not in sand	Shelled peanuts	Total[b]
			Upper	Lower	Upright	Upper	Lower	Upright			
Roots	Pegs										
C	C*	7	5.6	7.5	7.0	5.6	4.4	0.0	8.9	12.8	20.8
		10	18.2	6.8	9.6	8.8	8.3	7.5	7.9	18.8	33.0
		Av	11.9	7.2	8.3	7.2	6.4	3.8	8.4	15.8	26.9
—S	C*	15	7.1	9.4	13.3	21.5	15.3	6.2	15.4	28.3	45.7
		16	22.6	10.8	0.0	14.0	9.4	0.0	17.1	17.8	39.0
		Av	14.9	10.1	6.7	17.8	12.4	3.1	16.3	23.1	42.4
C*	C	9	33.1	11.8	16.8	21.1	25.7	18.5	29.6	30.3	69.0
		18	28.2	7.1	18.2	17.7	13.8	27.4	23.4	27.3	61.1
		Av	30.7	9.4	17.5	19.4	19.8	23.0	26.5	28.8	65.1
C*	—S	8	27.4	11.4	11.0	16.7	16.7	16.7	17.4	24.9	52.3
		14	36.4	11.1	9.8	20.7	15.7	25.0	26.2	24.9	64.4
		Av	31.9	11.3	10.4	18.7	16.2	20.9	21.8	24.9	58.4
C	—S*	5	6.4	8.0	8.1	8.2	6.6	3.6	9.6	13.8	24.0
		6	7.2	7.3	4.4	7.2	3.6	5.4	6.8	9.7	18.9
		Av	6.8	7.7	6.3	7.7	5.1	4.5	8.2	11.8	21.5
—S*	C*	12	27.0	7.8	16.7	18.7	16.2	18.5	25.4	27.3	58.5
		17	25.3	8.0	17.5	15.2	14.6	12.9	19.6	22.2	50.0
		Av	26.2	7.9	17.1	17.0	15.4	15.7	22.5	24.8	54.3
L. S. D. at 5% level			15.1	3.8	10.6	8.0	9.6	12.6	10.2	10.0	18.1
L. S. D. at 1% level			23.7	5.9	16.7	12.5	15.1	19.8	15.9	15.7	28.4

[a] Treatment after differentiation began. C is complete nutrient solution, –S is solution deficient in sulfur, and * is position S^{35} applied.

[b] The counts for the eight parts were totaled and the square root of that number obtained. Counts were made on pegs in the sand and hulls, but are not included for fear of contamination.

was harvested. The top portion of the central upright part of the plant was included in the last harvest. The material was separated in its various parts, dried and saved for assay. This left only short stubbles, and the harvest was essentially one-half at each time.

The various parts of the plant were ground, each part individually, mixed, and 0.8 gram used for each activity assay and sulfur evaluation. The A.O.A.C. method[6] of ashing with magnesium nitrate and precipitating the sulfur as barium sulfate was followed. The precipitate on the filter paper was put in a stainless steel planchet, ashed in a furnace, the ash uniformly dispersed in the planchet with 0.5 ml of ethyl alcohol, dried, weighed and counted in the proportional counter. The time period was the same as in the first experiment.

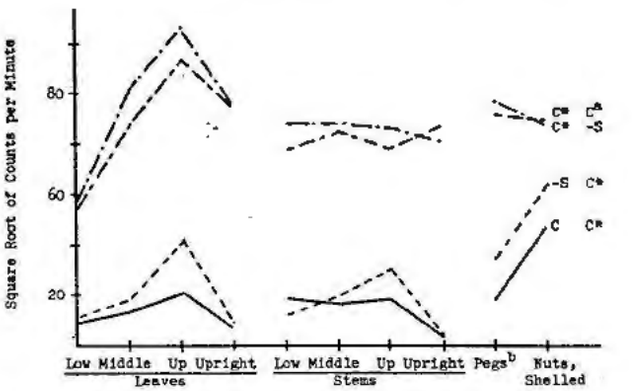

Figure 2. S^{35} content of peanuts, 1952. C is complete nutrient solution, -S is solution deficient in sulfur, and * is position activity applied. Letters on left, root treatment; on right, fruit. Pegs not in sand

RESULTS

The count values for the first experiment are given in Table I. They are expressed as square roots, at the suggestion of a statistician.

Large differences in counts for different parts of the plant as well as for different treatments are shown in Table I. In general much more activity was taken up through the root system than the fruit system. However, when the supply of sulfur for the roots was deficient from the early peg stage to the termination of the experiment, the fruit system absorbed more S^{35}, tending to compensate for the shortage in the root area. This difference was significant only in the counts for the upper stems, but differences in counts for other parts approached significance. The values for the first four treatments are given in graphical form in Fig. 2. This graph shows more clearly the relationships discussed above.

The second experiment was an effort to get more precise comparisons by decreasing the number of treatments and having another replicate. Furthermore, it was thought that the method of assay and associated operations were an improvement. Evaluations were made only on the plant material which had been harvested nineteen days after the S^{35} was applied.

The sulfur deficiency of the plants was late in the growing period and had no appreciable effect on the yield of dried material. The averages of the treatment yields ranged from 163 to 183 grams of dry material for the side last harvested. Evidently the supply of sulfur built up in the plant before differentiation in treatment was adequate for good growth. However, the late deficiency of sulfur did influence the sulfur content of the plant as shown in Table II.

The results of the assays are given in Table III. There is great variability for the same treatment in the individual cultures. However, a high or low result for a culture tends to be the same in the different parts of the plant. Nevertheless, the data clearly indicates that the root system absorbed more S^{35} than the fruit system. Again when there was a sulfur deficiency in the root zone the fruit system absorbed more S^{35}. This difference was significant for upper leaves and in some of the other parts of the plant the difference approached significance. Thus the results are rather similar to those obtained in the first experiment. Perhaps these differences can be more easily observed in the graphical presentation given in Fig. 3.

Of the total activity applied the proportion found in the last harvest is given in Table IV. The fruit system, where there was a sulfur deficiency in the root area, absorbed and transported to the last harvest about two and one-half times as much S^{35} as where sulfur was not deficient. The root system absorbed and transported to the same harvest about 25 per cent of the activity applied. In other words, the values for the fruit system, where there was a sulfur deficiency in the root area, were about an eighth of those for the roots.

Table II. Effect of Treatment on Mean Sulfur Content of Peanut Plants Expressed as Percentage (1954)

Treatment[a]		Leaves				Stems				Pegs not in sand	Shelled peanuts
		Upper	Middle	Lower	Upright	Upper	Middle	Lower	Upright		
Roots	Pegs										
C	C*	0.28	0.25	0.19	0.24	0.19	0.20	0.16	0.20	0.20	0.21
−S	C*	0.17	0.22	0.20	0.15	0.07	0.09	0.05	0.06	0.11	0.16
C*	−S	0.29	0.30	0.24	0.24	0.16	0.15	0.14	0.14	0.18	0.20
C*	C	0.29	0.29	0.24	0.26	0.15	0.16	0.14	0.17	0.19	0.23
L.S.D. at 5% level		0.04	0.04	0.04	0.05	0.05	0.03	0.04	0.04	0.04	0.03
L.S.D. at 1% level		0.06	0.06	0.06	0.08	0.13	0.06	0.07	0.06	0.06	0.04

[a] Treatment after differentiation began. C is complete nutrient solution, −S is solution deficient in sulfur, and * is position S^{35} applied.

Table III. Square Root of Counts per Minute for 0.8 Gram of Peanut Plant as Affected by Position of S^{35} Application Nineteen days before Harvest and by Nutrient Treatment (1954)

Treatment[a]		Pot no.	Leaves				Stems				Pegs not in sand	Shelled peanuts	Total[b]
			Upper	Middle	Lower	Upright	Upper	Middle	Lower	Upright			
Roots	Pegs												
C	C*	1	13.2	11.0	7.1	1.7	11.0	9.4	8.9	1.7	8.1	44.5	51.8
		7	18.6	9.1	6.3	7.2	16.1	16.2	16.3	5.5	20.5	44.6	61.2
		9	30.1	18.6	13.0	8.2	27.2	23.2	26.5	4.4	24.6	54.2	83.9
		Av	20.6	12.9	8.8	5.7	18.1	16.3	17.2	3.9	17.7	47.8	65.6
—S	C*	2	33.2	15.3	9.4	5.7	27.0	18.5	11.4	2.6	26.3	77.6	96.9
		5	49.6	22.0	12.2	7.5	37.0	24.6	14.2	5.0	35.7	62.4	102.6
		12	40.1	16.6	10.9	9.3	25.4	15.6	13.2	6.5	36.5	46.8	82.0
		Av	41.0	18.0	10.8	7.5	29.8	19.6	12.9	4.7	32.8	62.3	93.8
C*	—S	3	111.8	88.6	53.5	95.5	78.9	86.8	80.0	83.7	90.7	69.4	269.3
		8	135.4	105.4	61.7	102.7	97.8	94.5	100.3	84.8	107.7	98.0	317.4
		10	132.7	112.1	53.9	91.6	81.5	84.5	82.8	72.4	92.9	89.4	289.7
		Av	126.6	102.0	56.4	96.6	86.1	88.6	87.7	80.3	97.1	85.6	292.1
C*	C	4	120.8	88.4	50.5	95.1	86.3	91.0	86.4	92.6	104.2	91.2	291.4
		6	111.5	83.3	55.4	84.4	76.5	84.1	77.2	81.6	88.9	86.4	265.5
		11	110.8	89.0	59.8	101.7	73.9	80.6	72.4	86.2	79.8	87.8	269.9
		Av	114.4	86.9	55.2	93.7	78.9	85.2	78.7	86.8	91.0	88.5	275.6
L.S.D. at 5% level			17.7	13.0	7.0	12.2	16.9	11.9	17.2	10.1	17.6	25.2	37.0
L.S.D. at 1% level			26.8	19.6	10.6	18.5	25.6	18.0	26.1	15.4	26.7	38.2	56.0

[a] Treatment after differentiation began. C is complete nutrient solution, –S is solution deficient in sulfur, and * is position S^{35} applied.

[b] The counts for the ten parts were totaled and the square root of that number obtained. Counts were made on pegs in sand and hulls but are not included for fear of contamination.

The writer did not attempt to estimate the relative absorbing area of the roots as compared to the fruit system, but it would appear that the roots have many times the surface area for absorption. Furthermore the size of a pan was larger than that of a pot. Thus it would seem that the fruit system is a highly effective means of absorbing and transporting sulfur. This is in agreement with the conclusions of Thornton and Broadbent[10] for nitrogen.

COMMENTS AND CONCLUSIONS

Two experiments were conducted different years with two varieties of peanuts grown in sand culture. The objective was to determine the relative absorption and movement of S^{35}, as the sulfate, applied to the root system as compared to the fruit system. The results for the two experiments were similar.

There were large differences (Table III) for the same treatment on individual cultures. This was probably due to some physiological difference in absorption when no difference was observed in the growth of the replicates for the same treatment. Even though peanuts are a self-breeding crop and the seed used were from pure lines, this variability did occur. It has been known for a long time[7] that peanuts are quite susceptible to minor environmental influences. For that reason the entire data were presented to point out that even though there were wide differences between replicates, the trends were consistent. Furthermore, there were great differences in the tissues from different parts of the plant. This means that care must be taken in obtaining plant samples for assay.

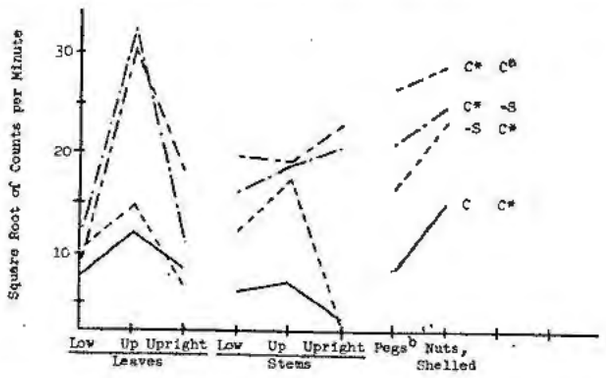

Figure 3. S^{35} content of peanuts, 1954. C is complete nutrient solution, -S is solution deficient in sulfur, and * is position activity applied. Letters on left, root treatment; on right, fruit. Pegs not in sand

Table IV. Per Cent of Total S^{35} Application Found in the Last Harvest of Peanuts (1954)

Treatment		Block 1	Block 2	Block 3	Average
Root	Fruit				
C	C*	0.6	1.2	2.1	1.3
—S	C*	3.3	3.2	2.8	3.1
C*	—S	4.8	30.3	29.8	27.3
C*	C	23.8	24.1	26.4	24.8

[a] Treatment after differentiation began. C is complete nutrient solution, –S is solution deficient in sulfur, and * is position S^{35} applied.

The sulfur freely moves to and from the fruiting system depending on where the activity was applied. In that respect sulfur is more mobile than calcium.[2] This would suggest that as long as the roots are well supplied with sulfur there will not be a critical shortage for fruit development. However, this does not mean that a deficiency of the sulfate ion in the fruiting area will have no effect on nut yield. In fact, the writer[4] has some evidence that it does.

Most of the absorption was through the root system. However, when there was a deficiency in the root area, the fruit system absorbed more sulfur tending to compensate for the root deficiency. That fruit absorption was about one-eighth of the root absorption.

In view of the small number of fruiting organs compared to roots, it appears that the fruit system per unit area is very effective in absorbing S^{35}. The results are further evidence that the peanut fruit system plays an important role in the intake and utilization of mineral nutrients.

From a practical point of view the results suggest that distribution and manner of fertilization may be important.

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Effects of Gamma Rays on Sprouting and Growth During Storage in Carrots and Potatoes*

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The maintenance of quality of certain vegetable crops during storage is a serious problem. Considerable damage is caused by sprouting, weight loss and rotting.

Prevention of sprouting has been paid much attention for years. The problem has been attacked in two different ways. Attempts have been made by plant breeding methods to produce varieties with improved storage quality. Much work has also been undertaken to find chemicals or plant growth inhibitors which can prevent sprouting and growth during storage, and an enormous list of reports has been published on the subject. A great number of plant growth inhibitors are known and, among several others, Turkey[3] has reviewed the work which has been done in this field.

Since larger doses of ionizing radiations are known to inhibit growth in several plants species, it should be of some interest to find out whether ionizing radiations could also be effective in inhibiting sprouting in crop plants during storage. Sparrow and Christensen[2] have already reported that gamma rays from Co^{60} have proved effective in reducing sprouting and weight loss in potatoes. Recently similar results have been obtained[1] with onions.

In our experiments samples of potatoes and carrots were irradiated and stored over the winter. The preliminary results of the experiments will be presented.

MATERIAL AND METHODS

In the fall of 1954, potatoes and carrots were irradiated in the reactor at the Joint Establishment for Nuclear Energy Research, Kjeller, Norway. The samples were placed in a special designed aluminium cylinder, 50 cm long and 10 cm in diameter. The inside of the cylinder was covered with a thin wall of cadmium. The irradiation was given in the reactor on a space just outside the reflector graphite where the potatoes and carrots were exposed to gamma rays. The material was exposed to the following doses: 5,000, 10,000 and 15,000 r. The potatoes were also exposed to a dose of 20,000 r.

After the radiation treatments the potatoes and carrots were stored in the following manner: The potatoes were stored in a dark cellar at a temperature of + 4 to + 6°C. Two replicas of each series of treatments were placed in small wooden boxes. One series of potatoes treated with Fusarex, a chemical growth inhibitor, was included in the experiments. The carrots were stored at a room temperature of + 12 to + 15°C. After 6 months the temperature increased to + 15 to + 18°C. This high temperature was used to promote maximum growth and rotting. The carrots were stored in moist sand in wooden boxes. Each series of treatments included 60 carrots which were divided into 4 replicas of 15 carrots.

RESULTS

Carrots

During storage, observations on rotting and measurements on growth were carried out 4, 6 and 8 months after harvest. No growth was observed in the carrots exposed to 10,000 and 15,000 r, as late as 8 months after harvest (Table I and Fig. 1). At a dose of 5000 r some sprouting was noticed, but a marked and significant growth inhibition, as compared with the untreated controls, was observed. After 8 months new root hairs appeared in the untreated controls, while the irradiated carrots had no root hair growth whatsoever.

Observations on rotting seem to indicate that a radiation dose of 5000 r had no influence on rotting throughout the experimental period. At doses of

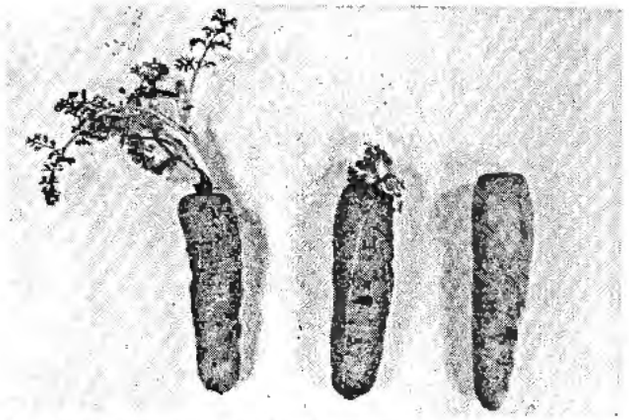

Figure 1. Representative samples of carrots from different treatments after 7 months of storage. From left to right: untreated control, irradiated with 5000 r, irradiated with 10,000 r

* This paper is published as Report No. 9 of the Institute of Genetics and Plant Breeding (Director: Professor dr. H. Wexelsen), The Agricultural College of Norway, Vollebekk Norway.

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Table I. Effects of Gamma Rays on Growth and Rotting in Carrots during Storage

Dose, r	Average length in cm of leaves at months from harvest			Per cent rotting at months from harvest		
	4	6	8	4	6	8
0	1.5	2.5	3.6	8	21	47
5,000	0.6	0.8	1.0	7	20	47
10,000	0	0	0	10	25	58
15,000	0	0	0	12	35	65

10,000 and 15,000 r an increased number of rotten carrots were observed after 8 months of storage. It seems as if the infections came from an initial rotting in the dead tissue of the growing point of the carrots. Further experiments will be carried out to test the significance of these observations, and to find out if other storage procedures or conditions may decrease rotting of irradiated carrots during storage (see Table I).

Potatoes

After 8 months no sprouts had appeared on the tubers irradiated with 5000–20,000 r or treated with Fusarex, while the untreated tubers showed a mean sprout weight of 2.8 grams. The length of the sprouts varied from 3 to 10 cm and the tubers had become shrunken and softer. The irradiated and Fusarex-treated tubers did not shrink or soften (Fig. 2).

The untreated potatoes had a loss in weight of 5.7%. When the weights of the sprouts are added, the loss in weight amounted to 8.7%. Fusarex-treated tubers had a loss in weight of 3.6%, while the irradiated tubers varied from 3.9 to 4.5% for the different radiation doses used. On the basis of this data the various treatments of 5000–20,000 r gamma rays and Fusarex showed significant less loss in weight as compared with untreated potatoes. No significant differences between the different treatments could be verified on the basis of this material.

Analysis of dry matter content was also carried out in the material. The various treatments seen to have no influence on dry matter of the tubers (Table II). All values of the different series had very close to 24% dry matter. At the start of the experiment the dry matter of the material amounted to 22%. Preliminary analysis of the ascorbic acid content was also carried out.

The values, listed in Table II, are derived from 4 parallel analyses, each included three tubers. Since no statistical analysis was carried out, little attention can be paid to the deviations found. It would be of great interest if a larger ascorbic acid content in the tubers treated with 5000 r of gamma rays could be verified. It may, however, be concluded that the analysis indicates that irradiation of doses up to 20,000 r of gamma rays has no harmful effect on the ascorbic acid content of potatoes.

We obtained no information on effects of irradiation on storage quality of carrots, but our experiments with potatoes may be compared with similar experiments by Sparrow and Christensen.[2]

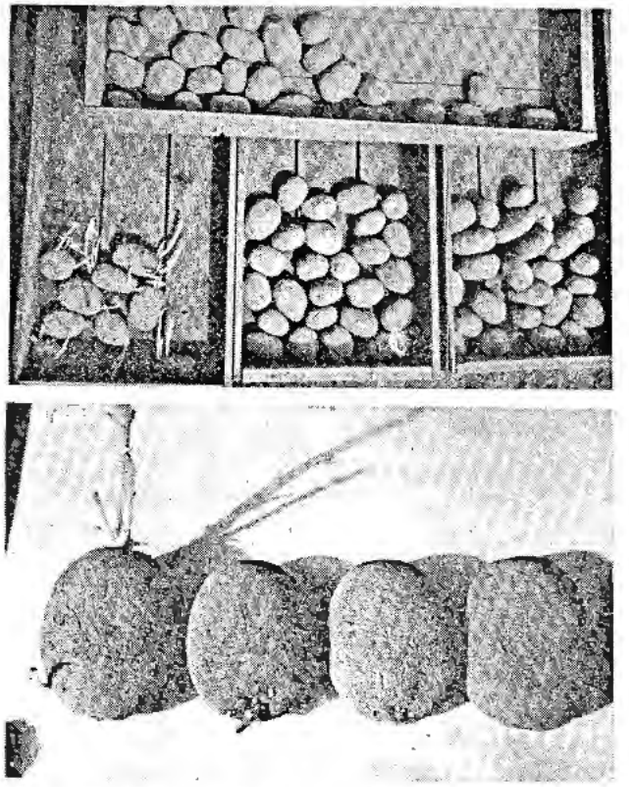

Figure 2. Stored potatoes 8 months after harvest given the following treatments: (a) from left to right, untreated, irradiated with 5000 r, irradiated with 15,000 r, and above, treated with fusarex; (b) representative samples from each of the lots in (a)

After 8 months of storage, sprouting was prevented and growth was completely inhibited by a dose of 5000 r in both experiments. The irradiated tubers showed less loss in weight than the untreated potatoes in our experiments, while Sparrow and Christensen[2] seem to have found no influence on weight.

A comparison of the results with potatoes and carrots indicate that a marked inhibition of sprouting is obtained with the same doses of gamma rays in both species. In potatoes 5000 r seems to be a little more effective than in carrots. The difference in storage conditions and temperature may contribute to this difference.

A taste panel of several persons found no unfavourable taste in the irradiated potatoes and carrots: They could not distinguish them from the untreated control samples.

Table II. Effect of Gamma Irradiation on Potato Tubers after 8 Months of Storage

Treatment	Mean sprout weight per tuber, gm	Loss in weight, %	Dry matter, %	Ascorbic acid, mg/100 gm
0	2.8	5.7*	24.0	5.2
Fusarex	0	3.6	24.3	7.4
5000 r	0	3.9	23.5	6.4
10,000 r	0	4.5	24.1	5.3
15,000 r	0	4.0	23.3	4.9
20,000 r	0	3.9	24.2	4.8

* 8.9 when weight of sprouts is added.

SUMMARY

Carrots and potatoes were irradiated with gamma rays and the storage quality was studied at different times after harvest. The carrots were exposed to 5000, 10,000 and 15,000 r of gamma rays. After 8 months of storage, doses of 10,000 and 15,000 r completely prevented sprouting and growth in the irradiated carrots. Carrots irradiated with 5000 r showed less sprouting and a marked growth inhibition throughout the experimental period.

Incidence of rot was fairly high in both the irradiated and untreated samples under the present unfavourable storage conditions.

Samples of potatoes were irradiated with 5000, 10,000, 15,000 and 20,000 r of gamma rays. All doses proved to be effective in preventing sprouting and growth in potatoes during the first 8 months of storage, the same result obtained chemically with Fusarex. Loss in weight was greater in the untreated potatoes than in irradiated and Fusarex-treated tubers. The radiation treatments showed no influence on per cent dry matter. No reduction in ascorbic acid content was noticed in the irradiated samples.

The effect of 5000 r seem to be equivalent to the effect of Fusarex in all the analyses which were carried out.

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Study on Spermatogenesis in Domestic Animals with the Aid of P^{32}

By R. Ortavant,* France

The rational use of male domestic animals for artificial insemination requires the use of a large quantity of semen of high fertility. Nevertheless, the conditions under which the animals should be raised, housed and fed, in order to achieve such an objective, are poorly defined, since contradictory findings and results have been reported. This situation is largely due to the failure of investigators to allow for the time which elapses between the moment when a given factor acts on spermatogenesis and that of the appearance of its effects on the ejaculate and the spermatozoa it contains. Indeed, while we have a few data pertaining to the transit time of the spermatozoa through the *ductus epididymidis* of domestic animals[1, 2, 3] we have none as to the duration of their spermatogenic cycle. Thus, prior to studying the effect of the various factors of interest in the production of sperm, we endeavored to provide answers to those two questions.

With this in mind, we have endeavored to label germ cells with P^{32}, since phosphorus is an important element in the composition of the spermatozöon. We injected intraperitoneally, 1.5 to 3 mc of P^{32}, in the form of Na_2HPO_4 into 25 Ile de France rams. The spermatozoa were then obtained either by electroejaculation[4] or, after castration, by separation from the *ductus epididymidis* by a technique derived from that of Dallam and Thomas[5] by crushing, filtering, and centrifugation. The separation of various phosphorus-containing fractions was carried out according to the method of Schmidt and Tannhauser[6] and according to the phosphorus content measured by Allen's technique.[7]

DURATION OF THE SPERMATOGENIC CYCLE[11]

As in the rat, the spermatogenic cycle (from parent spermatogonium to spermatozöon) in the ram (Table I) is rather complicated.

Howard and Pelc[8], following the injection of P^{32} in mice, found positive autoradiographs only for spermatogonium and spermatocytes at the preleptotene stage.[9] Furthermore, Howard and Pelc[10] showed that the P^{32} incorporated in desoxyribonucleic acid remained there for a very long time, after which it was passed on to the daughter cells. Since the spermatogenic cycle, in the ram, develops along lines similar to those observed in the monse, one may feel that the incorporation of P^{32} in the germ cells also takes place in an identical manner. Unfortunately, no autoradiograms of sufficient accuracy have been made to enable us to solve the problem in the ram, for the doses then to be injected are enormous. On the other hand, we have been able to follow the variations of the specific radioactivity of desoxyribonucleic acid in the spermatozoa as they come out of the testicle, in the proximal part of the head of the *ductus epididymidis*. We noted that, for 29 days following the injection of P^{32}, the specific radioactivity of desoxyribonucleic acid is very low, with a sudden increase on the 30th day, while that of the other fractions was consistently decreasing (see Table II below).

This would justify a belief that this increase in the specific radioactivity of the desoxyribonucleic acid coincides with the arrival of spermatozoa descended from spermatogonia or spermatocytes in the preleptotene stage, as tagged by the P^{32} immediately after the injection. The total computed duration of the

Table I. Simplified Scheme of the Spermatogenic Cycle in the Ram

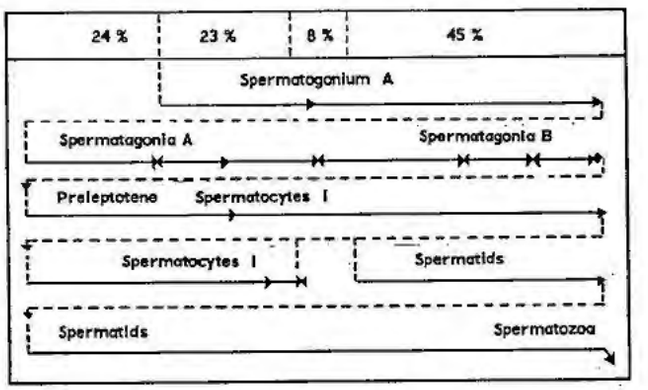

Table II. Variation of the Specific Radioactivity of the Desoxyribonucleic Acid of the Spermatozoa in the Proximal Part of the Head of the *Ductus Epididymidis*

Time after injection	*3 hr*	*4 d*	*7 d*	*15 d*	*25 d*	*29 d*	*31 d*	*37 d*	*45 d*
Radioactivity of DNA, cpm/γ P	0	0.04	0.07	0.07	0.06	0.10	2.6	2.2	1.9

Original language: French.

* Chargé de Recherches à l'Institut National de Recherches Agronomiques. With the technical help of C. Esnault.

Table III. Variation of the Radioactivity (cpm/gm) in the Head and Tail of the *Ductus Epididymidis*

Time elapsed since injection was made	*3 hr*	*4 d*	*15 d*	*23 d*	*25 d*	*29 d*
Head, proximal	33,000	33,000	18,200	8900	6000	5400
Head, distal	21,500	32,400	18,300	13,000	9260	8680
Tail, proximal	11,200	11,400	35,500	23,800	15,600	7800
Tail, distal	2500	6700	24,000	35,500	18,160	17,360

spermatogenic cycle in thus 39 to 40 days counting from the first division of the spermatogonia and 47 or 48 days counting from the formation of the parent spermatogonium. We found identical figures by an indirect method, determining the number of spermatids in the testicles of rams having a known daily output of spermatozoa.

TRANSIT TIME OF THE SPERMATOZOA IN THE *DUCTUS EPIDIDYMIDIS*[12]

A few hours following the injection of P^{32}, it is observed that, while the specific radioactivity of the desoxyribonucleic acid of the spermatozoa is low at the head of the *ductus epididymidis*, that of the other phosphorus bearing fractions is very high. As a matter of fact, it decreases progressively toward the distal end of the *ductus epididymidis*. On the other hand, a few days after the injection, radioactivity increases progressively in the other regions of the *ductus epididymidis*. Thus there is a displacement of the radioactivity front: the maximum, in the distal part of the tail of the *ductus epididymidis*, is registered approximately 20 to 23 days following the injection (Tables III and IV).

VARIATION OF THE RADIOACTIVITY IN THE SPERM COLLECTED

If the spermatozoa, instead of being collected from the epididymis following castration, are gathered at the opening of the *vas deferens* by electroejaculation, it will be noted that there is a progressive increase in tbe radioactivity of the sperm as time goes by. The radioactivity curve reaches a peak on the 14th to 16th day, then decreases very gradually (Table IV). It will thus be noted that the radioactivity front, under these experimental conditions, reaches the end of the *ductus epididymidis* a little earlier than it does in rams which are sexually at rest. This is due to the fact that the repeated collections cause a reduction of the reserve contained in the epididymis.

If further material is collected, it is then noted that the radioactivity of the fraction which contains desoxyribonucleic acid, which was very low, in turn reaches a maximum between the 43rd and 45th days. This coincides with the arrival, at the end of the *vas deferens*, of the spermatozoa detected on the 30th day in the proximal part of the head of the epididymis.

Table V. Variation of the Radioactivity of the Sperm (cpm/10^9 spz) Collected by Electroejaculation

Time after injection, days	*1*	*2*	*4*	*8*	*11*	*14*	*18*	*21*	*25 26*
Ram 59	220	506	822	1666	1530	2260	1644	1386	1544
Ram 67	22	128	222	660	2300	4000	3140	2900	1720

It can thus be computed that, in rams which are sexually at rest, the spermatozoa require approximately 21 days to travel the length of the *ductus epididymidis*. This is reduced to 14 days approximately, when frequent collections are made. These results agree with the figure found for the reserve contained in the epididymis of rams having a known daily output of spermatozoa. They are markedly higher than those determined by Phillips and MacKenzie,[1] and Gunn.[2]

CONCLUSIONS

According to our findings, two months or so elapse, in the ram, between the formation of the parent spermatogonium and the collecting of spermatozoa derived from it. Knowing at what stage of the cycle a given factor has an effect, it now becomes possible to foresee at what moment its influence will be apparent on the sperm collected.

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Table IV. Variation of the Radioactivity (cpm/mg) of the Ribonucleic and Phosphoprotein Residues of Spermatozoa in Various Parts of the Epididymis

Time elapsed since injection was made	*3 hr*	*18 hr*	*2 d*	*4 d*	*7 d*	*13 d*	*15 d*	*23 d*	*25 d*	*29 d*
Head, distal	1.4	2.2	3.3	16.4	14.4	16.2	19.2	14.4	11.4	10.6
Body, distal	0.3	1.2	2.4	6.4	3.7	12.6	12.5	12.7	8.8	9.0
Tail, proximal	0.10	0.8	1.8	1.6	3.6	8.8	10.7	13.0	8.8	10.2
Tail, distal	0.06	0.07	0.6	0.1	0.8	4.3	4.4	13.4	12.2	10.2

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Labelling Locusts with Radioactive Isotopes

By H. B. D. Kettlewell,* UK

Following successful attempts at marking Lepidopterous larvae with radioactive sulphur with the object of investigating population dynamics, larval death-rate, and dispersal,[1] an opportunity presented itself of applying similar principles to locusts (*Locusta migratoria* L. and *Locustana pardalina* Walk.). Accordingly, a series of laboratory experiments was carried out on locust hoppers and locust adults. In each case, phosphorus-32 was given and feeding permitted for twenty-four on forty-eight hours, and this was followed by their return to normal food; thereafter, each having been anaesthetized by carbon dioxide, the individual counts per minute were recorded with a Geiger counter, as also were those for their excreta and exuviae.

LOCUST HOPPERS

Large numbers of first and second instar hoppers were fed on hydroponic maize and grass, grown in solutions of phosphorus-32. Thus the isotope was given in an organic state. Also, sieved wheat bran was impregnated with labelled phosphoric acid and a strength of 10 μc of phosphorus-32 per gm of bran was found adequate. Uniformity of spread was checked by dyes. It was established that 40 gm of sieved wheat bran would take up 100 cm^3 of radioactive solution which, when mixed, gave a satisfactory spread of activity. Rapid drying at high temperature followed by stirring (a mask being worn) was essential; otherwise fermentation, resulting in deaths of locusts, took place. Known strengths of inorganic phosphorus-32 were thus ingested.

Individuals made radioactive during their first and second instars gave counts easily recognizable three to five weeks later, after they had become adult. There was no apparent difference in this respect between the *gregaria* and *solitaria* phases. The total phosphorus loss (exchange plus decay) after initial twenty-four hours feed was 80 per cent for the next fortnight.

For the first twenty-four hours following radioactive feeding, each hopper lost about half its radioactivity and, during this period, the excreta gave correspondingly high counts. Thereafter, the loss was negligible. Exuviae contained very little phosphorus-32.

There was no apparent difference between hoppers that ingested organic phosphorus-32 and those that ingested inorganic radioactive phosphorus.

At a temperature of 85–90°F, there were, in both cases, individuals which showed very low or no radioactive counts. This was found to be due to the fact that, during ecdysis, the hopper does not feed for a period of about twenty-four hours. A standard error can therefore be worked out for the proportion of hoppers which fail to become recognizably radioactive during their feeding time at a given temperature. This may account for the deficiency of radioactive releases reported by other workers (Putnam, L. G., personal communication) even when sampling is carried out immediately after release.

Among individuals feeding on radioactive hydroponic plants, a much greater uniformity of count was obtained by crowding, so that no one hopper fed exclusively on one leaf. The variance of the leaf counts was thus equalized.

Treated hoppers had a slightly higher mortality rate than untreated hoppers, and death usually occurred during ecdysis. The speed of metamorphosis appeared to be slowed down.

ADULT LOCUSTS

Similar feeds, both organic and inorganic, were given to adult locusts, and counts of up to 27,000 per minute at a distance of half an inch were obtained. With adults, the counts were much more uniform than with hoppers. Using a "DeVilbiss" No. 15 atomizer, adult *L. migrotoria* were also sprayed with phosphorus-32 in a wetting agent and their subsequent counts recorded.

As in the case of hoppers, but to a slightly lesser degree, phosphorus-32 was rapidly absorbed by adults, both as an organic and an inorganic salt, and was retained for a period of up to five weeks. Treated adults could be recognized at a distance of ten inches for a fortnight. The Geiger counts for excreta, high for the first twenty-four hours, fell rapidly to negligible proportions, the locusts losing 45 per cent of their radioactivity during that period.

Autophotography of adult locusts given a forty-eight-hour radioactive feed showed a concentration in the metathoracic pair of legs and in the ovaries and testes. The wings appeared to hold no phosphorus-32, as distinct from those adults, reared from radioactive hoppers, the wings of which showed up clearly on the films. This difference would permit double marking with the same isotope of both hoppers and adults within a given population.

In view of the fact that locust hoppers cast their skins frequently, external marking is of no use. This

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being so, there is no adequate marker available other than a radioactive isotope, and for work where a β-ray is sufficient, phosphorus with a half-life of 14.3 days is the most suitable element. There is no difference in the absorption of it by a hopper whether it is given in an organic state in grass and maize made radioactive or as an inorganic salt in bran. The bran feed is the obvious method of choice in the field, and large numbers of first and second instar hoppers can thus be made radioactive at comparatively low cost. Much could be learnt about hopper movement and death-rate by this method, the only one at present available. Its use, however, would entail the expenditure of much time and labour by specialized staff using costly apparatus. An attempt was made to circumvent this by an approach to Kodak, Ltd., with the object of obtaining a β-ray-sensitive 8-mm film trace, suitably insulated and of known length, which could be laid in the track of advancing hoppers. With random sample counts taken over this for fixed periods, subsequent developing would show the passage of radioactive hoppers. Unfortunately, this method is so far inapplicable for β-rays, as there is no strip sufficiently sensitive for so short a traverse. For the present, therefore, orthodox methods, involving capturing hoppers with a net and scanning the bag with a Geiger counter, would have to be used, though direct scanning of hopper concentrations could possibly be undertaken at night. Future developments in the direction of using a portable scintillation counter, with material emitting a γ-ray, may simplify this.

Unless some automatic recording device can be found, it seems that for labelling adult locults radioactive isotopes have no advantages over the well-tried colour paints, with which adults have been marked in the past for observing dispersal.[2] Untrained workers can see and record these, whereas radioactive locusts demand highly skilled technicians using special apparatus.

ACKNOWLEDGEMENTS

I wish to thank the following members of the University of Cape Town who gave me facilities: Prof. J. Day, Department of Zoology; Dr. A. M. Cormack, Department of Physics, for much technical advice; and Prof. E. Isaac and Dr. E. Schelpe, Department of Botany. I am also indebted to the Anti-Locust Research Centre for providing a grant for this work, and to the Nuffield Foundation for allowing me six months leave of absence. Full results have been deposited with the Anti-Locust Research Centre, British Museum (Natural History), London, S.W.7.

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Eradication of the Screw-Worm Fly by Releasing Gamma-Ray-Sterilized Males Among the Natural Population

By R. C. Bushland, E. F. Knipling and A. W. Lindquist,* USA

During 1954 screw-worms, *Callitroga hominivorax* (Cqrl.), were eradicated from the island of Curaçao, Netherlands Antilles, by means of a new tool in biological control—sterilized insects. Male flies, reared in the laboratory and sterilized with gamma rays from cobalt-60, were released in the field to compete for mates with normal males. It was possible to release sterile insects in greater numbers than existed in nature. As the females mate only once, those that mated with sterile males were incapable of reproduction. The sterile males competed so effectively that screw-worms were eradicated. This paper is an account of the research conducted on this method of controlling the screw-worm.

EFFECTS OF IRRADIATION ON INSECTS

It has been known for almost 40 years that insects can be sterilized by radiations. Runner[19] reported that cigarette beetles, *Lasioderma serricorne* (F.), laid infertile eggs after exposure to X-rays. Extensive studies on the effect of radiations on insects followed the discovery by Muller[13,14] that X-rays induced mutations in *Drosophila melanogaster* (Meig). In 1928 Muller reported that when untreated *Drosophila* females were mated to heavily treated males they laid eggs that failed to hatch.

Following Muller's discovery that chromosomal changes can be induced by X-irradiation, other geneticists and cytologists tested its effects on various insects. White[20,21] treated four species of grasshoppers with X-rays, and Carlson[4] X-rayed still another species of grasshopper. Whiting[22] irradiated *Bracon hebetor* (Say.). Reynolds[18] reported on the effects of X-irradiation on *Bradysia ocellaris* (O.S.), and Crouse[6] treated another species of fungus gnat, *B. coprophila* (Lint.). Koller & Ahmed[9] compared the effects of X-irradiation on *D. melanogaster* and *D. pseudo-obscura*. Thus, in addition to the extensive literature on *D. melanogaster*, biologists had observed the effects of irradiation on still another species of fruit fly, a parasitic wasp, a beetle, two species of fungus gnats, and five species of grasshoppers before our work on the screw-worm fly.

Muller,[15,16] Lea,[10] and Catcheside[5] have reviewed the literature of genetics and cytology dealing with the effects of radiations on insects. There seems to be general agreement that X-rays and gamma rays cause similar effects. If the germ cells of an insect are irradiated, chromosomal changes result, and the extent of the changes depends upon the amount of ionizing radiation. Extreme changes cause the cells to degenerate. Less extensive mutations may not prevent the sperm from fertilizing an egg, but the zygote may be incapable of maturing and usually dies in the embryonic stage. Geneticists call mutations that prevent the survival of the fertilized egg dominant lethal mutations.

Our work with the screw-worm fly has been to determine the dose of irradiation sufficient to cause dominant lethal mutations in all the germ cells. Technically speaking, the males so irradiated are not truly sterile, because they are still capable of producing sperm which fertilizes eggs, but for practical purposes they are called sterile because their progeny die as embryos.

BIOLOGY OF THE SCREW-WORM

The screw-worm fly (Fig. 1) is a calliphorid fly which is parasitic in the larval stage. The female deposits her eggs in a mass of about 200 on the edge of an abrasion in the skin of a warm-blooded animal. Flies oviposit on cuts, scratches, the navels of newborn animals, and even such inconspicuous wounds at tick bites. The eggs hatch in 12 to 24 hours, and the larvae feed on the living muscle tissue. The insects feed as a colony, eating a hole in the flesh. Infested wounds attract other flies to oviposit, and untended animals may be literally eaten alive. Almost invariably infested animals die if not properly treated with a larvicide. The larvae (Fig. 2) complete feeding in 5–6 days and then crawl from the wound and burrow into the soil to pupate. The pupal stage (Fig. 3) lasts about 8 days in warm weather. Adult flies emerge, work their way to the surface of the soil, and crawl up on the nearest vegetation to expand their wings and fly away. They mate when 2 to 4 days old, and at the age of 6 days the female is ready to lay eggs. The adults usually die of old age in about 3 weeks.

In nature the screw-worm is an obligatory parasite on warm-blooded animals. The flies oviposit only on living animals, and if the host animal dies before the larvae have completed two-thirds of their development, they cannot survive. The population is therefore limited, and the insects never reach such num-

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Figure 1. Female screw-worm fly

bers as are attained by flies that breed in carrion. Domestic animals that are properly cared for may become infested, but proper wound treatment with a larvicide prevents the survival of any maggots. In order to survive the species must increase in the wounds of neglected domestic animals and wild animals.

Screw-worms are easily reared in the laboratory. The flies are maintained in cages provided with water and are fed chiefly on honey. The adults also feed readily on exudate from ground meat during the pre-oviposition period. The females will readily oviposit when confined in shell vials containing pieces of fresh beef. The egg masses are transferred from the vials to moist filter paper and held in a covered Petri dish for hatching.

Although the larvae are obligatory parasites in nature, they can be reared in the laboratory by a modification of the procedure described by Melvin and Bushland.[12] A mixture of ground lean meat, citrated beef blood, and water with formaldehyde to retard putrefaction serves as the rearing medium. The larvae are reared at a temperature of about

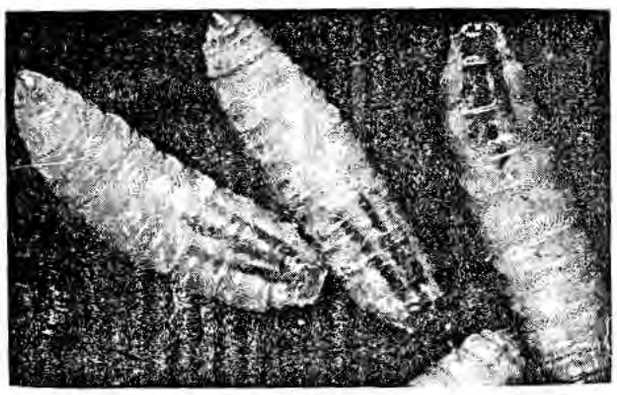

Figure 2. Full-grown screw-worm larvae

Figure 3. Screw-worm pupae

95°F and grow almost as well as they do in their natural hosts. The larvae crawl from the rearing medium and drop into sand trays to pupate. The pupae are sifted from the sand and transferred to screen cylinders, which are held in a cabinet at 80°F and 100 per cent relative humidity. The flies emerge after 7½ days.

The screw-worm is tropical and semitropical in its distribution, being found the year around only in those parts of the Americas where the winters are mild. In the United States screw-worms used to be limited in their winter distribution to regions bordering on Mexico. In the summertime they migrated up the Pacific coast area about as far as the Sacramento Valley, and in the Midwest they traveled as far north as Kansas before being killed by cold weather. The summer migration of the midwestern infestation extended northward rather than eastward, and screw-worms seldom were found very far east of the Mississippi River, and the population that migrated to Mississippi and Louisiana did not survive the winter.

In 1933 screw-worms were accidentally introduced into Georgia through shipment of infested cattle from Texas. The Georgia infestation spread into Florida, where the insects found a winter climate suitable for survival. During the summer months screw-worms spread over the Southeastern States, doing millions of dollars of damage each year. In average winters the cold kills screw-worms north of peninsular Florida. If the Florida overwintering population could be destroyed, screw-worms might be eliminated from the Southeastern States. It is believed that with proper precautions to prevent importation of infested livestock the area might be kept free of the parasite.

LABORATORY TESTS WITH IRRADIATED FLIES

The Entomology Research Branch's studies with irradiated flies have been directed toward the practical problem of eradicating the overwintering Florida population. When screw-worms first became established in Florida, the US Department of Agriculture, in cooperation with the State, made every effort to control them through good animal-husbandry practices and the use of larvicides to treat infested wounds. However, enough insects survived

in wild animals and neglected domestic animals that eradication was impossible. Some other supplementary procedure is necessary if screw-worms are to be eradicated.

In 1950 we began experiments with irradiated flies. The first tests[2] were with X-rays. Both pupae and adults were irradiated. When flies less than 2 days old were given a dose of 5000 roentgens, the males were sterilized but the females were not affected. Pupae were more susceptible to the sterilizing effects of the irradiation. When they were irradiated 2 days before flies were due to emerge, a dose of 2500 roentgens caused sterility in the males, but 5000 roentgens were required to sterilize females. The sterilizing doses of irradiation did not appear harmful to the pupae, since as many adults emerged from irradiated groups as from untreated controls. About the only adverse effect was that flies from irradiated pupae did not live quite as long as controls.

Mating experiments with caged flies established that males mated repeatedly if mates were available, but females mated only once. If a female mated with a sterilized male, she laid only infertile eggs. When mixed populations of sterile and normal insects were caged together, the irradiated males seemed to compete for mates about equally with normal males. The proportion of infertile egg masses was similar to the ratio of sterile to normal males in a caged population.

X-ray equipment was adequate to treat small numbers of insects, but it would be too expensive for large-scale irradiation of screw-worms. Therefore, experiments were made with gamma rays from cobalt-60. Laboratory tests were made in which screw-worms treated with 200-kilovolt X-rays were compared with others treated with gamma rays from cobalt-60. As for the preceding work, pupae were X-rayed at Brooke Army Hospital, Fort Sam Houston, Texas. The gamma rays were from an 11-curie distributed source loaned us by the Biology Division of Oak Ridge National Laboratory. The two types of irradiation[3] showed very similar effects. It was established that the minimum sterilizing dose was 5000 roentgens, which was best given to insects that had been in the pupal stage for 5 days at 80°F. Except for a somewhat reduced adult longevity, the sterilizing radiation seemed to be well tolerated by the insects.

GAMMA-RAY SOURCE FOR STERILIZING INSECTS

As the cobalt-60 source loaned by the Biology Division appeared satisfactory for screw-worm sterilization, arrangements were made for the Oak Ridge National Laboratory to construct a source especially for this purpose. This source has been described by Darden, Maeyens, and Bushland[7]. It consists of 70 curies of cobalt-60 in a lattice arrangement. The cobalt slugs are spaced in the wall of a brass cylinder 3½ inches in diameter and 12-inches long, which is held in a lead-filled steel shell. An aluminum irradiation chamber of about 900-cm³ capacity is suspended from a steel plug and is lowered into the brass cylinder. At the time the source was constructed, in 1953, the activity within the irradiation chamber was 235 roentgens per minute, with a maximum variation of ± 13 per cent.

The source in operation is shown in Fig. 4. It is set up on an elevated base in a locked concrete structure separated from the main laboratory buildings at Orlando, Fla. Entomological technicians do not need to approach closer than 3 feet from the unit to sterilize insects, and the source is considered entirely safe for skilled biological technicians, since the radiation level within the room is negligible except for the region directly over the shield. The operator, using a chain and pulley to lower the irradiation chamber within the source, has practically zero exposure to irradiatinn.

FLORIDA FIELD TESTS WITH IRRADIATED FLIES

From 1951 to 1953 a group consisting of A. H. Baumhover, A. J. Graham, D. E. Hopkins, F. H. Dudley, and W. D. New conducted field studies in Florida. Techniques were developed for estimating fly populations in the field and appraising the effects of releasing sterilized insects. Laboratory-reared screw-worms labeled with P^{32} according to the technique described by Radeleff *et al.*[17] were released and the numbers compared with natural populations.

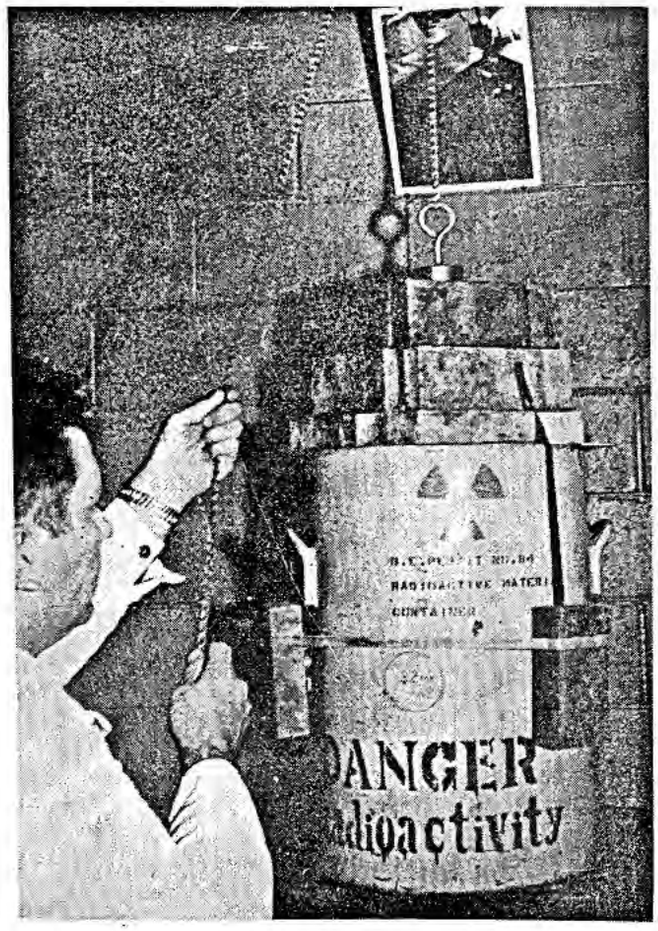

Figure 4. Cobalt-60 source

These field observations confirmed our earlier opinion that screw-worm populations, even where most abundant in Florida, did not exceed more than a few hundred insects per square mile.

Screw-worm flies could be caught in traps baited with decomposing liver, but the most reliable index to screw-worm abundance was obtained by collecting egg masses from artificially wounded and infested goats exposed to oviposition by flies of a natural population. Nearly all the females of the normal population were fertilized, as eggs from wounded goats hatched practically 100 per cent.

When males sterilized with gamma rays were released on Sanibel Island at the rate of 100 per square mile per week, the fertility of egg masses collected from wounded goats declined to less than 25 per cent, and in 8 weeks the natural population appeared to have been eradicated. However, after 12 weeks normal flies were again active. As Sanibel Island is only 2 miles off the coast of Florida and is part of a chain of islands, the test area was not sufficiently isolated to prevent migration of flies from adjacent untreated areas. Therefore, it was necessary to seek a truly isolated area to conduct a valid eradication experiment.

ERADICATION OF SCREW-WORMS FROM CURAÇAO

Late in 1953 the Veterinary Service of the Netherlands Antilles government requested advice from the US Department of Agriculture on methods of screw-worm control. The insects were extremely abundant on the island of Curaçao, about 40 miles off the coast of Venezuela, and did heavy damage to domestic animals, particularly goats. The small size of the island (170 square miles), the high population of screw-worms, and the isolation made Curaçao ideal for a field experiment to test whether screw-worms could be eradicated through release of sterilized flies. Details of this experiment are described in another paper.[1]

A. H. Baumhover and W. D. New of the Entomology Research Branch worked with B. A. Bitter of the Curaçao Veterinary Service in releasing flies and making observations on the island. Screw-worms for release were reared and sterilized at Orlando, Fla., by A. J. Graham, D. E. Hopkins, and F. H. Dudley. When pupae were five days old they were irradiated, packed with a little excelsior at the rate of 130 per kraft-paper sack, and shipped by air freight to Curaçao. Upon arrival in Curaçao the sacks were unfolded to make room for the flies, which emerged within a few hours. When emergence was complete, the adults were distributed by airplane over the island. An entomologist riding with the pilot tore open the sacks and dropped them at desired intervals as they flew over the island in flight lanes 1 mile apart. From the 130 pupae packed in each sack about 100 active flies emerged. Thus it was a simple matter to compute the rate of fly release. Releases were made twice weekly, the flight lanes being shifted ½ mile each time. The practical effect was that insects were scattered over the island in ½ mile flight lanes, as many per mile as were required to attain the desired release rate.

The screw-worm activity and the efficiency of the release of sterilized flies were measured by observations on egg masses collected from wounded goats. Eleven goat pens, each stocked with eight goats, were established at suitable locations over the island. Each week two goats in each pen were infested by making a small incision in the skin near the shoulder and implanting 100 newly hatched larvae. When the larvae were 3 days old they were killed with benzol. The wounds were attractive to flies from the time of infestation until several days after the larvae were removed. Thus, there were always at least two wounds at each goat pen to attract ovipositing flies.

In March 1954, prior to the release of sterilized flies, native screw-worms deposited about 15 egg masses per goat pen per week. All were fertile.

Sterilized flies were released at the rate of 100 males per square mile per week for 8 weeks. The fertility of the egg masses collected from wounded goats declined from the 100 per cent recorded prior to release to 85 per cent, but this reduction was not sufficient to affect the fly population, which actually increased during a period of favorable weather.

An experiment was then made in which one-half of the island was treated with sterilized flies at the rate of about 400 males per square mile per week, while the 100 rate was continued on the other half.

Female flies sterilized with 5000 roentgens could not oviposit normal egg masses, but some individuals deposited a few eggs that did not hatch. Those scattered eggs might have been confused with small egg masses deposited by normal flies. Females irradiated with 7500 roentgens were incapable of producing any eggs. To avoid any doubt as to source of screw-worm eggs, the sterilizing dose of 7500 roentgens was used during the eradication experiment. Eggs found were laid by normal females.

The rate of 400 males per square mile per week seemed to be so effective that the whole island was treated at that rate in an effort to attain eradication. This heavy release rate was started on August 8, and within 8 weeks normal fly activity had almost ceased. Observations on egg masses collected during this period are shown in Table I.

During the first 4 weeks the releases caused about 70 per cent sterility. This high sterility caused a marked depression in the number of insects in the subsequent generation. Since the release of sterilized insects was maintained at a high rate, there was an even greater percentage of sterility, which was reflected in the hatching records on the reduced number of egg masses collected.

Only two egg masses were collected after October 3, and both of them failed to hatch. They were taken on November 4 and November 11. The goat pens were maintained through January 6, 1955, and fly releases were continued at the same rate, but there was no normal fly activity on Curaçao.

Table I. Egg-mass Records in 11 Goat Pens during Release of Sterilized Flies over Entire Island of Curaçao

Week	Number or males released per square mile	Number of egg masses		Per cent of sterile egg masses
		Fertile	Sterile	
Aug. 8–15	491	15	34	69
16–22	224	17	38	69
23–29	175	17	36	68
30-Sept. 5	381	10	37	79
Sept. 6–12	451	7	42	86
13-19	701	3	23	88
20–26	450	0	10	100
27-Oct. 3	607	0	12	100

The United States representatives came home in January, but the Curaçao Veterinary Service has continued to watch for evidence of renewed screw-worm activity. Since there have been no cases of myiasis, it is concluded that screw-worms were eradicated from the island of Curaçao through release of sterilized flies.

SCREW-WORM ERADICATION IN THE SOUTHEASTERN STATES

We believe that the Curaçao experiment demonstrated that it may be practical to use sterilized flies in an attempt to eradicate screw-worms from the Southeastern States.[11] In ordinary winters screw-worms survive in Florida over an area of approximately 50,000 square miles. An eradication campaign in Florida would involve rearing, sterilizing, and releasing about 300 times as many insects as were required for the Curaçao experiment. We are now engaged in investigations to establish procedures for rearing, sterilizing, and distributing 50 million flies per week. We hope that our techniques for mass production of sterilized flies can be perfected so that within 2 years we can undertake an eradication campaign with reasonable confidence of success.

THE STERILIZATION METHOD ON OTHER INSECTS

In considering whether this method will be effective on other insects, one should take cognizance of some of the requirements for success.[8] A low natural population must exist or the population must be reduced by other means so that it is possible to release an excess of sterilized males. The insect must be easily reared in mass numbers in the laboratory. The mating behavior of the males must not be adversely affected by sterilization, and native females must be willing to accept sterilized males. Preferably the females should mate only once, but on theoretical grounds multiple matings should result in the production of infertile eggs in a ratio similar to that of released sterilized to native males.

Methods of measuring the insect populations per unit area are necessary, so that the numbers required for release purposes can be accurately estimated. Obviously the area in which eradication is attempted should be isolated or protected by quarantine or other measures against reinfestation.

Each insect presents numerous problems, and a large amount of research on the effects of irradiation, habits of the insect, population trends of the species, rate and extent of migration, and other problems is necessary before the feasibility of the sterilization method as a means of control can be determined.

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A Study by Isotopic Dilution of the Solution of Sparingly Soluble Phosphates in the Presence of an Anion Exchanger

By G. Barbier* and Mme P. Quillon,† France

The solubility of sparingly soluble phosphate fertilizers, in particular of crushed phosphorites and apatites, has been evaluated, up to now, by means of conventional reagents, which give but imperfect data on their efficiency, and may even lead to serious errors. It would be preferable to use the natural solvent for these fertilizers, namely the water which soaks the ground. However the solubility of these phosphates is so low that, unless huge volumes of water are used, the quantity dissolved represents only an insignificant fraction of the sample, and the solubility found may be that of a constituent having actually but very little importance.

In order to get around the difficulty, it is possible to operate in the presence of an anion exchanger, which may fix the larger part of the phosphoric ions during their passage into the solution, thus making it possible for the dissolution process to go on. Thereafter, a radioactive phosphate-bearing solution (P^{32}) is added without a carrier. Once equilibrium of the isotope distribution is obtained, the mass m and radioactivity r of any fraction of the solvent, as separated by filtration or centrifuging, then are measured. If R is taken to designate the total radioactivity, use of the isotopic dilution formula:

$$\frac{M}{R} = \frac{m}{r}$$

makes it possible to compute the total mass M of the phosphoric ions which are statistically distributed, according to kinetic balance, between the liquid and the solid phases of the system (uniformity of the isotopic composition, as implied by the formula, is not incompatible with the results indicated below, within the limitations imposed by the precision of the measurements).

In order for mass M, as determined by this method, to be equal to that of the phosphoric ion given up by the phosphate to the solution and the solid exchanger (assumed to be initially free from phosphoric ions) the system must fulfill two conditions:

1. All the phosphoric ions held by the exchanger must remain in motion, none must be fixed by a bond which would make it incapable of displacement, so that isotopic dilution extends to every one of these ions. This point has been verified[1] for a neutral calcic kaolinite first treated by a dilute solution of P^{32}-tagged phosphate under conditions which would allow the precipitation of calcium phosphates, then by a solution of $H_3P^{32}O_4$, without a carrier.

Here, on the other hand, are the results achieved in the presence of an amberlite which first had been purified by treatments with hydrochloric acid and dilute soda, then adjusted to the required pH. The conditions were: 0.5 gm amberlite agitated with 80 cm³ of a P^{32}-labeled calcium phosphate solution; pH = 7.2–7.5, then the addition of $H_3P^{32}O_4$ with an activity of 7107 cpm without carrier, followed by agitation over 24 hours.

P_2O_5 added, mg	0.4	0.6	1
P_2O_5 left in solution, mg	0.24	0.36	0.544
Activity of solution, cpm	4044	4189	3845
Computed value of M, mg P_2O_5	0.42	0.61	1.02

2. In addition, the phosphate to be dissolved must not itself give rise to isotopic exchangers or phosphoric ions in the solid phase. Some calcium phosphates, even when they are well crystallized, in particular hydrated dicalcium phosphate, when placed in contact with a radioactive phosphate-bearing solution, absorb an important fraction of the radioactivity, even when they are partly dissolved.[1] However, this isotopic exchange, in the crystals or on their surface, is of negligible significance for the phosphorites or apatites under study: after 8 days of agitation of an aqueous solution, the isotopically dilute mass of P_2O_5 in the solid phase (M less P_2O_5 in solution) represents approximately 0.02 to 2% P_2O_5 left in the solid phase. (The importance of isotopic exchange in the solid phase seems to vary with the same sign as the solubility of the phosphate).

The method was applied to five finely crushed natural calcium phosphates of various origins (90% of the particles < 50 μ) having a very uniform granulometric composition (samples turned over by the International Phosphates Studies Association):

1. 2.5 gm amberlite + 200 cm³ of 0.015 N $CaCl_2$; initial pH = 5.5 + 20 mg of phosphate; 8 days of agitation; radioactivity introduced: 500,000 counts per minute $H_3P^{32}O_4$ without a carrier.

Original language: French.

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Designation of phosphates	*A*	*B*	*C*	D_1	D_2
% P_2O_5	29.5	25.3	30.0	35.8	36.4
Isotopically diluted P_2O_5, % of P_2O_5 in the sample	66.9	44.1	25.4	6	7.2
Final concentration of solvent, P_2O_5, mg/liter	0.5	0.3	0.19	0.04	0.05

2. 1.5 gm amberlite + 95 cm_3 of 0.015 *N* $CaCl_2$ + 12 mg of phosphate; 2 months of agitation; then 300,000 cpm $H_3P^{32}O_4$ without a carrier; 4 days of agitation; initial pH = 5.25 progressively raised to 5.7.

Designation of phosphates	*A*	*B*	*C*	D_1	D_2
Isotopically diluted P_2O_5, % of P_2O_5 in the sample	81	69	39	8.3	6.7
Final concentration of solvent, P_2O_5, mg/liter	0.5	0.35	0.28	0.09	0.07

The quantity of isotopically diluted P_2O_5—namely mass *M* of the formula—closely matches, according to the preceding data, the amount given up by the phosphate. The major part of the latter, approximately 98%, is fixed by the amberlite, and this is why an important fraction of the phosphate was dissolved.

The above results, which plainly show differentiation between the phosphates of various origins, show that nearly all of them can be dissolved in a liquid similar to the solutions naturally found in the soil, at a relatively high P_2O_5 concentration (0.5 mg P_2O_5 per liter) provided the pH is sufficiently low.

On principle, the use of isotopes would not be indispensable for a study of this type. It might be possible, for instance, to attempt the elution of the phosphoric ions held by the acceptor by means of a reagent which does not dissolve the phosphate. It would also be possible to establish a relationship between the number of phosphoric ions held by the acceptor and their concentration in the solvent, but it would be necessary to make allowances for all the conditions which might modify the distribution of the phosphoric ions between the solid and liquid phases (pH, calcium salt concentration, etc.). In addition, suitable curves should be plotted for each amberlite sample. The use of the isotopes seems to be more convenient and safer.

Our experiments confirmed—as we have observed on the slag used for phosphorus removal[2]—that the sparingly soluble calcium phosphates may, in the soil itself, be subjected to a sort of digestion by solid acceptors of PO_4 ions, (clays, sesquioxides) which makes their PO_4 ions diffusible, and thus absorbable by the plants, with the reservation, of course, that, under the conditions of PO_4, Ca and OH ion activity in the medium, the solubility product of the calcium phosphate under consideration is not affected.

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In Situ Measurement of Soil Bulk Density

By J. A. Vomocil,* USA

Soil tilth, extremely important as a component of land productivity, is difficult to measure and define quantitatively. It is the summation effect of many factors, some independent, some not. Hence, a single measurement method should not be expected to depict tilth. Ultimately the plant itself provides the best index for evaluation of this factor. However, in agronomic research, there is a need for charaeterization of this property for evaluation of soil management practices, tillage methods, effects of field traffic on soil condition, and other related operations.

Due to the variability of some soils over short distances, the properties of these can be adequately represented only if sampling is thorough. Hence, a measurement method must be rapid to be satisfactory. Secondly, it must be capable of rapid determinations to such depths below the surface as are effected by management practices and which in turn effect crop growth. Of course, the determination must be such that results from it are related to productivity of the soil, either theoretically or empirically. Finally, for experimental work, the measurement method should not cause appreciable plot disturbance.

The high penetrating power of gamma rays and the availability of sources of these in the form of radioisotopes suggest the possibility of using them for measurement of soil bulk density. Admittedly, bulk density is not a completely adequate index of soil tilth, but if used in conjunction with other properties, it can provide a representation of relative condition.

THEORY

When electromagnetic energy flows through matter, its intensity is diminished. There are several mechanisms of dissipation, but these are unimportant here. The principle factor is that the diminution at some frequencies is proportional to the density of the medium. Some frequencies have such characteristics that their transmission is affected by few variables except density, and their transmission is great enough that they become suitable for practical measurement of density. X-rays and gamma rays fall into this class. Their conductance is in accordance with the Beer-Lambert Law.

$$\frac{I}{I_o} = ke^{-\mu x}$$

where I is the intensity of radiation transmitted by a thickness x, I_o is the initial intensity, and μ is the linear absorption coefficient which is the product of the absorption per unit mass (mass absorption coefficient), and the density of the medium. Obviously if one plots the log of intensity transmitted through a constant distance from a constant intensity source against the density of the medium, a straight line is obtained if the mass absorption coefficient is constant. Radiations can be selected whose mass absorption coefficient is very nearly independent of the chemical composition of the medium.

Berdan and Bernhard[1] used 60-kv X-rays in a trial with two soils and found that a separate transmission curve had to be plotted for each soil. X-ray transmission was found to be sensitive to other variables in the soil. These authors suggested the use of Co^{60} gamma-ray transmission for determination of density. At the same time, Belcher *et al.*[2] proposed the use of Co^{60} gamma-ray scattering and transmission for bulk density determination.

Co^{60} has definite advantages. Its half-life of 5.2 years is sufficiently long that corrections for decay are small and need by made only about monthly. The energies of its dichromatic radiations (1.16 and 1.32 Mev) are such that absorption is almost entirely by Compton scattering, and consequently transmission is independent of chemical composition within reasonable limits.[3,4,5] In addition, Co^{60} is relatively inexpensive and easy to obtain.

For gamma-ray soil densitometry, a source of gamma rays, such as a small piece of Co^{60} wire, and a detector, such as a Geiger-Mueller tube, rigidly fixed with respect to one another, are required. These may both be housed in one probe[2] or in 2 parallel probes.[6,7,8]

If both are housed in one probe, gamma rays are scattered back to the probe, and some are absorbed by the intervening soil before beam intensity is measured. Hence, the mechanism is complex, but Belcher *et al.*[2] obtained a linear relationship between intensity at the detector and wet bulk density of the soil. However, within the single probe, the source and the detector are necessarily separated by considerable lead shielding and consequently the thickness of soil whose average density is measured with the probe in a single position is 20 to 30 cm. In agronomy there is interest in thinner layers, such as plow soles. Using two probes overcomes this difficulty.

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INSTRUMENT CONSTRUCTION, CALIBRATION AND USE

Vomocil[7,8] has suggested a modification usable in agronomic work employing two probes and a battery operated rate meter. The instrument is portable and once suitably calibrated measures wet density in the field. Construction,[8] indicated in Fig. 1, is simple.

The two probes were 2.54 cm od aluminum tube with a wall thickness of 0.16 mm. They were fastened together near one end by two lengths of 5.1 × 7.6 cm oak wood so the distance between them was 30.48 ± 0.08 cm.

The cobalt-60 (one millicurie in May, 1953), consisting of an 8-mm section of 1-mm wire contained in a hollowed aluminum wafer (diameter 2.1 cm, thickness 0.18 cm), was housed in one probe. A second wafer, pivoted to the first by an off-center rivet, served as a lid. By making this container as thin as possible and tightly fitting 5-cm long plugs of lead above and below it in the probe, it was possible to provide some collimation of the beam toward the detector, shield personnel, and prevent movement of the source in the probe.

The other probe contained a side window glass Geiger-Mueller detector tube in a shield with a 3-meter coaxial cable. The outside diameter of the tube shield was 2.20 cm, so it fit snugly into the probe yet allowed it to be removed for transport and storage. A brass bushing was fitted to the end of the shield opposite the tube socket to provide a better fit into the aluminum probe.

Calibration was achieved by packing soil to a definite bulk density in a steel tank 42 cm inside diameter and 80 cm deep. The tank contained upright tubes to accept the probes. Border effects in this calibration chamber were found to be negligible.

As shown in Fig. 2, the plot of log transmitted intensity *versus* wet bulk density was not linear. Deviation from linearity, probably due to changes in geometry with density, does not affect the usefulness

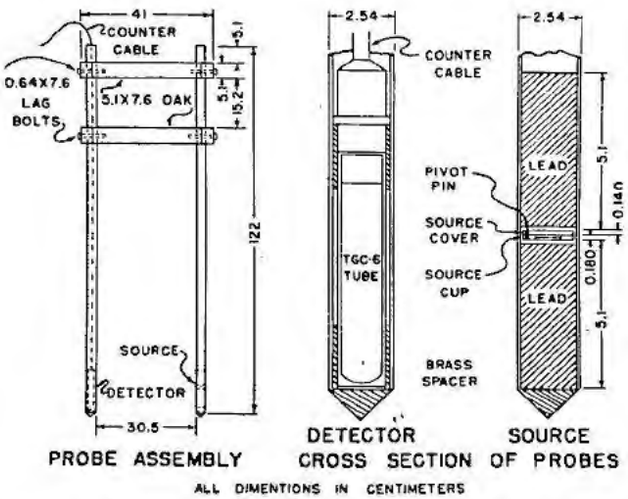

Figure 1. Construction of densitometer probes. All dimensions in centimeters

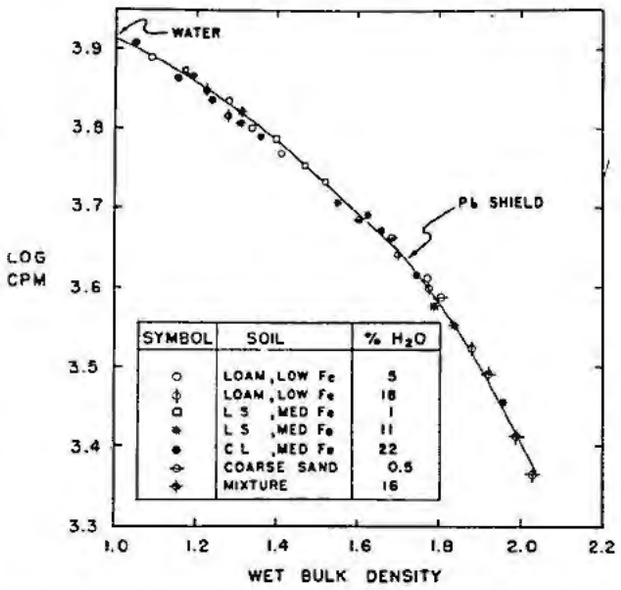

Figure 2. Calibration curve for densitometer. Transmission of gamma through soil

of the method. Theoretically, if no scattering or secondary emission occurred, the mass absorption coefficient for soil should be about 0.056 cm^2-gm^{-1}. Actually, this coefficient was found to increase from 0.023 to 0.030 as wet bulk density increased from 1.0 to 2.0 with the distance constant at 30 cm. The difference between the actual and theoretical may have been due to scattering and secondary emission. Bernhard and Chasek[6] report no change in the coefficient with density. The mass absorption coefficient for organic soils should theoretically be very nearly the same as for mineral soils.

For field use of the instrument, two holes were made into which the probes were lowered to measure the wet bulk density of the soil between them. Soil removed from these holes was saved for moisture content determination needed to convert wet bulk density into dry bulk density by the following relationship: $D_B = DBW/(1 + M_w)$ where D_B is the dry bulk density, DBW is the wet bulk density and M_w is $^1/_{100}$ of the moisture percentage on a dry weight basis. Since the holes for the two probes must be parallel and a fixed distance apart, a guide, described elsewhere[8] was used to prepare them.

As the radioisotope decayed, I_o changed slightly with time, and the rate meter batteries ran down, necessitating a periodic recalibration. This was easily accomplished by using two fixed points on the curve; for example, water with a density of 1.00 and the lead shield used to protect personnel from radiation hazard, which gave an attenuation equivalent to a wet bulk density of 1.72. The position of the entire calibration curve was shifted according to changes in counting rates for these points each month.

For an interprobe distance of 30 cm, 1 millicurie of Co^{60} gives a convenient counting rate range with normally encountered soil bulk densities. This is sufficient radioactive Co to create some radiation

hazard to personnel. The source was shielded with lead by placing 5-cm long lead plugs above and below it in the source probe, and by mounting a spherical shield 6 cm thick on the outside of the probe. This spherical shield served also to support the probes at a fixed depth when they were in the soil. It rested on the soil surface and the source probe slid down through it to the desired depth and was then clamped in position. When the probe was in the soil, the operator was shielded from the source by soil. Film badges were regularly used by operating personnel, and in two years' experience, no detectable dosage was reported.

The distance between the probes may be varied as desired to fit the experimental conditions. However, as the distance is increased, source size should be increased in order to keep within a satisfactory counting range. Commercial rate meters usually have four ranges: 0–100, 0–1000, 0–10,000, and 0–100,000 counts per minute. The 0–10,000 cpm range is probably the most convenient. The size of the source needed for any distance between source and detector can be approximated by the relationship:

$$I_o = \frac{4\pi I X^2 e^{\mu x}}{Ca}$$

where I_o is the source size, I is the desired counting rate in the same units as I_o, X is the desired distance between source and detector, C is the efficiency of the counting system (0.01 for Geiger-Mueller tubes), a is the cross sectional area of the active portion of the detector perpendicular to the beam, and μ is the linear absorption coefficient. This relationship is not exact because μ varies slightly with changes in distance, but the value for 30 cm may be used for approximation.

The gamma-transmittancy method enables one to make bulk density measurements quite rapidly compared to other procedures. Another advantage is that measurements may be made to considerable depth with less effort than required for other methods. Plot disturbance is very slight, only 2 holes about 2.5 cm in diameter are left in the soil when measurement is completed. Precision of the instrument is ± 2%, and results obtained with it compare favorably with those obtained with a 7.5-cm diameter core sampler.[8] Stones in the profile interfere in preparation of the holes and in interpretation of the data.

Some soil immediately surrounding the holes is no doubt compacted when the Veihmeyer tube is used to prepare holes for the gamma-ray densitometer but the distance this extends is probably very small compared to the distance between the probes, and consequently its effect on the measured density is small.

Full details of construction, calibration, and operation of the gamma ray soil densitometer are given elsewhere.[8]

INTERPRETATION OF RESULTS

The method proved accurate and rapid in field trials. Readings ranged from 2000 counts per minute for a wet bulk density of 2, to 8600 counts for a density of 1. Values obtained with this method were, in general, about 1.5 per cent lower than those found with the core sampler technique on the same soil. Under good conditions a crew of three could make 200 measurements a day with much less effort than would have been required with former methods and with much less plot disturbance. This rate was possible with some measurements being made at depths of 50 cm in the soil.

The most reliable interpretation of results was possible on a relative basis. That is, where two areas of soil similar in texture, horizon development and location, drainage, and depths were available, one under good management, the other under poor management, comparison of the density profiles from the areas indicated the magnitude and position of important differences in soil compactness.

Where such comparisons were not possible, soil air space, calculable from bulk density and soil moisture data, was a useful index for evaluation of soil physical condition. Significant correlation was found between yields of potatoes and corn and the average bulk density of the 10- to 40-cm depth region. This occurred only at relatively high densities (1.50–1.70) for loams. At lower average profile densities, correlations were not significant. The relationship between relative yield and average profile density appears to be parabolic, indicating that density enters twice as a factor in productivity. Significant decrease in yields of potatoes and corn occurred only after air space fell to about 12–14 per cent.

POSSIBLE FUTURE DEVELOPMENTS

There are several modifications which might be made to adapt the gamma-ray densitometer to a particular problem. For example, if it is necessary to measure the bulk density of relatively thin layers of soil, a detector with small physical dimensions might be used instead of the Geiger-Mueller tube. The least thickness of the measured layer, which increases as the distance between the probes increases, can be no smaller than the vertical dimension of the detector (about 5 cm for the tube used by Vomocil).[8] This dimension may be reduced by use of a different type of receiver, such as a scintillation detector. With this modification, the thickness of the measured layer could be further reduced by bringing the probes closer together. This, together with the fact that scintillation phosphors have a higher efficiency for gamma rays than Geiger-Mueller tubes do, would allow appreciable reduction in the size of source necessary.

For studies of soil dynamics under loading, it is conceivable that the source and detector could be lowered into permanently installed tubes and have no mechanical connection with one another. In this manner, continuous measurements could be recorded while loads are applied to and removed from the soil between the two tubes.

The effects of surface compaction have been referred to and evaluated in the work of Alderfer and Robinson.[9] The difficulties associated with using the core sampler technique anywhere in the soil are magnified in its use for measurements on crusts. A method for evaluation of the physical condition of surface crusts and compacted layers is sorely needed.

The gamma-ray densitometer, as modified by Vomocil,[8] is incapable of measuring this compactness due to loss of scattered radiation through the surface, and the averaging of transmittancy through layers about 8 cm thick. However, an instrument based on scattering and transmission of gamma rays could conceivably be devised which would be suitable for this purpose. If a collimated beam of rays were directed toward the soil surface at an angle, these would be scattering back or reflection toward the surface from various limited depths. If a detector were provided with a collimator before it, which was pointed at the soil at an angle toward the source, it would receive the radiation from a certain region in the soil plus leakage through the shielding. The intensity of the detected radiation should then be a function of the bulk density of the surface soil if careful consideration is given to over-all geometry. Such an arrangement seems theoretically possible, and may be worthy of some developmental effort. However, a number of important variables, such as the distance from the source-detector system to the soil surface and parallelism between the two, would have to be carefully controlled.

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Record of Proceedings of Session 14C

MONDAY AFTERNOON, 15 AUGUST 1955

Chairman: Mr. V. Kuprevich (Byelorussian SSR)
Vice-Chairman: Mr. A. L. Kursanov (USSR)
Scientific Secretaries: Messrs. E. O. Hughes, I. D. Rojanski and C. Polvani

PROGRAMME

P/10 Studies of special problems in agriculture and silviculture by the use of radioisotopes ..... J. W. T. Spinks

P/1049 Studies on plant nutrition, fertilizer and soil by the use of radioisotopes ..... S. Mitsui

DISCUSSION

P/104 Applications of radioisotopes to the study of soils and fertilizers: a review ..... L. A. Dean

P/112 Uptake and transport of mineral nutrients in plant roots ..... E. Epstein and S. B. Hendricks

P/460 Factors affecting the availability to plants of soil phosphates ..... R. Scott Russell *et al.*

DISCUSSION

P/694 The use of tracer atoms in studying the application of fertilizers ..... V. M. Klechkovski

P/695 Determination of the availability of soil phosphates and fertilizers with the aid of radioactive isotopes of phosphorus ..... A. V. Sokolov

P/716 Co^{60} in the study of the role of cobalt as microelement in the nutrition of plants ..... O. K. Kedrov-Zikhman

DISCUSSION

P/106 Utilization of radioactive isotopes in resolving the effectiveness of foliar absorption of plant nutrients ..... H. B. Tukey *et al.*

P/105 The use of radioactive isotopes to ascertain the role of root grafting in the translocation of water, nutrients, and disease-inducing organisms among forest trees ..... J. E. Kuntz and A. J. Riker

DISCUSSION

The CHAIRMAN: At this meeting we are to hear and discuss ten papers dealing with the results of research in agriculture and plant physiology.

There are two brief comments I should like to make myself.

No proof is needed of the great importance of the isotope method for the investigation both of problems already set by science, some of which are of long standing, as well as of those of recent origin. With the help of this method, it has, for example, been established that the oxygen liberated in photosynthesis originates in the molecule of water, not in that of carbon dioxide, as was previously assumed.

I would draw attention to the fact that the theoretical inferences have not as yet been drawn from the experimental data so far accumulated. In particular, I consider that the time has come to evolve a new theory of the mineral or, if you like, the root nutrition of autotrophic plants. Recent discoveries, for example the fact that plants utilize the carbon dioxide of the moisture in the soil and absorb carbon from organic substances in the soil through the extracellular ferments of the roots, indicate that the new theory will be universal and that the artificial distinction between autotrophic and heterotrophic nutrition will be eliminated.

Thanks to the isotope technique, we have been extremely successful in making new biological discoveries and making them in profusion. We are beginning to feel a little overwhelmed by this profusion on account of the many unrelated fields to which these discoveries relate. At all events, we have on

the one hand this wealth of facts and on the other, the comparative paucity of new ideas evolved by the biological sciences in recent years. We know, however, that scientific progress is largely determined by the evolution of ideas and by the concentration of research on the solution of new problems.

This fact is illustrated by the many investigations inspired by the ideas of the great English naturalist, Charles Darwin, and the eminent Russian scientist, Pavlov.

With the help of isotopes, complicated processes involving the participation of certain enzymes have been elucidated, the role of many simple substances and complex compounds in normal and pathological activity determined and new methods of influencing living matter discovered. But we are still too timid in exploring new fields and prefer to follow well-trodden paths and familiar themes. The time has probably come to attempt, with the help of the isotopic method, to investigate the processes of the synthesis of living matter, to determine the part played by the individual components of protoplasm in the operation of heredity, and to elucidate the causes and mechanism of asymetrical synthesis. Plant or animal viruses might be selected as a suitable subject with which to initiate these studies. A study of virus nucleoproteins would incidentally contribute to the solution of major problems of medicine and photopathology.

As the work of this Conference has shown, scientists in Canada, Japan, the USA, the USSR, the United Kingdom and other countries, about fifty in all, have unanimously and without previous consultation, come to the conclusion that certain scientific or practical problems are of outstanding importance and with equal unanimity have recognized the advantage the isotope technique offers for their solution.

This, of course, is no coincidence.

I trust that, like others taking place in the Palais, the present meeting will be to the benefit of all our countries.

Mr. J. W. SPINKS (Canada) presented paper P/10.

Mr. S. MITSUI (Japan) presented paper P/1049.

DISCUSSION OF P/10 AND P/1049

Mr. E. SAELAND (Norway): Recent studies in Norway have taken place concerning the uptake from rubidium-86 by the Norwegian spruce. May I ask Mr. Spinks whether similar experiments have been carried out with the corresponding Canadian species?

Mr. SPINKS (Canada): While I am not completely familiar with the Canadian work on rubidium-86, some work has been done on the red pine with it. Perhaps Mr. Mawson can make a better reply.

Mr. C. A. MAWSON (Canada): In the Canadian work, rubidium has been used as a translocation tracer. We have used rubidium to find out the rate at which material travels up tree trunks. The method has been to give an injection of radioactive potassium in order to saturate all surfaces interested in that kind of metal and to follow this up with rubidium. The reference in Mr. Spinks' paper to the speed at which translocation was taking place, arose from this work. It has only been used, as far as I know, as a means of measuring rate of translocation, and for that purpose it is very effective.

Mr. G. O. BURR (USA): Referring to the interesting paper of Mr. Mitsui, I should like to comment on the stoppage of translocation from the leaves by exposure of roots to H_2S. Our work indicates that the roots are a part of the general circulatory system and any blockage of their activity interferes with translocations in the top of the plant. Would Mr. Mitsui care to comment further on this subject?

Mr. MITSUI (Japan): In reply to Mr. Burr, I should like to say that the uptake and the upper movement and the downward movement simultaneously is the usual case for the ordinary plants. But I mentioned that in the case of water-logged soil, it sometimes happens that hydrogen sulfide comes into the root and goes up within the stem. In that case hydrogen sulfide inhibits aerobic respiration and in that way stops not only the upward movement but also the downward movement of the nutrients intaken from the roots and the assimilated products from the leaves.

Mr. E. EPSTEIN (USA) presented Mr. L. A. Dean's paper (P/104) by title only and then presented paper P/112.

Mr. R. SCOTT RUSSELL (UK) presented paper P/460.

DISCUSSION OF P/104, P/112 AND P/460

Mr. SCOTT RUSSELL (UK): This is not a question so much as a comment. It happens that there are many similarities between Mr. Epstein's work and that in which Mr. Middleton and I have been concerned. We therefore feel it appropriate to note that in our experiments with carrot discs, barley roots and intact barley plants, we have found that in short periods the nature of ionic interactions depends entirely on atomic size and valency in the same way as with a simple ion exchange resin.

Over longer periods (4–24 hours) the nature of the interactions varies greatly with the nutrient status of the tissue. Although our results have many features in common with those of Mr. Epstein, we are inclined to the view that all the results may in due course be explicable in terms of a series of exchange reactions which do not involve specific sites.

We are conscious of the fact that in detached roots and tissue slices, the distinction between active transport and simple exchange mechanisms must necessarily rest on indirect evidence. Although much information can be obtained with such material, we feel it is desirable that hypotheses should be con-

firmed by the use of intact plants or other systems in which unequivocal evidence of active transport can be obtained.

Mr. FROMAGEOT (France): I should like to ask Mr. Epstein a question. Do you know whether the "inner space" with reference to the sulfate corresponds to sulfur still in the form of sulfate or is it metabolically changed?

Mr. EPSTEIN (USA): I believe I understood the question. The only definition of sulfate being in an inner space, so-called, is that it does not diffuse out of the tissue and does not exchange with non-radioactive sulfate upon transfer of the tissue back into either water or non-radioactive sulfate. That is the definition for sulfate being in the inner space.

But now, in a sense, it has certainly been subjected to metabolic activity in passing into the inner space, so-called, because when you make conditions non-physiological, you do not get any sulfate into the inner space. Any sulfate that we then find is diffusable. In other words, it has not been actively transported. Therefore, a condition for sulfate getting into what we call the inner space is that metabolism be operative.

There is one more thing. There is really no evidence that the so-called inner space is one homogeneous sort of thing. The only definition is that the sulfate does not come out. But there may be a number of fractions of such sulfate.

Mr. K. KAINDL (Austria): I would like to mention that the connection of a similar occupation theory with the theory of self-reduplication of the living matter makes it possible to calculate the growth function of the living matter and, furthermore, of plants, as shown by theoretical work of our laboratory a few years ago.* It is very interesting that the paper of Mr. Epstein supports our more or less theoretical assumption. Similar ideas as given here in regard to competitive actions are also successful in the theory of the mechanism of growth regulators (cf. Bonner, USA) and we could prove it.†

Mr. MEDI (Vatican): I have a comment to make on this problem of the relationship between particle radiation and biological phenomena. It may well be that certain atoms in which the reaction is more effective may play an extremely important role in the processes of life itself. Accordingly, when confronted with phenomena including those described by Mr. Epstein, we should investigate the possibility of an effective collision between the radiation particles and the nuclei of the atoms I have just mentioned. It would probably be very useful to make selective experiments, i.e., to determine for which particles there is the greatest probability of an effective collision with the nuclei of the atoms which constitute the basic factors involved in biological reaction. Experiments of this type have been made which have given an indication of the line that should be followed.

Mr. EPSTEIN (USA): In regard to the comment by Mr. Kaindl, I would merely say that Michaelis is still very much with us. As for the last question, I do not believe that I understood it and it would not be wise for me to comment upon it.

Mr. V. M. KLECHKOVSKI (USSR) presented papers P/694 and P/695, as follows:

Fertilization is one of the most important methods of raising the yield of agricultural plants in modern agriculture. With the increased production and use of fertilizers, growing importance is attached to investigations directed towards a comparative evaluation of the different methods of using fertilizers in accordance with the biological characteristics of plants and with climatic, soil and other conditions.

Radioactive and stable isotopes are proving extremely helpful both in the study of theoretical questions relating to plant nutrition and in the solution of practical problems connected with the use of fertilizers. The tracer technique is being widely used in the Soviet Union in the study of methods of applying fertilizers. My paper reports some results of research carried out in the USSR with the help of the tracer technique into practical problems relating to the application of phosphorus fertilizers.

It is a well-known fact that the nutritional requirements of agricultural plants vary at different phases of their life. In order to evaluate the different methods of applying fertilizers, it is therefore important to know not only the total amounts of fertilizers utilized by the plants, but also the rate of utilization and the sources from which plants obtain their nutritional requirements at different stages of growth. A breakdown of this kind, which only became feasible with the development of the tracer technique, has led to a number of interesting discoveries which have an important bearing on the practice of fertilizer application.

The nutritional conditions of plants at different stages of growth are regulated not only, by the application of fertilizers at various times in the course of the vegetative process but also by the placing of the fertilizer in different positions in the soil. For example, a higher level of phosphate nutrition in the early stages of growth is secured by the local application of phosphates in the rows or craters in immediate proximity to the seeds.

One of the best forms of phosphorus fertilizer for application in the rows together with the seeds is granular superphosphate. Granular fertilizers are easier to apply and can conveniently be applied to the rows in small doses. In many soils, the phosphorus of a granular fertilizer is better assimilated by plants owing to the reduced absorption of phosphorus by the soil.

The advantages of a granular fertilizer, which are due to the reduced absorption of phosphorus by the

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* K. Kaindl. Versuch einer biophysikalischen Deutung des Pflanzenwachstums, Biochim. Biophys. Acta, *10*, 241-255. 1953.

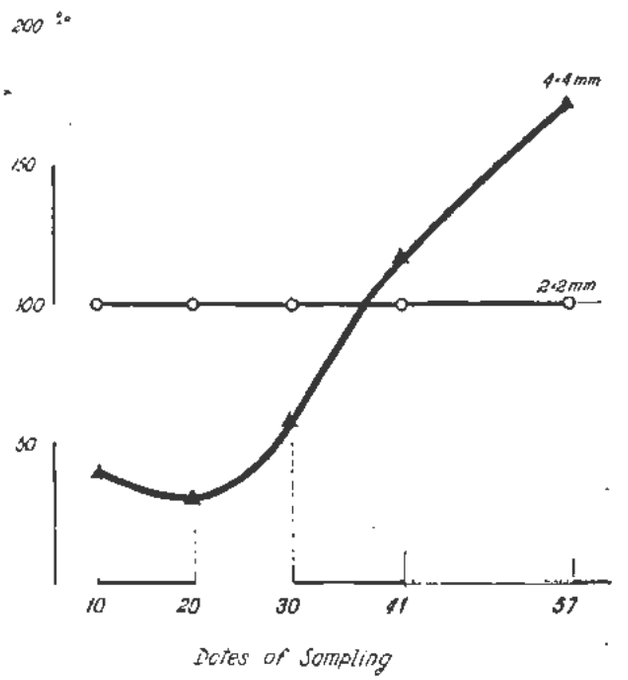
† Symposium on Growth Regulators, Wye (England), University of London, July 1955.

soil, can be most fully utilized by increasing the size of the superphosphate grains. If, however, the size of the grains is increased and the dose of fertilizer remains unchanged, the grains are more thinly distributed in the soil. As our experiments with labelled phosphates have shown, this leads to an uneven supply of phosphorus to individual plants, particularly in the early stages of growth when the root system is still poorly developed.

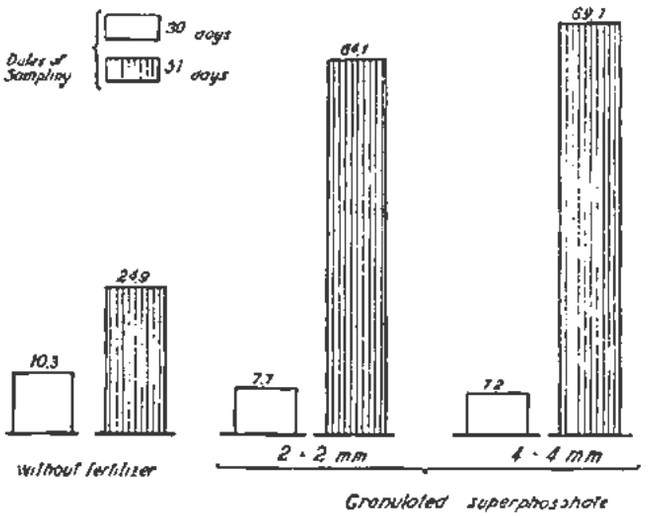
With the passage of time, as the root system enlarges and covers a greater area of soil, the density with which the grains are distributed in the soil becomes less important than factors affecting the fixation of the fertilizer phosphorus by the soil. Hence, in the later stages of growth, a better utilization by the plants of phosphorus from the larger grains is usually observed.

Slide 1 (Table I of P/694) shows the results of one of our experiments, in which the phosphorus uptake of plants from grains of various sizes was observed by means of an isotopic tracer. During the first 30 days of the experiment, the plants took up 2–3 times more phosphorus from the small grains, whereas they later showed a definite preference for the large grains. This relationship is shown particularly clearly in Slide 2 in which the amount of labelled phosphorus taken up by the plants from the small grains is taken as 100.

Our experiments established the fact that granular superphosphate fertilization has a strong influence on the assimilation of soil phosphorus by plants; it is significant that this influence varies at different periods of growth, as Slide 3 indicates. It may be inferred from these results that the main factor governing the increased utilization of soil nutrients in the experiments where granulated superphosphate



Slide 2



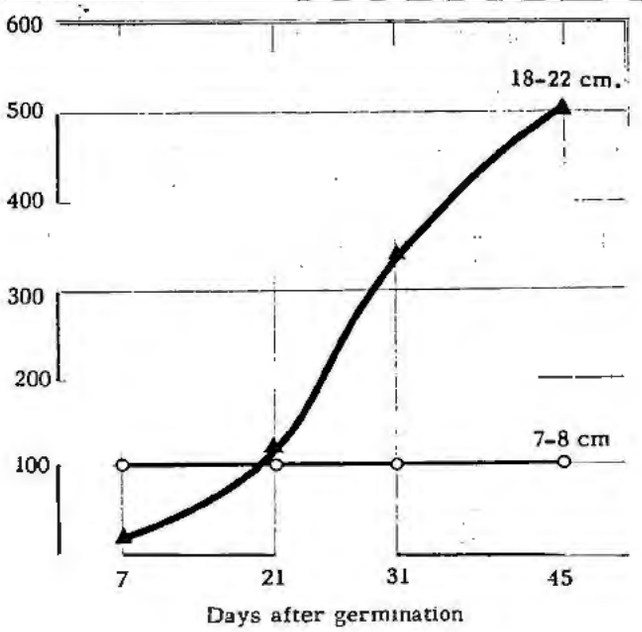
Slide 3. Assimilation of soil phosphorus by plants

was applied was the superior development of the root system in plants supplied with phosphorus in the early stages of growth by means of fertilizer.

The results of these experiments clearly reveal an important specific effect of the row application of fertilizer—it results in an immediate improvement in phosphorus nutrition in the early stages of growth and so creates conditions for a fuller utilization of soil phosphates by plants in later stages of growth.

With the help of the isotope technique, precise information was obtained on how the depth at which fertilizer is applied affects the scale and rate of its utilization by plants. Generally speaking, application of the main dose of fertilizer at some depth promotes a fuller utilization of the fertilizer by the plants than shallow application. But the comparative amounts actually absorbed of fertilizers applied at different depths change as a function of time. Experiments with labelled phosphates have shown that, in the initial stages of growth, plants take up more nutrients from fertilizers placed at little depth, but that this situation subsequently changes—in a number of experiments this occurred as early as two weeks after germination. This is illustrated by the results of a field experiment with oats which are shown in Slide 4. Here the amount of phosphorus taken up by the plants from fertilizer applied at a depth of 7–8 cm is taken as 100, the curve showing the amount of phosphorus taken up by the plants from fertilizer placed at greater depth, 18–22 cm.

The results of experiments with labelled phosphates have thus given us a better appreciation of the advantages of combining these two methods of applying fertilizers—application to the rows (or craters) at the time of sowing and the application of the main dose at some depth. With the same total dose of fertilizer, the combination of row and main dose application is conducive to the best utilization both of the fertilizers themselves and of the soil phosphorus, and to improved plant growth. The results of the field experiment given in Slide 5 (Table IV of P/694) testify to this.



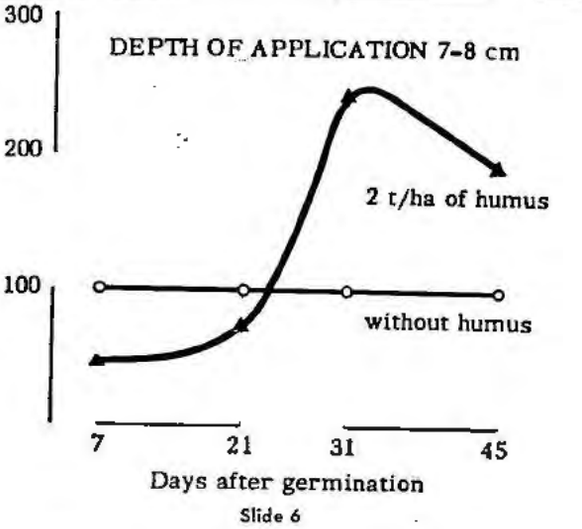
Slide 4

Experiments in which the isotope technique was used to make a separate, differentiated calculation of fertilizer up-take by plants where different methods of application were combined, have provided information on changes in the comparative utilization by plants of different sources of phosphorus depending on the other fertilizers used and, in particular, on liming.

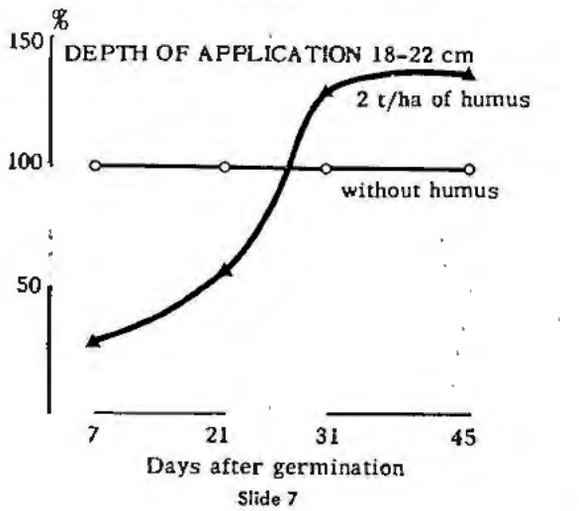
Large-scale production tests covering hundreds of thousands of hectares are being carried out in the Soviet Union on methods of combining the application of mineral and organic fertilizers with a view to increasing their effectiveness. The combination of phosphorus fertilizers with small quantities of an organic substance usually increases the phosphorus up-take of plants, particularly in soils with a high phosphate-fixation capacity.

Experiments with labelled phosphates have led to new discoveries in this field. Where these phosphates were applied together with an organic fertilizer, the effect on the up-take by plants of phosphorus from the superphosphate was found to vary at different times after application. In most cases, the beneficial effects of the organic substance increased with time; in the early stages of growth, instances of a definite decline in the use by plants of phosphorus from the organic-mineral mixture were even observed, as indicated by the figures relating to our field experiments given in Slides 6 and 7. These figures show that both where the fertilizer is placed at a depth of 7–8 cm and at a depth of 18–22 cm, the same phenomenon is observed—an increase in the beneficial effects of the organic substance on plant assimilation of phosphorus from the superphosphate with the passage of time.

The information obtained points to the desirability of trying out a system of applying fertilizers combining the application of a small quantity of phosphorus to the rows with the application of an organic-mineral fertilizer at depth.



Slide 6



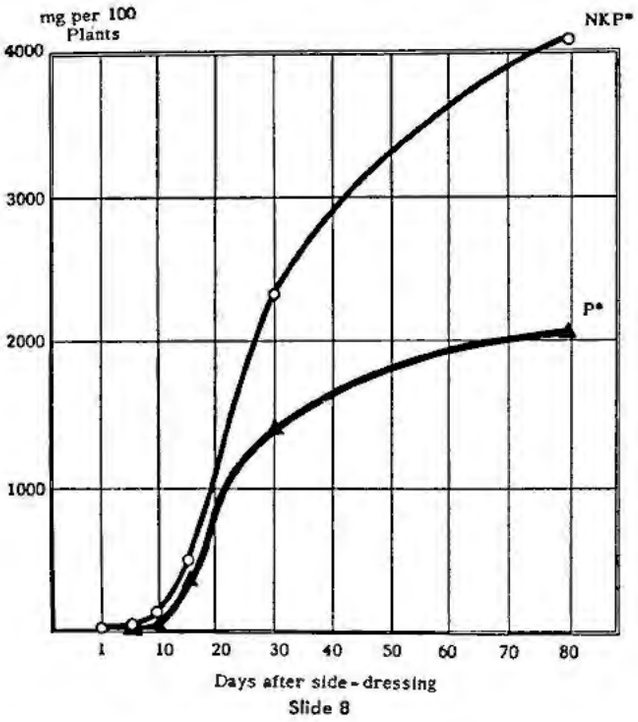
Slide 7

The use of isotopes has brought many new developments to the study of supplementary methods of regulating the nutritional conditions of plants by root and foliar fertilization. This method alone enables us to determine the interval between the time at which the supplementary fertilizer is applied and the time at which its intensive utilization by the plants begins.

Slide 8 shows the results of a field experiment with sugar beet. Information obtained from experiments of this kind permits a more accurate determination of the best time to apply fertilizers during the vegetative process, depending on actual physical conditions.

At the present time large-scale experiments covering thousands of hectares are being carried out in the Soviet Union with the foliar fertilization of sugar beet, cotton bushes and other crops. Here again, the tracer technique is proving helpful in the development of this new method and its introduction into general practice.

Many experiments have been made in which the entry into and movement within the plant of labelled



Slide 8

phosphorus applied by the foliar method have been closely followed. It has been shown that the labelled phosphorus introduced into plants by this method passes comparatively rapidly from the treated leaves into other parts of the plant and is soon involved in the metabolic process.

All these investigations are helping us to find the right answers to practical problems connected with the introduction of the most advanced methods of regulating the conditions of plant nutrition with a view to raising the yields of agricultural crops and improving their quality.

The paper submitted to the Conference by A. V. Sokolov (P/695) describes the results of research which is also of direct relevance to the practice of phosphorus fertilizer application.

A correct understanding of the relative assimilability of the various types of fertilizers is essential to the rational planning of their production and use. It is particularly important in the case of phosphates, the varying availability of which has an important bearing on the effectiveness of phosphorus fertilizers.

The use of an isotope tracer for the study of the degree of assimilability of the different forms of phosphorus fertilizers has permitted the development of a new method—the so-called selective phosphate uptake method.

This method is based on the principle of applying the forms of fertilizer to be compared not in different, but in the same pot or plot. In effect, therefore, the plant itself is being asked to indicate the form it takes up by preference when both forms are present at the same time in the same pot or plot. Under such conditions, the form which is more available for assimilation by the plant is the one most likely to be taken up by its roots.

The amount of phosphorus taken up by the plant from each form of phosphate is determined by labelling one of the forms; in one variant of the experiment, the control form is labelled, the form under investigation being unlabelled; in another variant, the control is unlabelled and the other labelled. The assimilability of the two forms of phosphate is therefore compared under absolutely ideotical conditions, the only difference being which of the two fertilizers is first given the radioactive label. The selective up-take method permits the detection of differences in the assimilability of phosphates which are not detectable by other methods of comparison.

The paper discusses the feasibility of evaluating the assimilability of soil phosphates by means of the isotope technique. Following methods similar to those used by Larsen, Fried and Dean, the author draws attention to the fact that the application to the soil of a soluble labelled phosphate alters the composition of the soil phosphates. The larger the dose of fertilizer, the greater its effect on the availability of the soil phosphates by the plants. The application of the smallest possible quantity of the labelled phosphate is therefore recommended—not more than 1 mg of P_2O_5 containing from 10–100 microcuries of P^{32} per kg of soil. The assimilable phosphate content of the soil can be calculated according to a formula similar to that proposed by Fried and Deao, if the labelled phosphate applied and the assimilable soil phosphates are utilized to the same extent.

In point of fact, however, the soil contains many different forms of assimilable phosphate, the degree of whose utilization by plants varies and depends on a number of conditions relating to the conduct of the experiment.

Further evidence of the arbitrary character of the results obtained by this method is provided by the fact that the relative proportions of fertilizer phosphate and soil phosphate used by plants change during the vegetative period, as a number of authorities have pointed out.

The isotope technique has been used in the chemical analysis of soils to determine the assimilable phosphate content. As we know, when the assimilable soil phosphates in the soil are isolated by means of different extraction techniques, a secondary precipitation and dissolution of the phosphates occurs. The final result of these processes is a balanced concentration of phosphorus in the extract—an exchange between the solid and liquid phases in the broad sense of the term. By introducing a radioactive isotope we can determine the amount of the substance in the solid phase which participates in this exchange reaction.

It might be assumed that soil phosphates capable of an exchange reaction with the phosphate ions of the solution represent forms assimilable by plants and that, if they are present in the soil, the latter can be said to be supplied with phosphorus. Experimental work has not borne out this assumption, as demon-

strated by the results of experiments in which the quantity of ordinary and labelled phosphorus passing into the different extracts and the quantity of labelled phosphorus taken up by the plants were compared under greenhouse test conditions.

I would point out that this conclusion is endorsed in the paper submitted to this Conference by R. Scott Russell *et al.* (P/460).

There is one more point to which I should like to draw attention. The use of labelled phosphates in field and greenhouse experiments provides a new method of determining the so-called coefficient of utilization of fertilizer phosphate without recourse to the usual differential method which is based on the tacit assumption that the utilization of soil phosphate by plants is the same whether fertilizers are applied or not. This assumption does not, however, correspond to the facts, as Mr. Spinks also points out in his paper (P/10). Where the dose of fertilizer is increased, the coefficient of utilization as determined by the isotope method differs substantially from the coefficient as determined by difference. Professor Sokolov's paper indicates that a similar discrepancy occurs if a number of soils with varying assimilable phosphate contents are used (Table IV of P/695).

The table shows the results of an experiment with soils taken from plots used for field tests over a period of years, to which varying doses of fertilizers had been applied with the result that their assimilable phosphate content varied. The figures make it clear that the larger the assimilable phosphorus content of the soil, the larger the amount by which the quantity of labelled phosphorus entering the plants from the fertilizer exceeded the phosphorus lost as determined by difference.

The table also shows that the discrepancy between the actual coefficient of fertilizer utilization and the coefficient as determined by difference is also affected by the method of applying the fertilizer. If the fertilizer is applied locally, this discrepancy is particularly striking owing to the mechanism of selective up-take.

All these facts point to the need for a far-reaching verification of existing concepts of the coefficient of phosphorus fertilizer utilization by means of experiments with labelled fertilizers carried out under varying soil and agro-technical conditions.

Mr. KEDROV-ZIKHMAN (Byelorussian SSR) presented paper P/716, as follows:

Radioactive isotopes are being increasingly widely used as tracers in the study of the most varied problems and in particular, in the investigation of questions of plant nutrition. The radioactive isotope of cobalt, Co^{60}, has, for example, played an important part in our study of the effects of cobalt on agricultural plants.

Research carried out by the Institute of Socialist Agriculture of the Academy of Sciences of the Byelorussian SSR and by the Soil-Liming Laboratory of the All-Union Institute of Fertilizers, Agro-technology and Soil Science of the Lenin Academy of Agricultural Sciences has established that, contrary to the widely held opinion, cobalt can have a beneficial effect on the growth, development and yield of agricultural plants.

In a greenhouse experiment on limed sod-podzol soil, cobalt had the effect of raising the total yield as well as the yield of kok-saghyz roots and of increasing rubber accumulation. I carried out this work in collaboration with O. E. Kedrova-Zikhman and the results were published as far back as 1942.

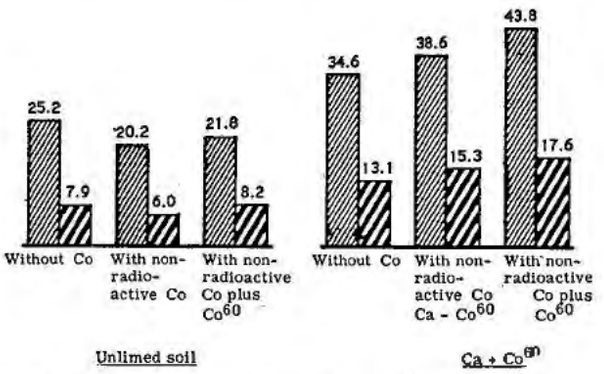
The results of this experiment were subsequently confirmed for red clover, flax, sugar beet and winter rye in greenhouse and field experiments carried out in collaboration with A. N. Protashchik and P. E. Rozenberg on sod-podzol and lowland peat-bog soils, and also in a field experiment with winter wheat carried out by A. P. Kevorkov. These experiments showed that cobalt has a beneficial effect in acid sod-podzol soils, mainly when limed, and on lowland peat-bog soils, even when unlimed.

The results of our experiments further showed that the beneficial effect of cobalt is greater in the case of the reproductive than of the vegetative organs of the plant, that it substantially accelerates the maturation of seeds, increases the fat content of the seeds and the fibre production of flax, and raises the sugar content in the roots of sugar beet.

With a view to making a more detailed study of the effects of cobalt on agricultural plants and in particular of the causes of the variations in its behaviour according to whether the soil is limed or unlimed, I have carried out a number of experiments with cobalt-60 since 1951 in collaboration with A. N. Kozhevnikova and, since 1954, with A. F. Agafonova also. These experiments consisted of greenhouse tests most of which were carried out in standard-size Mitcherlich pots and the corresponding laboratory tests, in particular, a radioactive assay.

In the greenhouse experiments, Co^{60} was applied to sod-podzol soil both limed and unlimed, in addition to ordinary cobalt. Both the ordinary cobalt and its radioactive isotope were applied in the form of a nitrate, the amount applied being 6 or 7 mg per pot in some cases and in others, 20 mg, the radioactivity varying from 160–480 μc. The experimental plants were barley, white mustard, turnips and radishes.

As the figures given in Slide 9 indicate, in this basic greenhouse experiment with barley, as in our previous greenhouse and field experiments with other crops, on unlimed soil neither the total yield nor the grain yield of barley showed any increase under the influence of cobalt and in most cases actually declined. (The large columns show the total yield, the small ones the grain yield.) On limed soil, on the other hand, cobalt caused a substantial increase both in the total yield and in the grain yield. Furthermore, the beneficial effect of cobalt was greater in the pots in which Co^{60} was applied in addition to ordinary cobalt than in the pots in which no radioactive Co was applied.



Slide 9. Effects of non-radioactive cobalt and Co^{60} on barley crop (6 mg per vessel). Vegetal experiment, 1951

A number of other experiments was carried out with barley, results of which confirmed that correctly selected doses of Co^{60} can increase plant yield.

In connection with these experiments, a radinactive assay was made for the purpose of investigating the processes of cobalt conversion in the soil, its nptake by and distribution among the various organs of the barley. For this purpose, the radioactivity was determined, first, of a certain quantity of the liquid accumulating in the saucers of Mitcherlich pots when the soil in the pots was watered and, secondly, of sample plants obtained from the greenhouse experiments.

The results of these experiments (Table IV of P/716) indicate that, on interaction with lime applied to the soil, soluble cobalt compounds form immobile compounds not readily assimilable by the plants. When a cobalt fertilizer is applied to a limed soil, both the fertilizer and the cobalt compounds in the soil are converted into a form which is less mobile and less assimilable (though nnt completely unassimilable) by the plants. If, however, a sufficient quantity of micro-fertilizers is applied, the nutritional state of the soil is improved in respect of cobalt. But the results of our investigations indicated that the concentration of cobalt compounds assimilable by plants is considerably less in limed than in unlimed soil. The figures given in Slide 12 (Table V of P/716) show that much less cobalt enters the plant from limed than from unlimed soil. It was further established that the cobalt content in the different organs of the barley varied and was susceptible to wide fluctuations depending on the concentration of mobile cobalt compounds in the soil. Another discovery was that the accumulation of cobalt was particularly high in the roots and lowest in the seeds.

The figures given in Slide 11 show that white mustard plants, which are extremely sensitive to any increase in acidity, developed very poorly in unlimed soil and they were therefore not used for further investigations. They developed well in limed soil, while in pots with a mixture of ordinary cobalt and Co^{60} (applied in the larger dose) a higher seed yield and a smaller total yield were obtained than where the same dose of ordinary cobalt alone was applied. The large columns show the total yield and the small ones, the seed yield.

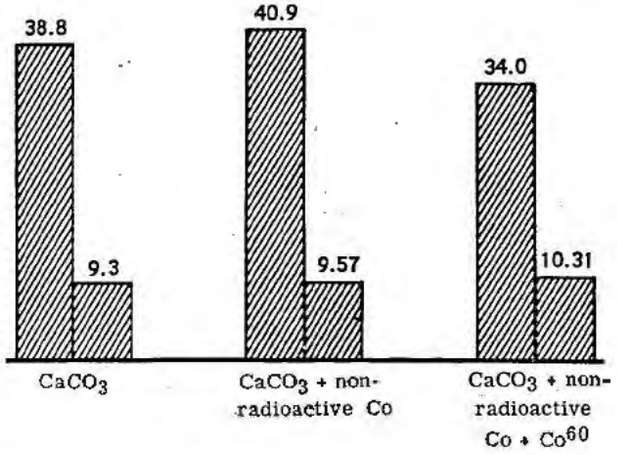
The radioactivity in the various organs of the plants in the mustard crop was assayed. On the basis of the counts recorded, it was possible to assess the relative cobalt content of individual plant organs. The figures obtained indicated that, in the case of both white mustard and barley, much less cobalt entered the plants from limed that from unlimed soil.

The results of our radioactive assay show that in plants grown in limed soil with a mobile cobalt content which, though generally speaking not high, is sufficient for normal development (as in the case of the barley), the maximum accumulation is in the roots and the minimum in the seeds. In unlimed soil with a high concentration of mobile-cobalt, however, the cobalt of the leaves was also very high, almost as high as in the roots.

In our experiments with turnips and radishes, the application of the larger dose of cobalt to the soil did not in most cases have any appreciable beneficial effect on the plant yield either in limed or unlimed soil. Under the influence of Co^{60}, there was a decline in yield in all cases, but the plants developed quite normally and were therefore used to carry out further experiments. In particular, the radioactivity of the radishes was assayed for the purpose of obtaining information on the processes involved in the movement of cobalt into the plant and of its distribution among the various organs.

Both in the experiments with limed and in those with unlimed soil, the highest cobalt content was found in the radicles. In the plants grown in unlimed soil, a high content was also found in the leaves.

Thus, the results of the experiments we carried out with different plants that are reviewed in this paper show that, where the snil contains concentrations of assimilable cobalt compounds which are small but sufficient for the normal development of plants, the cobalt mainly accumulates in the roots; where the concentrations are high, it also accumulates in the



Slide 11. Effects of non-radioactive cobalt and Co^{60} on white mustard crop. Vegetal experiment, 1954

leaves. These results point to the fact that cobalt participates in the physiological processes that take place in the roots—presumably in the formation of the enzymes of which it is a component, as the review by McIlroy also indicates.

An analysis of the results of our experiments with various plants also shows that the effect of both ordinary and radioactive cobalt on plants largely depends on the biological characteristics of the latter.

In conclusion, I feel I should point out that the use of the radioactive isotope of cobalt has been of great assistance in elucidating the problems we have studied. Co^{60} and the radioisotopes of other microelements should therefore find wide application in any future research on the interaction of compounds of various microelements with the soil and their effects on agricultural crops.

DISCUSSION OF P/694, P/695 AND P/716

Mr. H. B. Tukey (USA): Mr. Klechkovski, your paper, and that of Mr. Kursanov of the other day, are most stimulating. Would you care to comment and elaborate on the extent to which foliar feeding is being done in the Soviet Union and what are the forms of phosphorus and nitrogen being used there?

Mr. KLECHKOVSKI (USSR): In addition to research into foliar nutrition both with and without the use of radioactive isotopes, large-scale production tests of this method are being made in the Soviet Union. These tests, mainly with cotton and sugar beet, are being carried out over an area of thousands of hectares.

In experiments with cotton plants, it was noted that the foliar application of phosphorus led to some reduction in the abscission of ovaries and hence, to an increased yield. But in the large-scale production tests of this method in the cotton-growing areas of the Soviet Union, it was not always found to be justified on soils that were richer and had previously been heavily fertilized with phosphorus. The conclusion therefore seems to be that the results of this method will vary according to soil conditions. In experiments with sugar-beet, large-scale production tests are also being made of the foliar application of phosphorus and potassium side-dressings. One of the beneficial effects of foliar fertilization is to increase the sugar content of sugar beet, particularly if the treatment is given during the second half of the vegetative period.

As regards forms of fertilizer, superphosphates are mainly used in our large-scale production experiments. The fertilizer solution is sprayed from the air in applying the side-dressing to both cotton-plants and sugar beet. A study of the comparative effectiveness of the various forms of phosphate salts, too, showed potassium phosphate to be more effective than calcium phosphate. As regards nitrate fertilizers, urea and possibly ammonium nitrate would appear, on the basis of the information available to us at present, to be one of the best forms of nitrate fertilizer for foliar nutrition. The technique of foliar fertilization has been less studied with nitrate than with phosphate fertilizers.

Mr. KAINDL (Austria) I should like to ask Mr. Klechkovski whether he has carried out any experiments with cereals using foliar fertilization, and what was the result of these investigations?

Mr. KLECHKOVSKI (USSR): Experiments have been made with cereals, but we attach less importance to them since cereals react most strongly to an increased level of phosphorus nutrition during the early stages of growth and their needs are largely met without the use of fertilizers. Favorable results were, however, observed in experiments with the application of a phosphate side-dressing to winter wheat and rye in spring, particularly where phosphorus and nitrate fertilizers were combined. In experiments in which the isotope technique was used, it was shown that, with the surface application in spring of phosphorus fertilizers to sowings of winter rye and wheat, the phosphorus is taken up not only through the soil and the roots, but also in part directly through the leaves.

Mr. MITSUI (Japan): I was very much interested in the paper presented by Mr. Klechkovski, and particularly as regards granular fertilizers. We too have had many studies on the same problem, and in this connection I should like to ask what has been the maximum availability of phosphoric acid, in percentage ever experienced in the USSR?

Mr. KLECHKOVSKI (USSR): When the coefficient of utilization of granular phosphorus fertilizers is determined with the help of the usual chemical method by difference, curious results are obtained in some cases. Where small doses of phosphorus fertilizers are applied to the rows, the coefficient of utilization is sometimes over 100 per cent. This is due to the fact that the application of fertilizer to the rows increases the uptake of soil phosphorus, and calculation by difference (i.e., the determination of the coefficient of utilization by the usual chemical method) produces an obviously inflated figure. Experiments in which radioactive phosphorus is used give more accurate results; a figure of over 100 per cent is never obtained, the coefficient of utilization being in the region of 50 or 60 per cent and sometimes a little higher.

Mr. A. C. SCHUFFELEN (The Netherlands): I would like to ask Mr. Klechkovski if he has made any observation on the influence of the rainfall and rain distribution upon the placement of fertilizers. In my opinion, the depth of the placement must depend on the water conditions in the soil, and as the water condition changes each year it seems to me very difficult to say what is the best placement.

Mr. KLECHKOVSKI (USSR): I entirely agree with that comment. Water conditions in the soil undoubtedly have a considerable influence on the effectiveness of fertilizers applied at different depths. The depth at which the fertilizer is placed is particularly important where the moisture is insufficient

and the top layer of soil becomes parched. But in most cases the best results with phosphorus fertilizers are obtained by applying a small dose with the seeds, the bulk of the fertilizer being placed at a depth. This combination would seem to be the optimum method in most cases, even under widely differing soil conditions.

Mr. EPSTEIN (USA): I would like to ask a question of Mr. Kedrov-Zikhman. If you obtain positive effects in crops from the application of cobalt to the soil, do you regard this as adequate evidence for classifying cobalt as an essential plant nutrient?

Mr. KEDROV-ZIKHMAN (Byelorussian SSR): If what is meant is the agro-technical effect, the action of cobalt on yields, then the answer must be in the affirmative, since in a whole series of our experiments, both greenhouse and field, on cereal podzol soil, favorable results were obtained (the yield of wheat and rye increased by 5 centners per hectare and of beet root by roughly 30 centners per hectare). There can thus be little doubt of the beneficial effect of cobalt in regard to increasing yield.

As regards sources of cobalt fertilizers, attempts are being made to process cobalt ore with a view to obtaining pure cobalt compounds. Agriculture will, of course, have to count on using waste products, and tests with some of these have given good results. The foliar application of cobalt side-dressings to plants makes it possible to reduce the dose to well below that required to obtain the same effect with applications to the soil.

Mr. A. GUSTAFSSON (Sweden): In Sweden, as in the USSR, we try to increase crop production by a better utilization of soil nutrients. As regards nitrogen manuring, we have produced mutants which utilize high nitrogen dressings better than the mother line. I would like to ask you whether in your country strains of, for instance, barley are known which utilize microelements differently and whether plant breeding in this respect is carried on?

Mr. KEDROV-ZIKHMAN (Byelorussian SSR): In our investigations, in addition to the methods I have described, we have also applied cobalt fertilizers by the foliar method and by that of soaking the seeds, the effect on the seed growth and development and the plant yield being observed. These methods gave favorable results. In general, I would repeat what I have just told Mr. Epstein, namely, that according to the results of our experiments, cobalt fertilizers have a favorable effect on yields and will obviously find practical application in the future. Does that cover your point?

The CHAIRMAN: Perhaps you could discuss this matter after the meeting.

Mr. L. EHRENBERG (Sweden): I should like to direct another question to Mr. Kedrov-Zikhman. Your positive effects of Co^{60} irradiation on crop production are interesting. By irradiating barley seeds with low doses for X-rays—about 500 roentgens—we obtain, in our experience, in some years a considerable increase of production and in other years no effect at all. Have you had similar experience? If your answer is yes have you an explanation of this? Finally, have you estimated the gamma dose stimulating the material in this manner?

Mr. KEDROV-ZIKHMAN (Byelorussian SSR): There are two points which arise out of the results of our experiments. The first is that when cobalt was used as a fertilizer, the reaction of different plants varied both in regard to ordinary cobalt and to cobalt-60.

The second is that, in the studies of the effect of cobalt-60 on plants made in addition to our main investigation, we came to the conclusion that, if the correct dose was selected, successful results could be obtained with radioactive cobalt, but that unfavorable results were occasionally obtained, where the dose was too large. Moreover, cobalt-60 does not have the same effect on all plants. With the same dose, favorable results were obtained in experiments with barley, less favorable with white mustard, while with turnips and radishes the radioactive cobalt had a detrimental effect.

Mr. H. B. TUKEY (USA) presented paper P/106, as follows:

Roots are commonly accepted as the principal nutrient absorbing organs of plants. That other parts and other organs of the plant, such as stems, leaves, flowers, and fruits might also be able to take up nutrients has long been indicated by certain horticultural practices. Yet only recently has this truth been demonstrated experimentally, and in this demonstration radioactive isotopes have played a most important part.

Thus, by the use of the isotope technique it has been shown that nutrients may enter even through the bark of dormant fruit trees in mid-winter. Within 24 hours of application during February and March with temperatures at or below freezing (32°F), radioactivity was detected within the branches 18 to 24 inches both above and below the points of application.

Slide 12 shows a dormant peach tree injured by winter cold. Then the tree was sprayed in the dormant season in early spring. Slide 13 shows the result in growth. The branch on the left is from a tree so treated. The one on the right is from a tree not so treated.

It is, however, of still more importance that nutrients may enter through the leaves of plants. It is now established that the surface of a leaf is not the impervious structure described in many textbooks. Instead, it is structurally well equipped to absorb materials through both the upper and lower surfaces. In addition, the leaf area of a plant is considerable, one-tenth of an acre for a single 12-year-old apple tree or approximately ten times the spread of the branches.

Now it may be anticipated that foliar absorption of nutrient is affected by temperature, light, the pH

Slide 12

and the carrier of the treating solutions, and various additive chemicals, as well as the species of plant involved, the morphological nature of the absorbing organ, and the nutritional status of the plant. I must emphasize here that in this paper I am dealing primarily with horticultural crops, that is, fruits, vegetables, flowers and ornamental plants.

Briefly, and in general terms, phosphorus uptake is most rapid from an acid solution, whereas for potassium and rubidium it is more rapid from an alkaline solution. Absorption is facilitated and possible leaf burning avoided if recommended dosages of a wetting agent are added to the spray formulation. Both sides of the leaf will absorb nutrients. The petiole is especially efficient. The opening and closing of stomata is of little significance, since foliar absorption is observed during the night as well as during the day. Sucrose depresses uptake in some instances. Boron does likewise in some instances. The many factors which have been found to influence the absorption of foliar applied nutrients indicate, however, more than a mere passive entry of nutrients through leaf surfaces into the plant.

Urea is one of the most useful and well known nitrogenous fertilizers for leaf application. With some horticultural crops, as an apple tree, the entire requirement for nitrogen can be satisfied by a few appropriately timed sprays.

The first step in the utilization of the nitrogen in urea presumably is hydrolysis by the enzyme urease, splitting the urea molecule and giving ammonia and carbon dioxide, as follows:

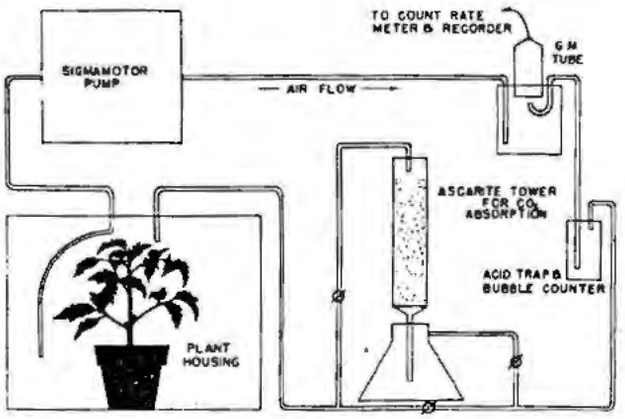
$$\begin{matrix} NH_2 \\ \diagup \\ C{=}O \\ \diagdown \\ NH_2 \end{matrix} + H_2O \xrightarrow{\text{Urease}} 2\ NH_3 + CO_2$$

As a measurement of urease activity, and thereby the rate of hydrolysis and possible utilization of urea, radioactive C^{14} urea may be employed and the rate of evolution of radioactive carbon dioxide determined in such an apparatus as shown in Slide 14. One can see here a tomato plant to which urea is applied in a known amount to a given area. The atmosphere of this closed system is then removed through the pump, passes on through a counter tube, revolves around, and the total count is secured in this way. We have done this in a great many plants with some very interesting results.

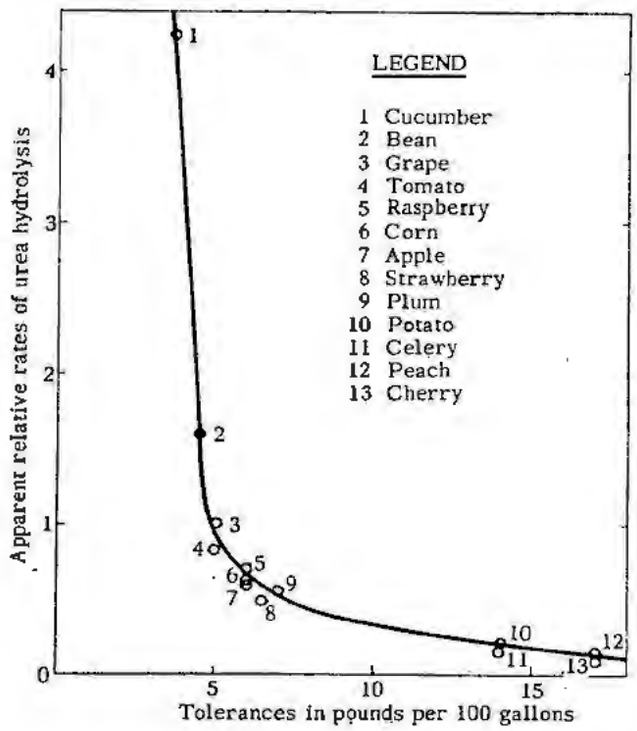
Using this technique, the apparent relative rates of urea hydrolysis (utilization) of foliar applied C^{14} urea have been determined for a number of vegetable and fruit crops. Further, these rates have been plotted against field and greenhouse tests of observed toxicity of urea to the foliage when applied at various concentrations.

From such studies it is possible to predict a tolerance of various plants (Slide 15). Thus, for plants which utilize urea most rapidly, such as the cucumber, the toxicity is at a low level concentration of perhaps two or four pounds per 100 gallons. On the contrary, for such plants as the peach and cherry, which utilize material very slowly, the concentration will be up to 15 and perhaps 20 pounds

Slide 13



Slide 14



Slide 15

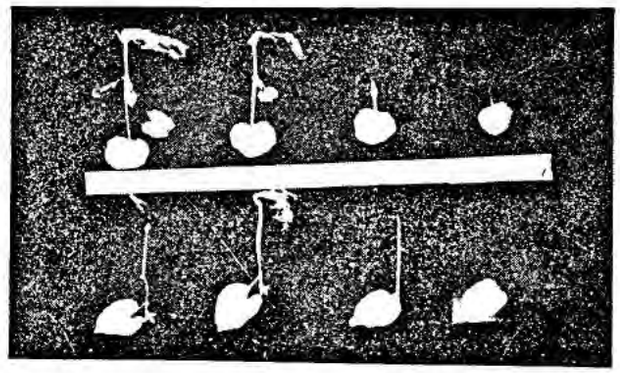
of urea per 100 gallons of water. Therefore, by this method we have a fairly easy method of determining the utilization of urea, what crops will respond and how they will react to the material.

Such highly mobile elements as nitrogen, phosphorus, potassium and rubidium when applied to aerial plant parts are readily translocated, both upward (acropetally) and downward (basipetally) (Slide 16) and at a rate comparable with that which follows root absorption.

Phosphorus accumulates rapidly in growing meristematic regions such as root tips, vegetative growing points, flowers, fruits and seeds, and even in the root nodules of bean plants (Slide 17) within just a matter of hours.

In fact, up to 95 per cent of the phosphorus applied to tomato may be utilized by the plant. Yet the total amount of nutrient taken up by the plant is of less significance perhaps than the critical time of application in the development of the crop. When P^{32}-labelled phospboric acid was applied to the soil in a band in the root area, and compared with sprays to the above ground parts during early flowering, it was found that the foliar sprays were 10 to 25 times more efficient than root applications in terms of phosphate uptake based on the percentage applied.

While all of this is true in the case of phosphorus, where the application to the leaf is 20 to 25 times more efficient than root application, the performance of calcium is quite different. But in the case of phosphorus, foliar feeding represents the most efficient method of applying fertilizers to plants that we yet know.

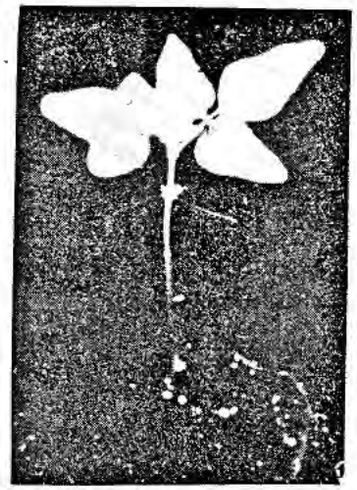


Slide 16

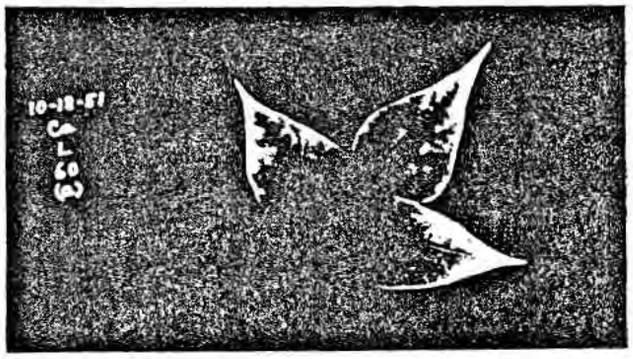
Calcium, strontium and barium do not move easily from the absorbing plant part, and downward transport is negligible, as shown in Slide 18 for foliar applications to a bean plant.

The absence of basipetal transport of radiocalcium is depicted also by the strawberry at the top of Slide 19 (Fig. 2 of P/106). The bottom picture is simply a photograph of the strawberry plant before the calcium was applied to the root. You will notice that the calcium moved across to the daughter plants but that there was little movement, if any, downward into the roots. These roots must of course receive their calcium of the root uptake from the soil itself. The pattern for phosphorus, potassium and calcium, as depicted in Slide 20 (Fig. 1 of P/106) which is an autoradiogram for the bean plant, is duplicated for both root and foliage without exception in 15 or 20 crops that we have used under many conditions.

To study further the movement of different nuclides within the plant, lateral shoots from two tomato plants were grafted as shown in Slide 21, so that the polarity of the two grafted portions were in reverse position to each other. To one plant of a grafted combination, P^{32} was applied through the roots by addition to the soil medium, as shown in the slide. Another combination was similarly treated, but with Ca^{45}. Assays were made at various hourly



Slide 17



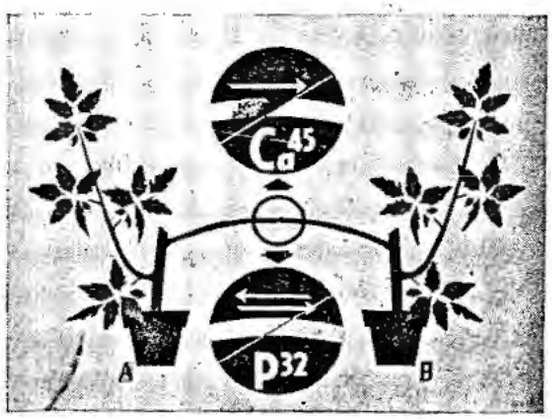
Slide 18

and daily intervals of portions of the plants on either side of the graft union. P^{32} was found to move rapidly across the union. To this, two plants were grafted together in reverse polarity. The phosphorus applied moves back and forth, as indicated in the diagram, between the two plants. As calcium was applied to a plant, it moved up. When it came to this point, it was effectively blocked. The accumulation of calcium was massive on the side to which it was applied, and it was negligible on the other side.

Since, as has been shown, nutrients may enter the leaf, it would seem plausible that under appropriate conditions they may be lost from the leaf. This hypothesis has been subjected to experimental evaluation by allowing plants to absorb isotopically labelled nutrient through their roots or through cut stems and then exposing them to simulated rainfall from a mist atomizer.

When measured quantities of radiophosphorus were applied for 48 hours through the bases of cut stems of bean, sweet potato, and poinsettia, and when the plants were then subjected to foliage leaching for 48 hours, up to 12.8 per cent of the absorbed P^{32} was lost from the plant. On the other hand, when the P^{32} was applied to the soil so that the material entered through the roots, no loss of P^{32} from the leaves was observed.

The result was that radiopotassium and radiorubidium were different. Following a 12-hour absorption of these ions by the roots, subsequent leaching for 4 hours was quite high, removing up to 71 per cent of the K^{42} and 14 per cent of the Rb^{86}.



Slide 21

These data show that loss of nutrients by leaching from leaves does occur. The results suggest that the leaching of nutrients from plants in humid areas or during periods of frequent heavy rainfall may be comparable to the losses of some ions from soil, and that poor setting of fruit, the occurrence of mineral deficiencies, and the poor cropping and poor maturity of plants, may be associated with the interesting phenomenon which has been revealed by radioactive isotopes.

Mr. A. J. RIKER (USA) presented paper P/105, as follows:

Radioisotopes have helped us to explore the vital sap systems of oak trees that carry water, nutrients, and sometimes disease-causing micro-organisms. These results confirm those reported elsewhere and in some of the exhibits which we have seen here at the Palais. They have been especially helpful in exploring the root grafts which so frequently join nearby trees in the black oak group. This work was done particularly by my associate Dr. Kuntz, and by Messrs. Beckman, Berbee, Parmeter and Drake. We secured technical advice about the isotopes from Dr. Burris.

Solutions of iodine-131 or of rubidium-86 were used. These were introduced into northern pin oaks—*Quercus elipsoidalis* Hill—as follows. A roofing paper cone was attached to a tree and made water-tight with roofing cement. Water was placed in the cone and a chisel was placed under the water. With a hammer blow, a cut was made into the last 3 annual rings of wood. Then the isotope was mixed into the water.

The rate of movement was detected with a portable instrument. Sometimes we climbed the tree with an extension ladder.

Sometimes we cut branches with a pole pruner to secure sample branches. In northern pin oaks with bright summer sun and low humidity the isotopes moved upward between 1.5 and 3 feet per minute. They were usually well distributed in the tops of oaks 35 feet high within 20 minutes. In bur and white oaks the rate was as fast, but the distribution was limited. Radioactivity appeared only in narrow, vertical streaks originating in the chisel cuts. These twisted with the grain of the wood.

During the night the upward movement was greatly reduced, being only 0.05 to 0.1 ft/min.

On a day with dense clouds over the sky, movement was reduced to 0.4 feet per minute. If the leaves were wet with dew or rain it was only 0.2 feet per minute.

While the trees were dormant, movement was quite slow, about 0.03 feet per minute, and mostly on the south side. It stopped altogether when the trees were frozen.

The downward movement of the isotopes during the summer and their detection in neighbouring trees led to a study of root grafting. The root grafts appeared to be quite common among oaks in the black oak group, but rare among oaks in the white oak group. Grafts occurred between roots of different sizes (see Fig. 1 of P/105). To guide the excavation of the roots, with functional grafts to other trees, isotopes were extremely useful. Potassium bromide-82 with its short half-life and strong gamma radiation was especially useful in selecting experimental grafted roots. Comparable studies were made with dyes and poisons because of the expense of isotopes (see Fig. 2 of P/105).

In one representative experiment, where trees were approximately 10 feet apart, treatment of one tree disclosed that 5 nearby trees were grafted to it. When these in turn were treated, 21 additional trees proved to be joined. When these were treated in turn, 10 more were joined. Thus, 36 trees were joined directly or indirectly to the first. Underground, these trees apparently were a united community.

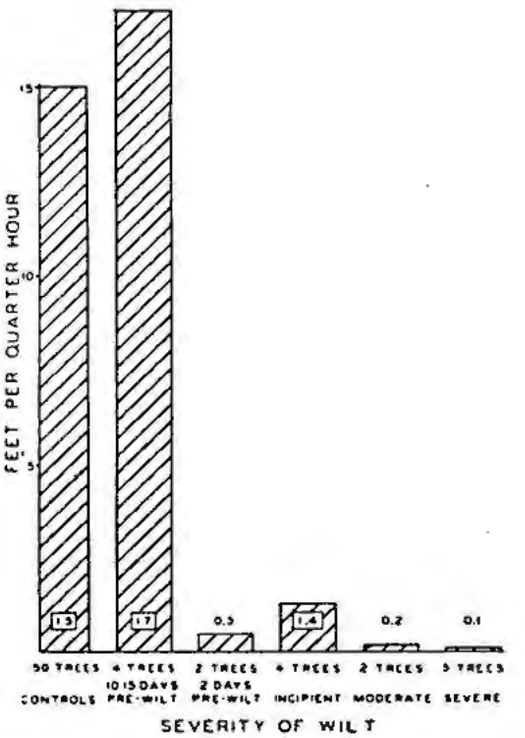
After the root systems of 3 nearby northern pin oaks were partly excavated and washed out, by water from a fire-fighting truck, 28 root grafts were observed between the three trees (see Fig. 3 of P/105). Many additional grafts were found between roots of the same tree.

When a tree was cut, much of its root system commonly was adopted by neighboring trees through root grafts (see Fig. 4 of P/105). This has interesting implications for the growth of trees which would take too long to discuss now.

The direction of sap flow through root grafts between trees was interesting. Grafted roots were cut, connected with rubber tubes, and the isotope introduced. In limited studies made during the summer in the daytime the flow more often passed from the dominant to the suppressed tree than the reverse.

In a similar way, isotopes have clarified the way the disease called oak wilt develops and spreads. It is caused by *Endoconidiophora fagacearum* Bretz. From the air the spread of oak wilt about infected trees is especially clear. From the ground one often sees in succession a dead tree, a dying tree and an apparently healthy tree.

We have compared the sap flow in an infected tree with that in a healthy tree (Slide 22). We found that the flow became reduced by 85 per cent 3 to 4 days before foliage wilt appeared. It was reduced by 99 per cent when leaves were wilted. The reason for the wilting is apparent from microscopic examinations. The sap conducting vessels are open in a healthy tree, but in a diseased tree these vessels are closed by tyloses which the fungus has stimulated to grow into the vessels. These tyloses prevented the flow of sap and the movement of isotopes in the sap stream.



Slide 22. Summary of upward movement by radioactive Rb_2Co_3 in wilting northern pin oaks

While the vessels were still open the sap stream was able to carry spores throughout the open ducts. This was determined in limited experiments with spores of the oak wilt fungus that were doubly treated with isotopes. They were treated first with silver-110 nitrate then thoroughly washed. Then they were treated with sodium iodide-131 and again thoroughly washed. The spores with insoluble silver-110 iodide-131 were killed, of course, and were heavier than living spores. But their movement with the sap stream through small branches and into leaves was easily followed with a counter. Such movement of the fungus through root grafts was doubtless responsible for the spread of the disease to nearby neighboring trees. The distance over which experimental trees had root graft connections varied with species, age, and circumstances. The maximum distance observed with northern pin oaks was 52 feet. The local control of oak wilt was indicated by the movement of infection through root grafts.

With valuable trees the root connections were broken by digging ditches or by mechanical means. Sometimes we used a tractor drawing a blade 36 inches long going through the ground to break the root connections. A depth beyond 36 inches in our plots passed the point of diminishing return.

With trees of less value, a ring of healthy trees 25 feet from infected trees were killed with poisons or by other means. Numerous trials have shown both methods to be effective. The details of what to do have depended on circumstances and costs.

With effective local control we have practically succeeded in removing sources of infected material and in preventing further spread of the disease.

The importance and frequency of root grafting were quite different with different species and under different circumstances. When it has occurred frequently it seemed to have important implications both for healthy and for diseased trees.

DISCUSSION OF P/106 AND P/105

Mr. TUKEY (USA): My question is addressed to Mr. Riker. Both he and Mr. Spinks have mentioned the cross-transfer of nutrients within a tree. Does cross-transfer, or lack of cross-transfer, differ from species to species and from plant to plant?

Mr. RIKER (USA): Yes, it seems to vary greatly, depending upon the species. In the trees in the red oak group, including those on which we worked, the cross-transfer was usually very easy. However, in trees in the white oak group there was no cross-transfer. The material entered the tree at a single point and went up to the top of the tree in a relatively straight line, diverging only as the grain of the wood twisted.

Mr. RIKER (USA): I wanted to ask Mr. Tukey whether he has any information about the elution of organic materials from the leaves of trees and, likewise, whether the washing out of mineral salts or organic materials from the leaves of trees had any effect upon winter injury?

Mr. TUKEY (USA): Our work has been almost entirely with mineral nutrients. However, I know there are organic materials that come from leaves. My information comes from Dr. Sven, Odense (Denmark), whose laboratory I recently visited. I know—I think that this is no divulgence of any information preliminarily—that he has prepared a paper for the International Horticultural Congress to be given in September in Holland, and he has shown me that there are really very large amounts of organic materials which are leached from fruit trees.

Mr. K. KAINDL (Austria): I would like to make some additional remarks on the very interesting paper of Mr. Tukey.

In our laboratory, foliar application of primary potassium phosphate was investigated and some parameters influencing the intake of the nutrient were studied. In two respects, our results are different from those represented here. We were able to prove that a decrease of temperature from 23°C to 16°C gives an increase of the intake of about 50 per cent. Another discrepancy is shown by the result of the intake of the nutrient by the upper and lower part of the leaf. We obtained the contrary result but I agree that the stomata are of no importance. Maybe this discrepancy is founded on the different test plants. We used *Solanum nigrum, Galinsoga parviflora* and wheat.

The main purpose of our investigations was to make evident the functional connexion between the stage of development of a plant and the intake through the leaves. We found that a significant increase of the intake occurs in the early stage and at the vitality maximum—that means at the point of the highest growth rate.

This is only a rough account which has to be modified when the concentration of nutrient varies over some order of magnitude, but I do not want to go into details here.

Finally, I would mention that the combined foliar soil fertilization may produce the highest crop, as our experiments with *Solanum nigrum* showed.*

Mr. TUKEY (USA): Might I reply to that comment about temperature. I would agree that if temperature is a matter of drying out, then I would think there might be less uptake at a low temperature. However, our experience is that if we maintain the humidity, then the increase in the temperature gives, as I indicated here, a greater increase with each increase.

* K. Kaindl: Untersuchung über die Aufnahme von P^{32} markiertem primärem Kaliumphosphat durch die Blattoberfläche; Die Bodenkultur, 7, 4, 324–353 (1953). Also Foliar Fertilization with Phosphatic Nutrient Labelled with P^{32}, Second Isotope Conference, Oxford 1954.

Session 15C

RADIOACTIVE ISOTOPES IN PHYSIOLOGY AND BIOCHEMISTRY

LIST OF PAPERS

(Continued overleaf)

LIST OF PAPERS

(Continued)

Radioisotopes in Animal Physiology and Nutrition - Mineral Metabolism

By C. L. Comar,* USA

Radioisotope contributions to animal physiology already comprise a considerable literature well known to workers in the field. The study of certain important problems of calcium and phosphorus nutrition has been complicated by difficulties of interpretation of single dosage studies. These difficulties have arisen primarily because of the exchange reaction of such ions between blood and bone. These same exchange reactions, however, have led to an understanding of ion movement within the body that could not have been gained without the use of radioisotopes.

This paper presents some recent findings in regard to mineral nutrition with particular emphasis on calcium availability and metabolism in domestic animals. To serve as a basis for understanding the interpretations, a brief review is given of current concepts of ion movement between circulating fluids (blood) and the skeleton. Important corollaries of such concepts are concerned with the hazard and mechanisms of deposition and removal of certain bone-seeking radioisotopes.

Documentation of unsupported statements will be found in the bibliography classified by subject matter. The contributions of the authors listed in the bibliography will be readily apparent to the reader and are gratefully acknowledged. Space does not permit discussion of the fine details, particularly in regard to bone metabolism. Rather, an oversimplification is made to present the general principles with emphasis upon those over-all reactions that are the most important.

SKELETAL METABOLISM

The Exchange Concept

In this discussion, exchange is considered as the movement of ions from the blood to replace ions in the bone and vice versa. This process in which no change of mass occurs is contrasted with that of bone accretion or growth, which does result in production of new bone. Note the reference of Neuman and Neuman.

Bone may be considered to consist of the bone mineral (bone crystal) and an organic matrix (osteoid). The bone crystals contain calcium, phosphate, and hydroxyl ions arranged in a hexagonal lattice structure that gives an X-ray pattern characteristic of the apatite minerals. The organic matrix is comprised of collagen fibers between which occurs a mucopolysaccharide identified as chondroitin sulfate. Certain substances present in blood are deposited in the organic matrix; these include beryllium, carbon, magnesium, phosphate, sulfur, lanthanide rare earths, and actinide rare earths. Such osteoid seekers will not be considered further in this paper.

It has been demonstrated that the surface ions of the bone crystals are in equilibrium with the body fluids bathing these crystals. Also, certain ions in the blood may become bound to or bound in the bone crystals by isoionic or heterionic exchange; these ions include fluorine, phosphate, calcium, strontium, radium, lead, hydrogen, sodium, and uranium. Attention will be primarily directed toward calcium from the point of view of nutritional studies, with mention of strontium in connection with hazards of internally deposited boneseekers. It should be noted that strontium ions can exchange with calcium ions of the bone mineral; the statements to follow in connection with calcium will also hold for strontium except when otherwise noted.

It is emphasized that the bone crystals are very small. This means that the surface areas of bone mineral in the body are very great, and explains the importance of surface reactions in governing movement of bone-mineral seeking isotopes.

A simple model, which depicts calcium ion movement in the body, is presented in Fig. 1. It is considered that the circulating calcium in the blood comes into contact with the surfaces of a certain portion of the bone mineral and exchanges with the surface calcium. New calcium enters the blood from the gastrointestinal tract and calcium is lost by urinary and fecal excretion. In the growing animal, some of the blood calcium is removed to form new bone.

ENTRY OF Ca* AND Sr* INTO BONE

Let us now consider the stoichiometry and kinetics of the exchange reaction. A model is presented in Fig. 2. We can regard the bone as an ordinary ion exchange column with solution (the body fluid containing calcium ions) constantly being recirculated through the bone. For purposes of illustration we

* Including work by C. L. Comar, Oak Ridge Institute of Nuclear Studies, Inc.; F. W. Lengemann, University of Tennessee; W. E. Lotz, Oak Ridge Institute of Nuclear Studies, Inc.; R. A. Monroe, University of California at Los Angeles; and P. M. Johnston, University of Arkansas.

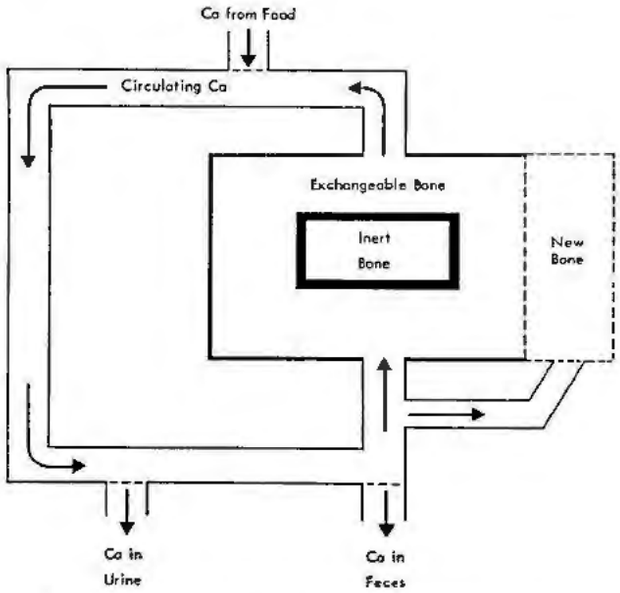


Figure 1. Simple model of calcium ion movement in the body

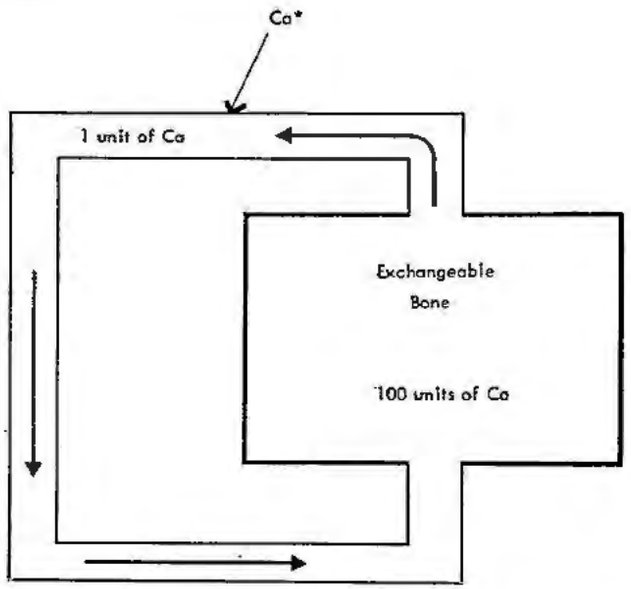


Figure 2. Model of exchangeable bone as an ion exchanger with body fluids being recirculated

can assume that the amount of exchangeable calcium in the bone is at least 100 times that in the body fluids. This ratio will vary with age and animal species but would hardly be expected to be less than 100 and usually would be much higher.

If labeled calcium ions are introduced into the blood they will then enter the exchange reaction and behave in exactly the same way as do the calcium ions already present. Evidence indicates that equilibrium is attained rapidly. At equilibrium, the specific activities in blood and exchangeable fraction of bone will be equal. Thus at equilibrium:

$$\text{Specific activity in exchangeable bone} = \text{Specific activity in blood} \qquad (1)$$

or

$$\frac{\text{Ca* in exchangeable bone}}{100} = \frac{\text{Ca* in blood}}{1} \qquad (2)$$

This means that shortly after the labeled calcium ions enter the blood, a great proportion of them will have entered the bone by essentially a physical process.

EXCHANGE VERSUS ACCRETION

As already implied, labeled calcium in the blood can enter the skeleton by the process of new bone formation. The rate of new bone formation can be readily estimated from growth data. It is also known that bone resorption and bone accretion can occur simultaneously in different parts of the skeleton; thus, net increase in bone weight will give an underestimate of the entry of labeled calcium by accretion.

It is of importance to estimate how much of a single dose of labeled calcium enters the bone by the exchange process and how much by bone accretion. Such an estimate can be made from the disappearance rate of labeled calcium from the blood. Figure 3 presents a typical disappearance curve for labeled calcium after intravenous injection into a month-old calf. This curve can be represented by the equation:

$$C(t) = 42e^{-2.1t} + 32e^{-0.38t} + 2.2e^{-0.070t} + 3.7e^{-0.0039t}$$

where $C(t)$ = percentage of dose in plasma at time t in minutes, a = percentage of dose removed by rate

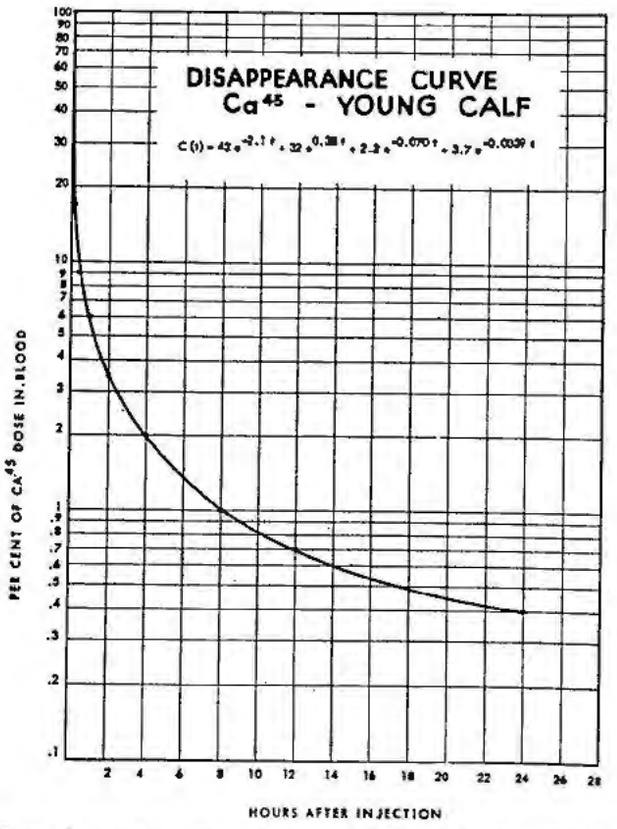


Figure 3. Disappearance of intravenously injected calcium-45 from blood of one-month-old calf

process described by the given term, and b = rate content for the process. From this equation it is calculated that the initial rate of disappearance of Ca* from the blood was about 102 per cent; this means that the normal calcium ions are being removed and replaced at the same rate. It can be calculated that if the blood calcium of this calf were being used for bone formation at this rate, then the animal would be forming about 1200 grams of bone per day. From growth studies it is known that such an animal would increase its skeleton by about 15 grams per day. Studies have shown that the endogenous loss of calcium would not exceed an amount equivalent to 5 grams of bone per day. Even other factors, such as the amount of bone resorbed and replaced, seem unlikely to account for the discrepancy between $15 + 5 = 20$ and 1200 grams of bone per day. Thus, one is led to the conclusion that under these conditions the major deposition of labeled calcium in the bone is caused by an exchange reaction. One must hasten to add that the extent of this exchange and the regions of bone where it takes place are governed by physiological factors. Indeed, such biological variables as vascularity, blood flow, crystal size, proportion of exchangeable bone, and hormone effects are all important; indirect correlations may well be attained between rate of growth occurring and amount of a single dose of Ca* appearing in the bone. Nevertheless it is difficult to avoid the conclusion that the major mechanism by means of which the labeled calcium ion leaves the blood and deposit in bone is one of exchange.

It should be emphasized that *after* bone and blood have reached equilibrium, then bone accretion will be responsible for further increases in the skeletal content of labeled calcium. For example, from Fig. 3 it would be judged that after eight hours, the major process of labeled calcium deposition was that of growth. However, it must be remembered that about 99% of the labeled calcium was already deposited in the bone by the eighth hour after administration.

Figure 4 presents a Ca^{45} autoradiagram of the metatarsal of a young calf; the bone was removed at two minutes after intravenous injection. This autoradiogram demonstrates the rapidity of deposition and also the correlation between deposition and areas of physiological function.

REMOVAL OF Ca* AND Sr* FROM BONE

Once the labeled calcium gets into the bone, whether by exchange or accretion, it can be removed only by resorption of bone or by exchange.

The amount removed by resorption will be governed primarily by the rate of growth of bone. Only that part of the labeled calcium that becomes located in a region of resorption will be subject to removal by this process. The material liberated by resorption enters the circulation and then follows the usual pathway. Figure 5 presents autoradiograms of Sr^{90} in the metatarsal of steers sacrificed one day and three years, respectively, after oral administration

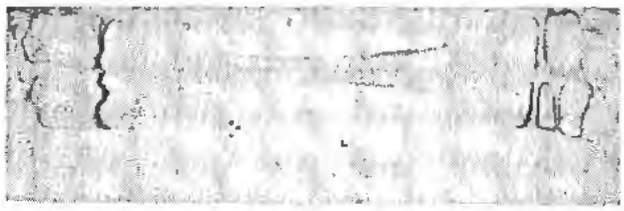


Figure 4. Autoradiogram of metatarsal of young calf sacrificed at two minutes after intravenous injection of calcium-45

of the radioisotope. This demonstrates removal by resorption and also emphasizes the high degree of retention where resorption does not come into play.

The removal by exchange can be discussed in terms of Fig. 1. At equilibrium the specific activities in blood and the exchangeable bone will be equal. As the specific activity of the blood is lowered by intake of dietary calcium and excretion of blood calcium, there will be a net movement of Ca* from the bone to maintain equal specific activities. As will be discussed later, the amount of stable calcium entering this process is small compared with the amount of skeletal calcium. Thus, removal as is actually observed is very slow indeed. There are other factors that tend to decrease this removal rate. Some of the labeled calcium originally deposited in the exchangeable part of bone will become unavailable for exchange; this occurs by new growth covering the crystal surface or by diffusion of the Ca* away from the crystal surfaces.

EFFECTS OF EXCHANGE ON TRACER STUDIES

A direct effect upon tracer studies may be illustrated by Fig. 6. If 100 units of stable calcium ions enter the blood, whether by injection or by absorption from the tract, they will come in contact with bone surfaces and undergo exchange. Assuming no bone accretion 100 stable calcium ions will be contained in the blood leaving the bone to go through the process of endogenous excretion or recycling for other metabolic uses. If, however, 100 units of labeled calcium ions enter the blood, they will be accumulated in the bone surfaces and, for the most part, replace stable calcium ions; these stable ions then follow the same path as do all the calcium ions in the blood leaving the bone. This behavior is not based on any "isotope effect" due to a differential behavior of Ca^{45} ions in blood or bone; it merely results from the attainment of equilibrium in a system of high calcium concentration in bone and a low concentration in blood. Expressed mechanistically, the stable calcium ions contact the bone and replace like calcium ions; whereas, the labeled calcium ions contact the bone and replace unlike stable calcium ions because there are so few, if any, labeled calcium ions in the bone to be replaced. Support for this concept may be found in Table I, which presents the comparative fecal excretion of calcium as measured simultaneously by chemical calcium balance and radioassay of orally administered $Ca^{45}Cl_2$. Note that the per cent of Ca^{45} excreted is lower than the per cent of calcium excretion.

NUTRIENT AVAILABILITY

Concept of Endogenous Loss

It is important to be able to measure fecal endogenous calcium and phosphorus excretion because this information allows direct estimation of (*a*) the proportion of the element in a given feed that is unavailable to the animal, and conversely, the net digestibility; (*b*) the maintenance requirements; (*c*) the actual body losses as they may be affected by such variables as feed levels and age; and, (*d*) the over-all turnover of these minerals in the body. This turnover is important from the standpoint of removal of hazardous bone-seeking elements.

Over the past several years, endogenous fecal calcium and phosphorus excretions in cattle have been determined singly and simultaneously by means of variations of the isotope dilution technique. The following general procedures have been found to offer the best compromise of ease and reliability:

1. The animal is conditioned to the metabolism stall and to eat normally when placed on experiment. This stall in arranged for separate and quantitative collection of feces.

2. When the animal is reacting normally, as judged by food consumption, nature, and amount of excreta, the solution containing P^{32} and Ca^{45} is injected either subcutaneously or intramuscularly. Usually, 1 mc of Ca^{45} and 0.3 mc of P^{32} per 100 pounds body weight are given.

3. On the sixth day after dosage a blood sample of about 50 ml is taken.

4. On the seventh day after dosage the fecal collections are started. Records of total excretion are kept over the next five days, and from each day's collection a representative sample is taken for analysis. Alternatively, a composite fecal sample representing the five days' excretion can be used for analysis.

5. On the eleventh day after dosage another blood sample is taken.

6. Samples are analyzed for total calcium, total phosphorus, and for Ca^{45} and P^{32} by the differential absorber method.

Calculations are made as follows:

$$\frac{\text{Average specific activity of feces}}{\text{Average specific activity of plasma}} \times \text{daily fecal Ca} = \text{endogenous fecal calcium} \quad (4)$$

$$\frac{\text{Ca intake-fecal Ca} + \text{endogenous fecal Ca}}{\text{Ca intake}} \times 100 = \text{net digestibility} \quad (5)$$

Attention is called to a calculation from these data of total digestive juice calcium (TDJ Ca). Note the reference of D. Laszlo and co-workers. The TDJ Ca is considered to comprise all the calcium that reaches the intestinal tract from within the body and tissues. If it is assumed that the TDJ Ca mixes with the food calcium and that absorption takes place from this mixture, then this equation is valid:

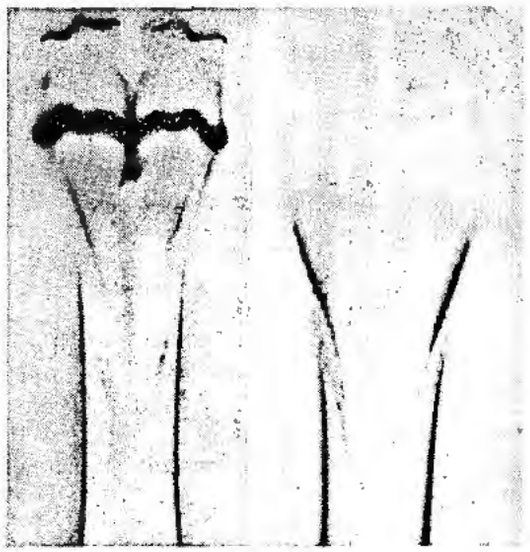


Figure 5. Autoradiograms of metatarsals of cattle sacrificed at one day (left) and three years (right) after strontium-90 ingestion

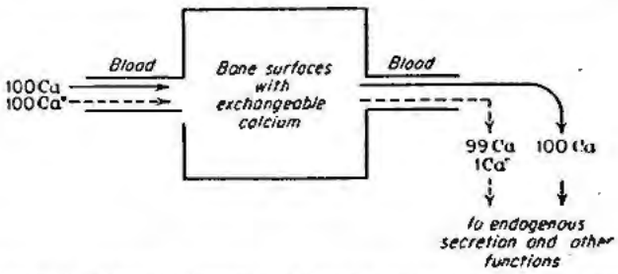


Figure 6. Schematic illustration of how the exchange of calcium ions between blood and bone causes an apparent difference in the behavior of calcium-45 and stable calcium ions in the system

$$\frac{\text{TDJ Ca}}{\text{Ca intake} + \text{TDJ Ca}} = \frac{\text{average specific activity of feces}}{\text{average specific activity of plasma}} \quad (6)$$

Thus, the TDJ Ca can be calculated and also the amount of TDJ Ca that is reabsorbed in the gastrointestinal tract. It will be noted that the endogenous fecal calcium is that part of the TDJ Ca that is actually excreted.

It should also be noted that the TDJ Ca can readily be calculated from the endogenous fecal loss and the net digestibility. The equation is as follows:

$$\text{TDJ Ca} = \frac{\text{endogenous fecal calcium}}{1-(\text{net digestibility})/100} \quad (7)$$

Table I. Comparative Fecal Excretion of Dietary Calcium and Calcium-45 by Cattle

Age	No. of cattle	% Ca intake excreted	% Ca^{45} intake excreted
10 days	3	7	3
1 month	3	17	7
6 months	5	76	62
2 years	8	82	70
Mature	5	103	70
Aged	4	128	83

The data in Table II illustrate some results of such experiments with both calcium and phosphorus. In this study six calves were divided into two groups as comparable as possible. These animals were raised on an adequate normal farm ration, except that one group was placed on a low-normal calcium intake whereas the other group received higher amounts. The animals were about four months of age at the start of the experiment. After about six months it was determined that there were no significant differences in the fecal endogenous calcium loss between the groups. The values in Table II were obtained at about 15 months after the start of the experiment. It should be noted that the calcium and phosphorus intakes listed were those at the 15-month period and had been proportionally increased as the animals grew.

It is clear from the fecal endogenous values that the animals had adapted to the lower calcium diet by significant reduction in the loss of this element from the body. This loss reduction is also reflected by the total digestive juice data. The phosphorus values appeared more variable and showed no trend. Diet did not seem to affect digestibility. The net digestibility for calcium ranged from 21 to 32 per cent whereas the values for phosphorus ranged from 71 to 78 per cent.

SUPERIORITY OF MILK AS A CALCIUM SOURCE FOR CALVES

Previous studies have indicated that milk is a better source of calcium than are most other foods. This is of practical importance since there is a tendency to reduce costs by feeding milk replacements rather than fresh milk to dairy calves. Also, there is an interest in raising the percentages of roughage in calf rations. The following experiment was undertaken to confirm and to determine the extent to which milk is actually superior.

The experimental plan and results are summarized in Table III. In two of the trials, as designated by the footnote in the table, the balance trials were run on the same animal before and after being weaned. For the other comparisons, pairs of animals were picked to be comparable insofar as possible. Three weeks before the balance trial, except for the two-day-old calves, one animal from each pair was weaned from the milk diet to one of the orchard-grass hay and commercial concentrate. Conventional calcium balance trials were run and net digestibilities were determined, as previously described, by means of calculated values of fecal endogenous loss. The results show clearly that the calves were able to absorb and retain more calcium from milk than from hay and concentrate.

It was of interest to determine whether the same effects occurred in aged animals. In an 11-year-old dairy cow the net digestibility of calcium was determined to be 8 per cent in a diet of corn, soybean oil meal, and hay supplying 30 grams of calcium daily. After the animal had been placed for a few days on a ration of two parts hay, one part corn, and two parts dry skim milk supplying 32 grams of calcium daily, the net digestibility was determined to be 23 per cent.

MOVEMENT OF CALCIUM IN DEVELOPING CHICK EMBRYO

Studies have been under way to investigate the movement of calcium among the various components of the hen's egg during embryonic development. Particular attention has been given to the source of the embryonic calcium. In general, the procedure has been to inject Ca^{45} into the dense albumen of the

Table II. Summary of Fecal Endogenous and Total Digestive Juice Calcium and Phosphorus in Cattle as Adapted to Dietary Levels

Animal no.	*1*	*2*	*3*	*4*	*5*	*6*
Body weight (kg)	253	350	348	258	357	322
Intake (gm/day)						
Calcium	26	20	17	58	43	44
Phosphorus	26	20	17	43	32	34
Fecal endogenous loss (gm/day)						
Calcium	2.6	2.6	2.4	3.9	3.4	4.0
Phosphorus	6.4	—	7.1	8.0	—	4.6
Total digestive juice secretion (gm/day)						
Calcium	3.6	3.9	2.9	5.2	4.4	5.8
Phosphorus	29	—	27	34	—	16
Apparent digestibility (%)						
Calcium	15	19	3	17	13	22
Phosphorus	53	—	33	60	—	57
Net digestibility (%)						
Calcium	26	32	17	24	21	31
Phosphorus	78	—	74	76	—	71

Table III. Digestibility of Calcium as Affected by Diet and Age of Calves

Diet	Age	Body weight (kg)	Net digestibility of calcium
Milk	2 days	33	100
Milk	2 days	31	100
Milk	2 months	79	89
Hay and grain	2 months	61	12
Milk*	4 months	95	91
Hay and grain*	4½ months	110	33
Milk	5 months	132	72
Hay and grain	5 months	144	18
Milk*	7 months	152	84
Hay and grain*	7½ months	165	47

* Same animal.

fertile egg. At about 24-hour intervals during hatching, eggs were sacrificed and samples of yolk, albumen, shell, blood, extraembryonic fluid, and total embryo were analyzed for Ca^{45} and total calcium.

The specific activity curves are presented in Fig. 7. The albumen in this experiment represents the original source of labeled calcium and therefore had the highest specific activity (SA) at all times. Experience showed that the Ca^{45} was uniformly distributed in the albumen, certainly by a few days after injection. The definite decrease in SA of the albumen that occurred after six days must have resulted from a loss of calcium from this component with a replacement by calcium of a lower SA from some other component.

It is next of interest to consider the SA of the shell, which showed a peak at about nine days and a rapid decline thereafter. It is probable that the Ca^{45} ions

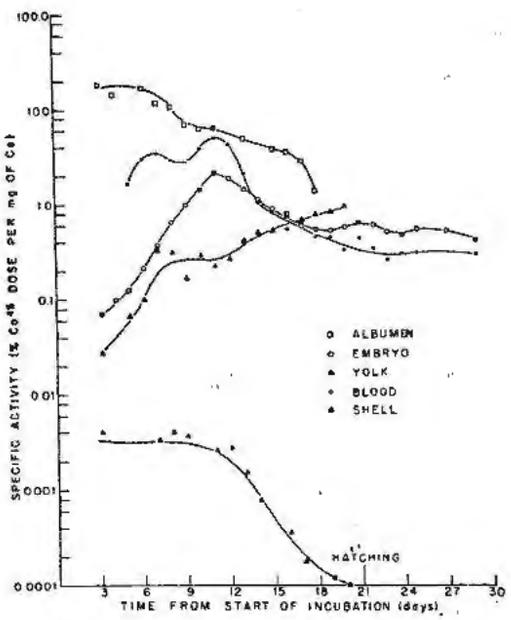


Figure 7. Time pattern, during hatching, of specific activities of calcium-45 in the various components of the hen's egg. Calcium-45 was injected into the albumen just before start of incubation

entered the shell by exchange. It must be remembered that the values for the shell in Fig. 7 represent the SA of the total shell and that the SA of the localized inner surfaces was probably much higher. It is also logical that this inner surface would be selectively removed at the time when the shell begins to supply calcium. Thus, the SA of the calcium initially furnished by the shell would be much higher than indicated by the values in Fig. 3. In terms of amount, the shell accumulated approximately 11 per cent of the dose by the eighth day.

The SA of the total embryo showed a steady rise until the eleventh day and then declined until hatching. In general this means that before the eleventh day, a larger proportion of the calcium ions of the embryo had originated in the albumen pool than after the eleventh day. The SA of the blood followed the same general pattern except that it was higher than the SA of the total embryo between 5 and 14 days and slightly lower thereafter. Up to the eleventh day the blood SA curve was very similar to that of the albumen; this curve is consistent with the water movement that occurs during the first 9 to 10 days of development. During the sixth to ninth day the albumen loses a considerable amount of water to the embryo and extraembryonic membranes and it is logical that the SA of the fluid portion of the blood would reflect that of the albumen because of the mechanism of blood formation. After the 11-day peak the blood SA fell rapidly. This probably can be accounted for by the fact that this is a period of intense osteogenesis with large amounts of calcium being used; also, since the total amount of calcium in the blood is low at 13 days, the blood SA is very sensitive to any replacement of calcium that occurs.

The SA curve of the yolk was characterized by a plateau between the seventh and twelfth days; thereafter it gradually rose until the twentieth day at which time the yolk is drawn through the umbilicus into the body of the chick. The increase in yolk SA during early incubation was probably a result of diffusion of calcium ions from the albumen to the yolk, and the curve closely resembles that for water movement from the albumen to the yolk that occurs

during this time. During the period of time when the SA of the yolk is rising, the yolk is separated from the albumen only by the vitelline membrane. Later the yolk sac and the extended area vasculosa intervene, and additional barriers to diffusion are established. It would appear that the yolk is therefore cut off directly from its source of Ca^{45} and any further rise in the SA of the yolk would have to be the result of the absorption of labeled calcium from the blood. This situation seems to be indicated by the rise seen in the SA of the yolk between the eleventh and fifteenth days. During this time it appears that the blood is giving up some of its calcium that is being stored in the yolk for future use. The continued rise of the SA of the yolk after 15 days may in part be due to the imbibition of extraembryonic fluids by the embryo and to the possibility that some of these fluids find their way to the yolk sac via the vitelline duct. The SA of the extraembryonic fluid was relatively high, about 1.4 at 19 days. This high SA of the extraembryonic fluid probably can be accounted for by the fact that there is an apparent mingling of the contents of the albumen sac and the amnion and chorioallantois during the last week of incubation.

In summary it may be stated that calculations indicate that 85 to 95 per cent of the embryonic calcium accumulated over the first ten days was derived from the yolk. After ten days the shell started to contribute calcium to the embryo. The movement of calcium among various egg components was correlated with accepted morphological and physiological concepts.

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Isotopes in Permeability Studies

By Hans H. Ussing,* Denmark

The use of isotopes in biological studies has an old tradition in Denmark. Thus it was there that Hevesy did his famous experiment with the uptake of radioactive lead by horse beans. Also the first application of radioactive indicators to the study of animal metabolism took place in Copenhagen when Hevesy in collaboration with Christiansen and Bomholt studied the behaviour of radioactively labelled bismuth in rats.

Among those who at a very early date realized the enormous potentialities of the tracer method was the great Danish physiologist August Krogh. In 1935 he collected a team with the object of applying heavy water and deuterium-labelled compounds in biological research. During the war the isotope work of course became increasingly difficult. As the war was drawing towards its end, Krogh—who was then in exile—put forward a plan of a concentrated attack on the important problems of the behaviour of inorganic ions in biological membranes. The plan assumed that the cyclotron of the Institute of Theoretical Physics should produce the isotopes, whereas the biological work should be performed at the Laboratory of Zoophysiology. The plan met with the approval of Professor Bohr and Professor Rehberg who had succeeded Professor Krogh as head of the Laboratory of Zoophysiology, and the present author was put in charge of the biological part of the project.

The use of isotopes turned out to give an insight in the kinetics of biological permeability which seemed entirely out of range only 25 years ago. As an example of the results which can be obtained through the use of isotopic tracers in membrane studies we may take the analysis of the so-called active ion transport processes in the amphibian skin, a study which, among other things, led to the clarification of the origin of the bioelectric potentials in the skin, the intestine[24] and the urinary bladder.[13] But first we must make clear the distinction between active transport and passive diffusion.

ACTIVE TRANSPORT AND DIFFUSION

Active transport may be defined as a transport across cellular membranes taking place as a consequence of work being performed by the cells. The distinction between active transport and diffusion usually presents little difficulty for uncharged molecules. A behaviour in accordance with Fick's law of diffusion is taken as proof that the passage through the membrane is passive. The behaviour of ions, however, is governed by the combined effects of concentration gradient and electric potential gradient. Under certain conditions a third force enters into the picture, namely the solvent drag force, arising from net flow of solvent through the membranes. The expression describing the passage of an ion through a living membrane may thus become extremely involved, notably if the membrane, as usually is the case, consists of several layers of different properties, placed in series.

A great simplification is obtained, however, if one considers not the net amount passing through the membrane, but the "flux ratio"; the proportion between the total diffusion stream going in and that going out. These two figures are readily obtainable using isotopic tracers for the ion. If two different isotopic tracers are available for the ion in question, or if the difference between influx and outflux can be obtained by chemical analysis, influx and outflux can be obtained together in one tracer experiment.

It can be shown that for a passive ion diffusing through a membrane the following equation is valid,[21,19]

$$M_{in}/M_{out} = \frac{c_o}{c_i}\frac{f_o}{f_i} \exp\,(zFE/RT) \qquad (1)$$

where M_{in} is the influx, M_{out} the outflux, f_i and f_o are the mean activity coefficients, and c_i and c_o the concentrations of the considered ion in the inside and outside compartments, respectively. E is the electric potential difference between the inside and outside solutions, z is the charge of the ion, F is Faraday's number, R the gas constant and T the absolute temperature. The solutions are assumed to be well mixed.

The above equation is valid irrespective of the membrane structure as soon as a steady state is obtained. If the behaviour of an ion is in accord with this equation, we must conclude that it is not subject to active transport. If the equation does not apply, the ion in question cannot move freely and accordingly must cross the membrane, wholly or in part, chemically combined with another molecule.

ION MOVEMENTS IN THE FROG SKIN

Now we can turn to our specific problem: the ion movements through the isolated surviving amphibian skin. As early as in 1857 Du Bois-Reymond observed

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that the isolated frog skin maintains a potential between its inside and outside. Later it was found that this property depends upon the presence of Na or Li ions. In the thirties of this century another surprising property of the frog skin attracted the interest of physiologists. In 1935 Huf found that the isolated surviving frog skin, when in contact with Ringer solution on both sides, performs an active transport of sodium chloride from the outside solution to that bathing the inside. Shortly afterwards Krogh[11] observed that salt-depleted frogs are able to take up salt from the surrounding medium, even when the latter is as dilute as 10^{-5} molar with respect to NaCl. Still more surprising perhaps was Krogh's finding[12] that the mechanism is specific to Na. Neither K nor Ca were taken up from solutions of their chlorides.

If we now apply Equation 1 to the behaviour of Na and Cl in the frog skin, it is readily seen that the transfer of Na is bound to be due to active transport. If, for example, the potential difference across the skin is 60 mv (the inside positive relative to the outside) and the solution on both sides is Ringer's, the equation indicates that the influx ought to be one tenth of the outflux. Experiments show, however, that the reverse is more nearly true. The influx is higher, and often 10 times higher, than the outflux.[14]

The fact that active transport in contrast to passive diffusion is dependent on metabolic processes is clearly demonstrated in recent experiments on dinitrophenol-poisoned frog skins, performed by Dr. Schoffeniels in our laboratory. Table I shows some of the results. The skin was placed with Ringer's as inside solution and $^1/_{10}$ Ringer's as outside solution. It is seen that in the control periods the influx is much larger than the outflux despite the fact that the active Na transport has to overcome both the concentration difference and the electric potential difference. Thus in the first example the actual flux ratio is 3.66 whereas that calculated under the assumption of passive diffusion according to Equation 1 is 0.011. During DNP-poisoning, however, the active transport stops and Na starts leaking out in accordance with the concentration difference. The sign of the potential reverses, the outside solution becoming positive as it should if Na leaks faster than Cl. The flux ratio found during poisoning is very closely equal to that calculated for passive diffusion. Thus DNP which is known to uncouple metabolism from the formation of high-energy phosphates and thus from doing useful work, will also "declutch the sodium pump", and leave the Na ion to diffuse like any passive ion.

Table I. Influence of Dinitrophenol upon the Active Transport of Sodium through the Isolated Frog Skin

Inside medium, Ringer's. Outside medium, 1/10 Ringer's. pH of bathing solutions, 8.3. M_{in}, influx; M_{out}, outflux of Na, measured with Na^{22}, respectively. E, potential difference (sign refers to inside solution). For each experiment is given first a control period, *C*, and then an experimental period after the addition of DNP to give a concentration of 1×10^{-2} mM (Schoffeniels, E., in preparation).

	M_{in}	M_{out}	E	M_{in}/M_{out}	M_{in}/M_{out}
	$\mu mol \cdot cm^{-2} \cdot h^{-1}$		mv	found	calculated
C	0.34	0.093	62	3.66	0.011
DNP	0.25	1.57	−11	0.16	0.15
C	0.445	0.145	82	3.07	0.025
DNP	0.049	0.56	− 6	0.087	0.126
C	0.228	0.008	72	28.5	0.017
DNP	0.032	0.216	− 7	0.148	0.132

For the chloride ion the situation is different. The electric potential difference might well provide the force necessary to transfer chloride ions from outside to inside solution. Detailed studies of the transfer kinetics of chloride, using the isotopes Cl^{38} and Cl^{36} have, as a matter of fact, shown that chloride in the isolated frog skin behaves in complete agreement with Equation 1.[9,15] The investigation of the chloride permeability further showed that the higher the potential difference the lower were both influx and outflux of chloride ions. This observation was, at least qualitatively, in agreement with the working hypothesis advanced some years ago[20,21] that the electromotive force giving rise to the skin potential was created by the active transport of the positive sodium ions from the outside to the inside solution, whereas the passive movement of chloride ions created a variable short-circuit for this potential. This hypothesis evidently requires that a high chloride permeability should be associated with a low potential difference and vice versa.

THE SHORT-CIRCUITED FROG SKIN

It is clear that the above working hypothesis goes beyond the statement that the transport of Na is an active process. The active transport of an ion species may or may not create a potential difference, depending on the way the process is carried out. Thus a process by which a K ion is carried one way in exchange of a Na ion carried the other way would not in itself create a potential difference. Furthermore it would be quite conceivable, indeed likely, that other cellular processes than the active Na transport would contribute to the electric asymmetry of the skin. Notably we have to consider those metabolic ions which, like H^+ and HCO_3^+, have been considered important in some hypotheses advanced to explain the skin potential. For these ions the use of tracers, so important in the study of the transfer of Na and Cl, seemed to be out of question. The problem can, however, be attacked in the following way: Suppose that the skin potential were short-circuited, so that both sides of the skin were at the same potential, and that, further, solutions of identical composition were bathing both sides. Under such conditions no net transfer of passive ions would take place. Ions which are subject to active transport would, however, continue to flow onesidedly, and the current flowing through the short-circuit would be the resultant of all the net transport processes. Actually, determinations of the current that can be

drawn from the more or less completely short-circuited frog skin have been made previously by Francis,[5] Stapp[18] and Lund and Stapp,[16] but these determinations were not correlated with ionic movements. An experimental setup was therefore constructed[23] making possible the simultaneous determination of short-circuit and Na transport. The absolute transport rate for Na through the frog skin is so low that the demonstration by simple chemical analysis of the current/active transport relationship would meet with nearly insurmountable difficulties. The tracer method, on the other hand, allows the determination of ion transport rates with a very good accuracy. The experimental procedure is the following: The skin is placed as a membrane separating two lucite tubes containing Ringer's solution. The potential difference across the skin is measured between calomel electrodes, making contact with the bathing solutions close to the skin. Another pair of electrodes, placed in considerable distance from the skin, are connected in series with a battery and a micro amperemeter. The current in this latter circuit is now adjusted so that the potential drop across the skin is reduced to zero. It is obvious that this "clamping" of the potential at zero accomplishes a total short-circuit of the skin. (It goes without saying that as soon the potential-clamp is removed, the potential difference across the skin is reestablished by the metabolic processes). Since the amperemeter is placed in series with the skin, the short-circuit current generated by the skin can be read directly. As long as the potential difference is artificially maintained at zero, the electromotive force of the skin has only to overcome the internal skin resistance whereas the resistance of the bathing solutions and the rest of the outer circuit is overcome by the applied battery-voltage.

During the short-circuiting it is now possible to measure outflux and influx of the ion species under investigation. Thus the Cl^- influx and outflux can be measured with Cl^{38} and Cl^{36}, the Na fluxes with Na^{22} and Na^{24} etc. For K, influx and outflux have to be measured separately in parallel experiments since only K^{42} can be used as tracer for this ion.

Both in the case of K and of Cl influx and outflux turned out equal, indicating that these ions did not contribute to the production of electric current. For Na, however, the result was different. Table II shows some typical results.

Table II. Short-Circuit Current and Sodium Flux Values for a Number of Short-Circuited Frog Skins (*Rana temporaria*) (Ringer Solution on Both Sides)

	$\mu a/cm^2$			
	Na in	*Na out*	*Δ Na*	*Current*
I	20.1	2.4	17.7	17.8
II	11.1	1.5	9.6	9.9
III	40.1	0.89	39.2	38.6
IV	62.5	2.2	60.3	56.8
V	47.9	2.5	45.4	44.3

Both influx, outflux and electric current are expressed in $\mu a/cm^2$ and thus can be compared directly. It is seen that the influx is somewhat larger than the current whereas the outflux is very much smaller. In other words: the sodium ion continues for hours and hours to move onesidedly from the outside to the inside compartment in spite of the fact that the concentration is the same on both sides and that the potential difference is maintained equal to zero. But the really striking result is that within the accuracy of measurement, the net Na flux (which is influx minus outflux) is exactly equal to the electric current generated. Thus the active transport of Na is the sole source of the electric asymmetry of the frog skin.

The identity between active Na transport and electric current output holds true even when the skin is treated with hormones and drugs which are known to influence profoundly the skin potential.

It has already been mentioned that DNP inhibits the active Na transport, and so do all metabolic poisons like cyanide, narcotics, etc. Very strong inhibition is obtained with eserin, tetraethylpyrophosphate and other anticholinesterases.[8] Stimulation of the transport rate is obtained with atropin[8] and neurohypophyseal extracts.

MODE OF ACTION OF NEUROHYPOPHYSEAL HORMONE

As a matter of fact the short-circuited frog skin in connection with isotopic tracers provides an excellent object for the detailled study of hormone and drug effects. In the case of the neurohypophyseal hormone effect, for example, the isotope experiment suggested that the hormone acted by increasing the pore size of some layer which presented a considerable resistance to the movement of Na ions. In a somewhat simplified form, the reasoning behind this conclusion was the following: In the short-circuited skin system with identical solutions on hoth sides, the Na-flux values, M_{in} and M_{out}, may be considered as rate constants for the forward and back reactions of the process of Na transport. Thus we have $K=M_{in}/M_{out}$, where K is the equilibrium constant for this process. But according to a well known law, we have $-\Delta F = RT \ln K$ where ΔF is the free energy change. Thus the net driving force in the process of Na transfer, E_{Na}, is determined by the equation

$$E_{Na} = RT \ln (M_{in}/M_{out}) \qquad (2)$$

It now turned out that although the neurohypophyseal hormone increased the current and the Na transport, often by more than 100 per cent, the flux ratio and thus presumably the driving force remained practically constant. Therefore the hormone effect was likely to be a drop in resistance to Na. Incidentally, the resistance to Cl as measured with isotopes is not effected by the hormone, so it was unlikely that the effect consisted in say making the membrane thinner. But a drop in resistance can be brought about in many ways. At the outset the hypothesis of

an increased pore size was preferred due to certain peculiarities in the water permeability observed during hormone action. Thus the diffusion rate for heavy water through the skin of frogs and toads is unaffected by the hormone although the rate of osmotic transfer of water through the skin goes up by a factor of 2–5 when minute amounts of the hormone are added to the solution bathing the inside of the skin.[10] The phenomenon was explained as follows: The rate of *diffusion* of water through a pore membrane of given thickness is determined by the total diffusion area available. If n is the number of pores and r is the pore radius, the diffusion rate or water flux, M_w, is given by $M_w = k_d n r^2$, where k_d is a constant. The rate of *osmosis*, however, is assumed to be a bulk flow of water, which is determined by the laws for laminar flow and not by the laws for diffusion. For cylindrical pores, according to Poisseuilles law, the flow rate depends upon the radius to the 4th power. The net osmotic flow of water, Δ_w, therefore is determined by $\Delta_w = k_o n r^4$, where k_o is another constant.

Since the diffusion rate depends upon the radius to the 2nd power, whereas the osmotic flow depends on the radius to the 4th power, it is obvious that a small increase in r which gives a hardly perceptible increase in the diffusion rates of D_2O will give a large increase in the rate of osmotic flow. Still, the peculiarities in the water permeability might have had a different explanation. Thus Caprano and Bernini[2] assumed that the hormone induced an active transport of water inward through the skin. It is, however, possible to deduct certain consequences from the pore theory of hormone action, consequences which can be tested experimentally. Let us consider a membrane with narrow pores through which a net flow of water is established. The driving force may be for instance a difference in hydrostatic pressure, or the presence in one of the bathing solutions of an osmotically active substance which cannot enter the pores. If we now consider a molecular species which *can* diffuse through the pores, it is clear that the linear rate of flow in the pores will influence the diffusion, so that molecules diffusing in the direction of flow will be speeded up and those moving in the opposite direction will be slowed down. If, on the other hand, the membrane is no pore membrane but constitutes a continuous phase, one must assume that water molecules and other molecules pass by dissolving in the membrane phase. Therefore the osmotic transfer of water cannot influence the diffusion rates of other substances, which must diffuse at the same rate both ways. The presence of pores in a membrane thus can be tested by determining simultaneously the diffusion coefficient of a test substance in both directions during osmotic or hydrostatic water flow. In order to determine the two diffusion coefficients one simply needs two batches of the test substance labelled differently with isotopic tracers. In our particular case it is desirable (1) that the test substances are of at least the size of the Na ion, (2) that they are not lipoid soluble (because lipoid soluble substances may penetrate by dissolving in the membrane material between the pores), (3) that the molecules are small enough to penetrate and (4) that they are not likely to be subject to active transport. For the time being experiments along this line are at progress in our laboratory and some of the results[1] are presented in Fig. 1. The test substances used were thiourea, labelled with S^{35} and C^{12}, and acetamide, labelled with C^{14} in position 1 and in position 2.

The results are given as the logarithm to the ratio between the diffusion coefficient for influx (k_{in}) and that for outflux (k_{out}), because, in analogy with what had been said in connection with Equation 2, the solvent drag force on the test molecule must be considered equal to RT ln (k_{in}/k_{out}). This driving force is plotted as a function of the rate of osmotic water flow through the test object, namely the skin of the common toad (*Bufo bufo*), placed with Ringer's solution on the inside and $^1/_{10}$ Ringer's on the outside. The proportionality between osmotic water flow and the solvent drag force is very apparent, demonstrating that, indeed, the movement of the test substances as well as the water is confined to narrow pores. It is further seen that all hormone-treated skins show a pronounced solvent drag effect, whereas the untreated skins show only little drag effect. These experiments demonstrate three things: (1)

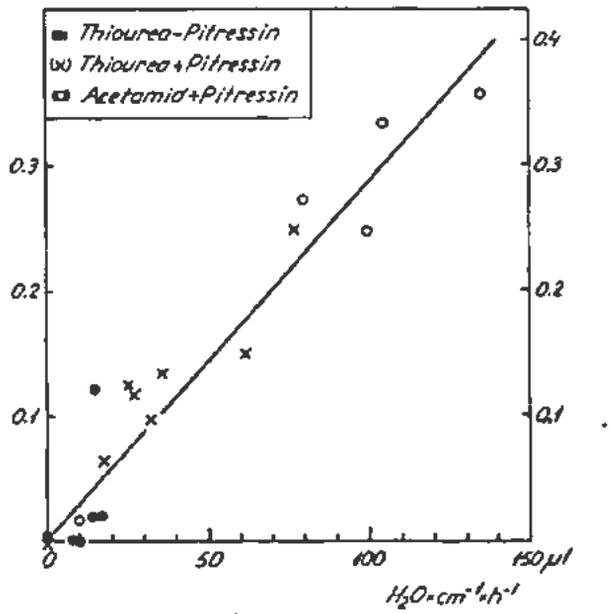

Figure 1. Showing the effect of the rate of osmotic flow of water through the isolated toad skin upon the solvent drag force exerted on the test substances thiourea and acetamide. Log (k_{in}/k_{out}), the logarithm of the ratio between the inward and outward diffusion coefficients, is taken as a measure of the drag force: k_{in} and k_{out} are determined simultaneously with two batches of the test substance labelled differently with radioactive tracers ($S^{35}C(NH_2)_2$ and $SC^{14}(NH_2)_2$, and $CH_3C^{14}ONH_2$ and $C^{14}H_3CONH_2$). Inside medium Ringer's; outside medium, 1/10 Ringer's. All experiments with acetamide, O, and part of those with thiourea, X, are performed on skins treated with pitressin (the antidiuretic neurohypophysial hormone) which increases violently the rate of osmotic water flow

osmosis through a living membrane can be of the type of bulk flow, (2) the layer acted upon by the hormone is provided with pores and (3) the hormone acts by increasing the pore size, and this increase in size is sufficient to account for the effect of the hormone upon the active Na transport.

MECHANISM OF ACTIVE TRANSPORT

Up to this point we have taken the active transport as an observed fact without considering the underlying mechanisms. We have eliminated concentration difference and electric potential difference as driving forces, because, in the short-circuited frog skin we have Na transport in the absence of concentration— and potential difference. Solvent drag force can also be eliminated because we can have active Na transport in the absence of any net water flow through the membrane. Thus by elimination we come to the conclusion that the operating force is of chemical nature. That also would he in agreement with the high specificity of the Na transport mechanism which refuses to transport as closely related ions as K, and Rb. It is true that Li can be transported to some extent by the skin[25] but Li piles up in the epithelium cells and finally inhibits the transport mechanism.

Some authors have advanced hypothesis according to which active ion transport is associated with electron transfer, so that for one electron passing from substrate to oxygen, one ion is transferred.[17,3] In the case of the Na transport of the frog skin, however, such hypotheses can be ruled out since, under suitable conditions the number of Na equivalents transferred exceed the number of equivalents of oxygen consumed.[26] Several hypotheses depend on the operation of "membrane carriers", that is, substances associated with the membrane which, by complexing with the ion to be transported allows it to enter the membrane phase at one boundary and to be released at the other. In order to accomplish active transport either the splitting or the formation of the complex must depend on energy drived from the cell metabolism. For instance, the carrier may have a high affinity to Na in its reduced form and a low affinity in the oxydized form, or the high affinity form may be phosphorylated and the low affinity form dephosphorylated etc. A third possibility is that the complex does not simply diffuse through the membrane but that it is pulled through by a contracting molecular chain.[6,4] One interesting property of carriers is that even when they are not linked to a source of metabolic energy, so that they can give rise to active transport, they may bring about abnormal diffusion kinetics.

Thus if the carrier-Na complex is uncharged, its movements must be independent of the potential difference across the membrane. If, further, the Na concentration is high enough on both sides to saturate the carrier, so that no carrier molecule leaves either boundary except in company with a Na ion, the net Na transfer approaches zero, although it would be possible by the use of isotopes to demonstrate a Na flux of about equal size in both directions (exchange-diffusion).[22] Finally, if the carrier is an acid, the carrier would make possible a one-to-one exchange between H and Na ions, so that H^+ would pass from a solution of low pH into one of high pH whereas, for each H ion transferred, one Na would have to pass in the opposite direction, even if it were against a concentration gradient. The latter situation seems to be established when the Ringer's solution bathing the outside of the frog skin is made acid to give a pH of 3, whereas that of the inside solution is maintained at pH 8. Under these conditions the active Na transport is inhibited. A detailled analysis shows however, that there is a flow of electric current due to diffusion of H ions inward, whereas the Na outflux becomes larger than the influx (Schoffeniels). Thus despite the fact that the Na concentration is the same on both sides, the potential energy present in the form of the pH difference drives the "sodium pump" backwards. The operation of a carrier molecule without metabolic energy supply would explain these observations.

Admittedly there still remains much to be done before we fully understand the active Na transport of the frog skin. But the recent rapid progress in this field is almost exclusively due to the use of isotopic tracers.

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The Role of Radioactive Isotopes in Investigating the Physiology and Biochemistry of Digestion

By K. S. Zamychkina and D. E. Grodsensky, USSR

The great advantage of the method of labeled atoms is that it affords the possibility of carrying out chronic experiments on an intact animal. In this respect the combination of the isotopic method with Pavlov's method[1] is especially stimulating and prominsing, viz., for the experiments on animals with chronic fistulae of the digestive glands.

The evidence presented in this communication demonstrates the advantage of the method in solving various problems of physiology and biochemistry of digestion.

EXCRETION OF RADIOACTIVE PHOSPHORUS WITH THE DIGESTIVE JUICES AND THE CHEMISTRY OF PHOSPHORIC BILE COMPOUNDS

The first experiments in this direction dealt with the excretory function of the digestive glands; radioactive phosphorus being used as an indicator.

The study of the excretory function of the digestive glands is based on the theoretical concepts developed by Soviet physiologists[2] concerning the excretory function of the glands and the participation of the gastro-intestinal tract in the intermediate metabolism.

Experiments were carried out on dogs with chronic fistulae of the digestive glands: gall-bladder,[3] intestine,[4] parotid and submaxillary gland, pancreas and isolated ventricle.[5]

The excretion of radioactive phosphorus with bile was also investigated in patients with a temporary fistula of the bile duct.[6]

Indicator doses of radioactive phosphate were administered to dogs intravenously and *per os*, and in humans *per os*.

The time of appearance of radioactive phosphorus was thereafter determined in the respective digestive juice as well as the ratio between the jnice and blood-serum activity at various intervals after the administration of the isotope. The kind of compounds in which radioactive phosphorus was excreted has also been determined.

In all the digestive juices radioactive phosphorus detected within the first few minutes after its injection into the blood stream. Except for bile, maximum activity was noted during the first hour after the administration of the preparation.

In all the juices, except bile, the total phosphorus concentration was about 2-3mg% of which a large part was inorganic phosphate. Radioactive phosphate is thus mostly excreted as inorganic phosphate. Bile was an exception, first, because its phosphorus level was high as compared with other juices; in liver bile of dogs up to 120 mg and in bladder bile up to 200 mg%. Radioactivity of the bile reached its maximum only 24 hours after intravenous or *per os* administration of radioactive phosphorus. This was observed both in experimental dogs and in human patients.

The curves of specific activity of bile in dogs at various intervals after administration of $Na_2HP^{32}O_4$ are presented in Fig. 1. The course of specific activity as a function of time has a regular character although the total phosphorus content determined colorimetrically varied rather greatly from one sample to another. The maximum of specific activity of bile was recorded 24 hours after the administration of phosphate. A comparison of the total quantity of radioactive phosphorus, excreted into the intestine with the amount of radioactive phosphorus detected in the faeces shows that phosphorus excreted with bile is taken up again in the intestine. These results obtained at the very heginnings of the isotope method suggested a more detailed study of the composition of the phosphoric bile compounds and of their fate after the excretion of bile into the intestine.

Information on the chemical composition of the bile in particular of its phosphorus ompounds may be found in any monograph dealing with this question as well as in general textbooks of biochemistry and physiology.[7] However all this information belongs to the end of the nineteenth and the beginning of the twentieth century. Thus in Sobotka's monograph, Hoppe-Seyler's data on the dog bile are cited, which have been obtained as far back as the seventies of the past century.

The insufficient interest paid to the bile phosphorus compounds is due firstly to the fact that bile was considered chiefly as an excretion, secondly, that due attention was not paid to the physiological significance of excretion of large amounts of phosphorus compounds into the intestines, and thirdly that the classical methods of investigation of the composition and concentration of phosphoric bile compounds did not yield satisfactory results.

It is the inadequacy of these methods that interfered with our attempt to develop a method of

Original language: Russian.

chemical analysis of bile.[7] The same difficulties were encountered by the French students—Polonovski, Bourrillon,[8] Etienne-Petitfils and Kahane[9] as described in their recent publications on bile chemistry in which somewhat modified classical methods of bile extraction with organic solvents were used.

The chemical method as checked by the isotope dilution method showed that only about 1% total phosphorus is to be found in the bile as inorganic phosphorus. Most diverse methods were used for extraction of organic phosphoric compounds from the bile by means of different solvents and their combinations. Because of solubility anomalies it was not possible to establish with certainty the relationship of the organic phosphoric compounds to phospholipids, or to solve the question as to whether we are dealing in the bile with substances belonging only to this class or with phosphoric organic compounds of another nature.

It might have been supposed that organic phosphorus compounds of the bile are not in a free state but combined with other bile components, in particular, with bilirubin whose coupling with phosphorus compounds has been suggested by earlier evidence.[10] To check this assumption, it was necessary to find methods for breaking the bonds of organic phosphorus bile compounds with its other components.

Besides the nature of the organic phosphoric bile compounds it seemed necessary also to determine in a long term experiment the rate of synthesis of these compounds in the dog liver as well as their excretion with bile.

This problem was solved by combining the method of partition paper chromatography with the radiometric method.[11] The experiments were carried out as follows. Dogs were treated *per os* with radioactive phosphorus in the form of disodium phosphate with milk. Bile was collected at half-hour intervals during 6–7 hours on the first day after the administration

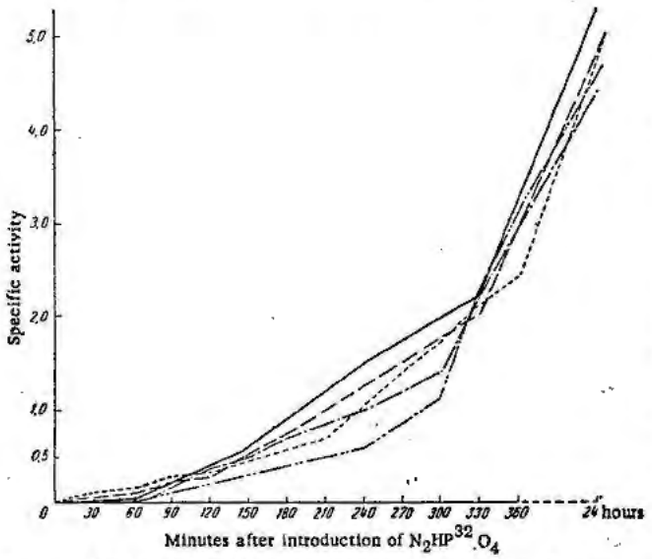

Figure 1. Specific activity of total phosphorus of dog bile after per os administration of $Na_2HP^{32}O_4$. Individual curves correspond to the experiments carried out on different dogs

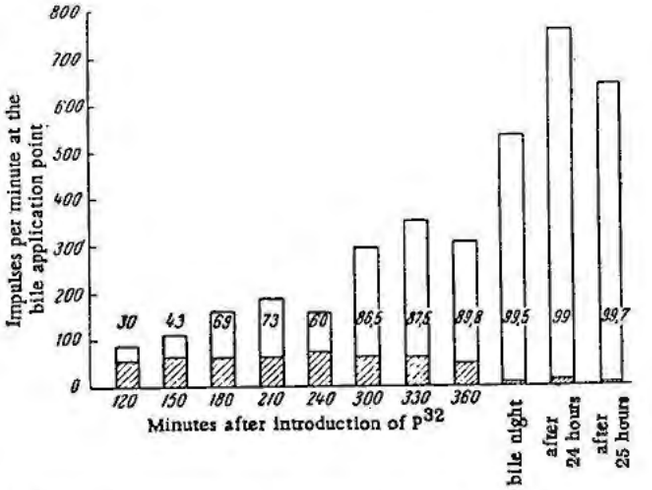

Figure 2. Chromatographic partition of radioactive bile phosphorus. Lower part of columns, radioactivity due to inorganic P^{32}; upper, to organic P^{32}. Figures, % of organically combined P^{32} as related to total radioactivity of the spot. Beneath the columns, time of bile collection after administration of $Na_2HP^{32}O_4$

of radioactive phosphorus and on the next day. A few spots of bile were put on the chromatographic paper collected at different intervals after the administration of radioactive phosphorus. The radioactivity of each spot was determined with the aid of bell-counter; descending chromatography—in a solvent mixture of butanol-water-ammonia. Control chromatographic experiments showed that inorganic phosphate and glycerophosphate as tested in this mixture remain at the point of application.

Residual radioactivity at the point of application of the drop and the spreading of radioactivity along the chromatogram toward the front of the solvent were determined after chromatography of the bile by the same experimental procedures. When a strict determination was necessary of the outline of the spots radioautographs of the chromatograms were taken.

From the data of spot radioactivity prior to chromatography and the residual radioactivity at the application points of the spots after chromatography, calculations were made (in per cent) of the amount of radioactive phosphorus excreted with bile in the form of inorganic compounds at various time intervals after *per os* administration of the phosphates to dogs. The amount of stable phosphorus in the spots was determined colorimetrically after incineration of the respective parts of the paper dissected along the outline of the spots. Specific activity could be calculated from these data.

The results of such bile analysis (Fig. 2) showed the following:

1. Binding of inorganic phosphorus in the liver proceeds rather rapidly. In the bile collected 2 hours after the administration of radioactive phosphates, up to 30% of its total activity is found in the organic phosphoric compound, and after six hours up to 85% of all radioactive phosphorus of the bile is combined with organic compounds. On the next day,

when the maximum of specific activity is reached, up to 99% of all radioactive phosphorus of the bile is incorporated into the organic phosphoric compounds. On subsequent days the same ratio is to be found.

It appears from these data as well as from chemical determinations that with bile only about 1% of inorganic phosphorus is excreted. The specific activity of inorganic phosphorus was maximal during the first periods after the administration of radioactive phosphorus and then decreased approaching the specific activity of organic phosphoric compounds.

2. All bile chromatograms of the dog showed two radioactive spots: one at the application point of the bile, and the other close to the front of solvent (R_f=0.8). The first spot, as mentioned, was easily identified as an inorganic phosphate. To identify the second spot a series of experiments were carried out. The most important of them were as follows:

(*a*) Bile was hydrolyzed with 0.2*N* KOH for 24 hours at 37°C. All the radioactivity remained at the application point. Chromatography of the bile hydrolysate in a mixture of ethyl alcohol-trichloracetic acid-water as well as methyl alcohol-formic acid-water showed that the radioactive spot of the hydrolysate had the same R_f as that of sodium glycerophosphate. Identical results were received in hydrolyzing bile with barium hydroxide. Lead glycerophosphate was isolated from the bile hydrolysate: most of the bile radioactivity was contained in this salt.

(*b*) Chromatography was carried out simultaneously of radioactive dog bile and phospholipids extracted from the liver of the same dog by an alcohol-ether mixture. The spots thus obtained had an identical R_f suggesting a similarity in nature between the substances extracted by an alcohol-ether mixture from the liver and the organic phosphoric compounds of the bile.

(*c*) Radioactive bile was treated with various organic solvents with subsequent simultaneous chromatography of both, whole bile, and of fractions isolated from it. Among the methods of bile treatment applied were those of Polonovski and Bourrillon[8] and of Etienne-Petitfils and Kahane.[9] The phospho-organic compound isolated by means of these methods had the same R_f as the main spot of whole bile. However, much less phospho-organic substance was extracted by these methods than could be found by direct chromatography of whole bile.

The above investigations showed that phosphorus of the dog bile is secreted, mainly as phospholipid, namely lecithin, as evidenced by the identification of its hydrolysis products, viz., choline and glycerophosphate.

Until lately the literature data on the presence of phospholipids in the bile have been based on the experiments carried out at the beginning of the century by Hammarsten.[12] And yet in 1937 Jones and Sherberg[13] concluded that the bile of the dog, pig and cattle is free from any lecithin and neutral fats.

Our investigations carried out by the isotope method and proving the presence of phospholipids in the bile, provide the following additional evidence. Firstly, phosphorus of phospholipids constitues up to 99% of total phosphorus of the bile. Secondly, phospholipids synthesized in the liver are rapidly detected in the bile and the increase in the specific activity of the bile suggests that between the process of phospholipid formation in the liver and their excretion into the bile there is no great lapse of time. Thirdly, as far as chromatographic data are concerned, phospholipids discovered in the bile and those extracted from the liver have the same properties.

It is hardly conceivable that phospholipids are an excretion product and that their presence in the bile is due to an excretory process. The question arises as to the fate of organic phosphoric bile compounds after they get to the intestine, and first of all, whether they are taken up in the intestine, and in what form.

THE FATE OF ORGANIC PHOSPHORIC BILE COMPOUNDS ADMINISTERED *per os*.

To determine whether any intestinal absorption of organic phosphoric bile compounds occurs, experiments were carried on in the following way. One millicurie of radioactive phosphorus as sodium phosphate was administered *per os* to a donor-dog with a Shiff gall-bladder fistula. On the next day radioactive bile was collected from the donor-dog. In this bile radioactive phosphorus was almost entirely combined with organic compounds. The recipient-dog was fed with bile (mixed with milk) as such or as phosphoric organic compounds extracted from it. In the control experiments the dogs were treated with inorganic radioactive phosphorus added to inactive bile and milk.

Thereafter, at various time intervals, the radioactivity of blood serum of dogs, of the serum inorganic-phosphorus fractions and of lipid fractions were determined. Along with this the total, inorganic and lipid phosphorus content of the serum was estimated. From these data the specific activity of each phosphoric fraction was computed using the standard method:

$$\text{Specific activity} = \frac{\text{cpm/ml} \times 100}{\text{mg } P^{31}} \times \frac{\text{Total number applied, cpm}}{\text{Total weight of animal, gm}}$$

Typical radioactivity curves of the serum and of inorganic serum phosphorus received from control dogs are illustrated by the graphs of Fig. 3. All these curves appear quite similar in all the experiments.

An analysis of the curves shows that inorganic phosphorus applied to the intestine is taken up by the blood and during the first hours following administration circulates in the blood plasma in the form of inorganic compounds. This is shown by the fact that the radioactivity curve of the inorganic phosphorus fraction of the serum almost coincides with that of total radioactivity of blood serum. It is

only after 24 hours that these curves begin to diverge when organic phosphoric compounds enter the blood.

The picture was different when radioactive bile was introduced into the intestine. Radioactivity of the total serum phosphorus was much higher than that of the inorganic phosphate fraction (Fig. 3).

This means that in contradistinction to the control serum radioactivity is not due to the radioactivity of inorganic phosphorus. It has been shown in fact by special experiments that radioactivity of the serum is largely due to the radioactivity of the lipid fraction extracted from the serum by an alcohol–ether mixture (Fig. 4).

It appears from a detailed analysis of the curves that the absorption of organic phosphoric compounds from the bile administered to the intestine proceeds slower than that of inorganic phosphorus in the control experiment. The disappearance from the blood and utilization of absorbed organic phosphoric compounds are also slower than in the control experiments.

It will thus be inferred from the above data that phospholipids secreted with bile into the intestine are taken up by the blood without phosphorus being split off.

It seemed worth while therefore to find out by means of experiments *in vitro* whether a mixture of digestive juices from dog duodenum fistula or the juice from the Thiry intestine fistula are able to split the organic phosphoric bile compounds. After various intervals of incubation of the intestinal juices or of a mixture of digestive juices with radioactive bile the radioactive inorganic phosphate was estimated in the samples by means of the radiochemical or radiochromatographic method. This did not increase during the incubation of the samples; that is, under the conditions of our experiments the phospholipids of the bile have not been split in test-tubes by the digestive juices at issue.

Thus owing to the isotope method there is no more doubt that the bile phospholipids are taken up from

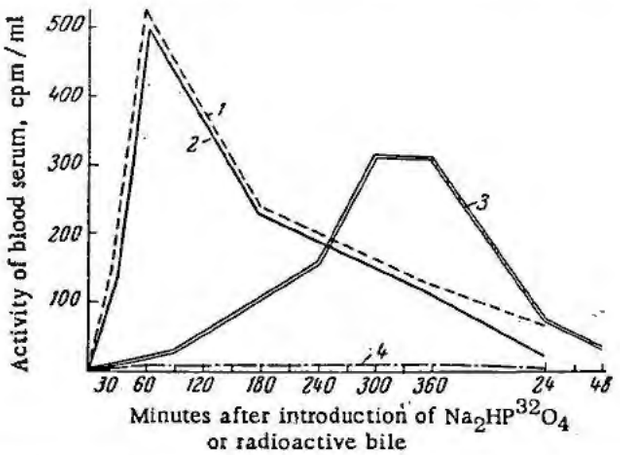

Figure 3. Radioactivity of total and inorganic blood serum phosphorus after per os administration to a dog of $Na_2HP^{32}O_4$ (curves 1, 2), or radioactive bile (curves 3, 4). Curves 1, 3, radioactivity of blood serum; curves 2, 4, that of inorganic blood serum phosphorus

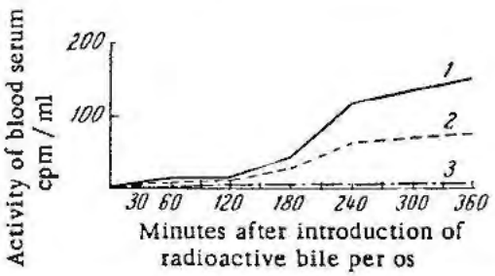

Figure 4. The turnover of phosphoric bile compounds in the organism. Radioactivity of total (curve 1), lipid (curve 2) and inorganic (curve 3) phosphorus of dog blood serum after per os administration of radioactive bile

the intestine without inorganic phosphorus being split off.

A revision is therefore justified of the existing views on the mechanism of absorption from the intestine of lipids in general[14] and of phospholipids in particular. The necessity of such a revision is also indicated by the data of Swedish authors[15] obtained with labeled glycerol and fatty acids.

The turnover of bile acids and of bile pigments was a question of much interest even as far back as the 19th century. Studies on cholesterol turnover have also been carried out.[16] The time has come to raise the question of the existence of a turnover of the phosphoric bile compounds (see Fig. 4).

PATH OF ORGANIC PHOSPHORUS COMPOUNDS

The purpose of these series of investigations was to receive an answer to the following question: does bile collected in a dog through a fistula in the gall-bladder contain the molecules of organic phosphoric compounds preliminarily administered to the dog *per os*?

The answer was received by means of the isotope-dilution method. The experimental procedure was as follows. During the following day after administration *per os* to a dog of inorganic phosphorus the specific activity of the bile usually keeps constant. On this background highly radioactive bile from a donor-dog was administered to a dog *per os*. In other experiments non-radioactive bile was administered. Would phospholipids of the administered bile pass from the intestine directly without change to the recipient-dog then it might have been expected that upon administration of radioactive bile an increase in the specific activity of the phosphoric bile compounds will take place while with non-active bile the specific activity of the bile in the recipient-dog will decrease. In the first case the specific activity should have increased owing to the penetration of highly radioactive phospholipids into the bile, and in the second case it should have decreased owing to the non-active phospholipids.

In Figs. 5 and 6 the results of these experiments are presented. It will be seen from the graphs that the specific activity of phosphoric bile compounds of the recipient-dog after *per os* administration of both radioactive and non-radioactive bile, does not change

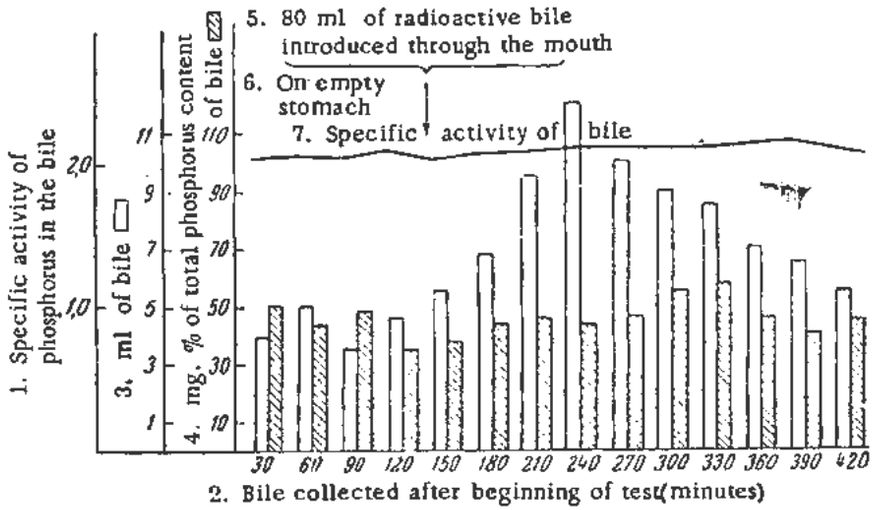

Figure 5. Absorption of bile inorganic phosphorus and phospholipids from the gall-bladder before and after administration to a dog of *radioactive* bile (measured while the specific activity of bile is stable)

although the total amount of excreted bile increases as well as its total of phosphorus content.

It appears from the above that bile phospholipids taken up in the intestine do not pass directly to the bile, and the stimulating effect of the administered bile is connected with the increase in synthetic liver activity, expecially with regard to phospholipid synthesis (see Fig. 5).

ABSORPTION OF NON-ORGANIC PHOSPHATE AND PHOSPHOLIPIDS OF BILE FROM THE GALL BLADDER. EFFECT OF ATROPINE

The discovery of the fact that bile phospholipids may be taken up in the intestine without being split suggests the possibility that such an absorption might

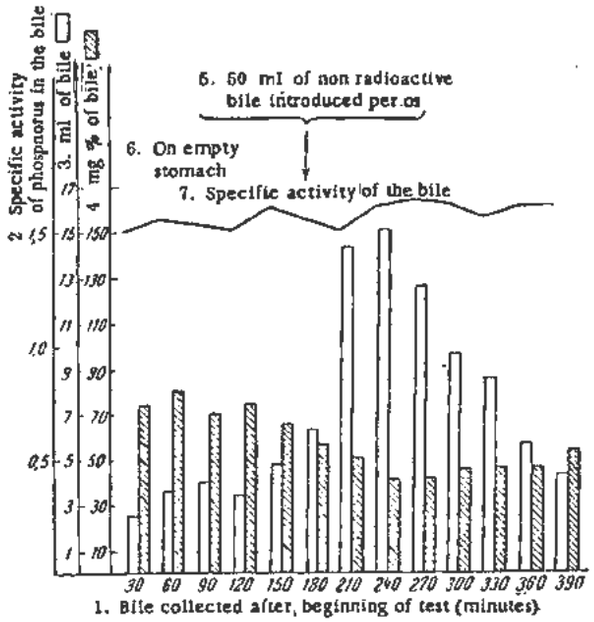

Figure 6. Absorption of bile, inorganic phosphorus and phospholipids from the gall-bladder before and after administration to a dog of non-radioactive bile (measured while the specific activity of bile is stable)

presumably occur also in the gall-bladder where accumulation of bile as well as several changes in the composition of individual bile components are known to take place.

Radioactive bile was collected from the donor-dog and introduced in acute experiments into the gall-bladder of another dog after preliminary ligation of the *d. cystici* and drawing off the bile from the bladder. In chronic experiments radioactive bile was introduced into the gall-bladder through a fistula. In these dogs the common bile duct was preliminarily ligated. After the administration of the bile the radioactivity of blood serum was tested. Not in a single case was there noted the transfer of radioactive phospholipids to the serum (Fig. 6).

In another series of experiments a solution of inorganic phosphate with known activity of radioactive phosphorus was administered to the gall-bladder. The transfer of radioactive phosphorus to blood was followed up for 5 hours. Toward the end of this period the remaining activity in the gall-bladder was estimated. It was found that, during the 5-hour period, up to 80% of the administered inorganic radioactive phosphorus was taken up from the gall-bladder.

The absorption of radioactive phosphate from the gall-bladder is regulated by the nervous system. This was shown by experiments on dogs subcutaneously injected with atropine (5 mg/kg body weight). Atropine was injected 30 minutes prior to the administration of radioactive inorganic phosphate to the bladder. In this case the curve of phosphorus uptake from the bladder differed greatly from the previous one (Fig. 7). Only 10-20% of the administered radioactive phosphorus was absorbed during the 5-hour period.

The above experiments make only the beginning of a systematic revision with the aid of the isotope method of our present knowledge about the absorption processes in the gall-bladder.

PHOSPHORIC BLOOD COMPOUNDS IN JAUNDICE

The secretion with the bile of a relatively large amount of phospholipids into the intestine and its subsequent absorption is, in our view, a normal physiological process related to the regulation of phosphorus metabolism in the organism and possibly to the transfer of phosphoric compounds from the liver to other organs. A disturbance of the normal process of bile excretion into the intestine should therefore result in a disturbance of phosphorus metabolism within the organism.

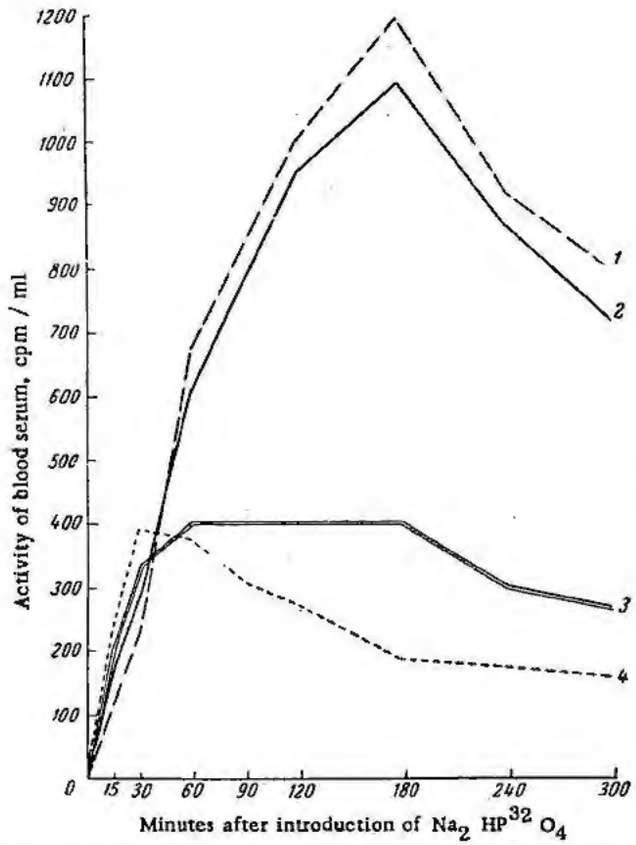

Figure 7. Phosphoric blood compounds in jaundice. Radioactivity of blood serum after $Na_2HP^{32}O_4$ administration to the gall bladder of a dog (acute experiments). Curves 1 and 2, without atropine; curves 3 and 4, after preliminary administration of atropine

It was found indeed that in diseases associated with jaundice, the phospholipid content of the blood is increased. The disappearance of jaundice is paralleled by a decrease in the phospholipid content of the blood plasm.

In patients suffering from parenchymatous hepatitis the phosphorus content of the blood increases along with the increase in bilirubin level: in high level bilirubinemia the phosphorus content attains 50–60 mg%. A decrease of the bilirubin content of the blood is paralleled by a decrease of the blood phosphorus level. In patients suffering from cholecystitis without jaundice the amount of phosphoric compounds in the blood does not exceed the normal level of 28–32 mg% (Fig. 8).

To answer the question why jaundice is accompanied by a change in the content of phosphorus compounds of the blood, experiments were carried out on rabbits. Prior to ligation of the bile duct, during the period of progressive jaundice and until the death of the animal, determinations of the concentration of total, inorganic and lipid phosphorus as well as of bilirubin, were carried out both in whole blood and in blood serum.

Figure 9 shows that during the progress of jaundice after ligation of the bile duct the phosphorus content of blood serum increases 2–3 times, and that of lipid phosphorus 4–5 times. The inorganic phosphorus content of the serum does not change. Just as in jaundice patients, the bilirubin curve is parallel to that of serum phosphorus (Fig. 9).

These results point to the conclusion that the normal level of phosphorus compounds in the blood is regulated first of all by the secretion of phosphorus compounds with the bile into the digestive tract. If this way is cut off, as, e.g., in mechanical jaundice, the regulation of lipid metabolism in the organism is disturbed. This is due not only to a disturbance in the digestion and absorption of lipids in the intestine but also to the fact that the secretion of phosphoric compounds into the intestine is discontinued.

It may be suggested from the increase in the lipid phosphorus level of blood serum that when the bile flow, which is rich in phosphoric compounds, is cut off these compounds enter the blood with bile pigments.

In order to ascertain the changes brought about by mechanical jaundice in the uptake and utilization of phosphorus the isotope method was applied.

By means of a gastric probe, radioactive phosphate was administered to fasting control and "jaundiced" rabbits and the specific activity of various phosphoric fractions of the serum periodically determined.

A comparison with the control (Fig. 10) shows the following differences in the absorption and utilization of phosphorus in the "jaundiced" rabbits:

(*a*) At an early period after the administration of radioactive phosphorus to jaundiced rabbits the specific activity of total serum phosphorus is higher than in the control. This difference becomes especially pronounced if we consider the fact that the inactive phosphorus content of the serum is increased so that the labeled phosphorus transferred from the intestine is being more than normally diluted by the inactive phosphorus circulating in the blood.

(*b*) Contrary to the control, radioactive phosphorus is found in the lipid fraction already during the first hours following its administration, and after 24–48 hours the maximum of specific activity of this fraction is attained. It will be noted that despite the increase of the serum phospholipid level the specific activity is not lower, and eventually even higher than in the control. It may thus be assumed that the transfer of inorganic phosphorus from the intestine to blood is increased in jaundice and that the newly

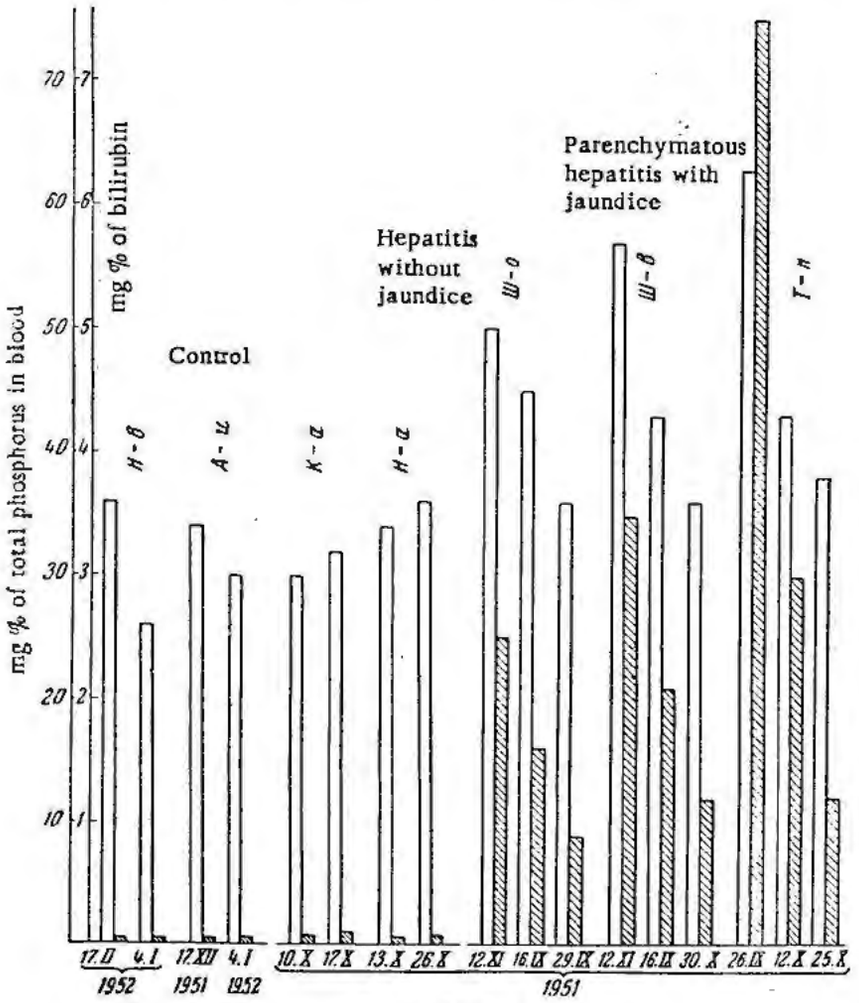

Figure 8. Total phosphorus and bilirubin content of patient's blood with and without jaundice. Unshaded columns, total phosphorus; shaded, bilirubin

synthesized phospholipids enter the blood at a high rate and in increased amounts. It is quite possible that these data might happen to be of practical importance with further elucidation of the mechanism of metabolic disturbances, particularly of phosphorus metabolism, in jaundices of various origin (see Fig. 10).

ABSORPTION OF PHOSPHORUS FROM THE INTESTINE DURING EXPERIMENTAL HEPATITIS IN THE ABSENCE OF JAUNDICE

As mentioned above, in patients suffering from liver diseases not accompanied by jaundice, the total, inorganic and lipid phosphorus content of blood serum remains normal. It seemed worth while to ascertain whether or not hepatitis is accompanied by any changes in the absorption of phosphorus from the intestine.

Hepatitis was induced in dogs through administration of carbon tetrachloride. This is known to be the common way of inducing experimental hepatitis. A new model of experimental hepatitis was also developed. Morphological investigations on rats and dogs show indeed that sodium salicylate applied to the gastro-intestinal tract in the course of 10–12 days at a rate of 0.1–0.2 gm/kg body weight induces the development of toxic hepatitis. While the animal was alive hepatitis was diagnosed by functional tests and *post mortem* by histological methods. The histological examinations were carried out by E. A. Rudik-Guntova.[17]

In addition to dogs with experimentally induced hepatitis some other animals were examined in which hepatitis was caused by infections from the bile paths. In most cases, these were dogs with a fistula of the gall-bladder and an inflammatory focus around the fistula.

The experimental procedure was as follows. Healthy and affected dogs were treated *per os* with radioactive phosphorus. This was administered to fasting animals in the form of milk solution of disodium phosphate the 250 ml; whereafter the specific activity of total blood serum phosphorus was followed up.

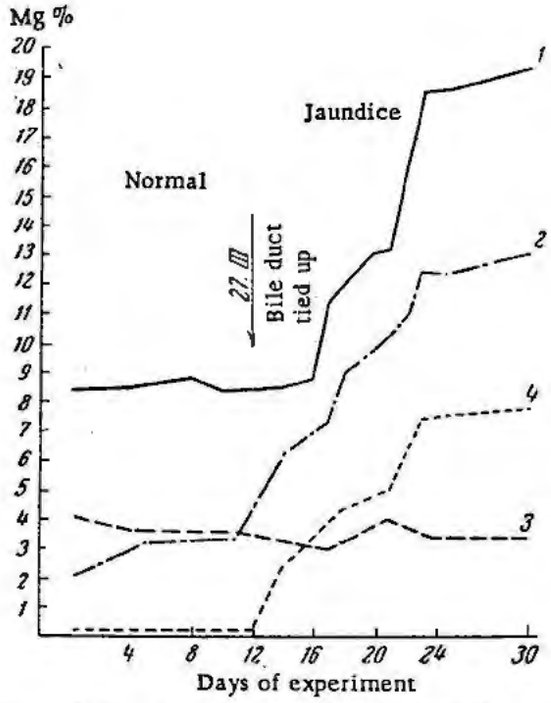

Figure 9. Blood serum content of total (curve 1), lipid (curve 2), inorganic (curve 3) and phosphorus and bilirubin (curve 4) prior to and after ligation of the common bile duct in the rabbit

The results are shown by the graph, Fig. 11. A distinct difference is to be seen between the specific activity of blood serum of healthy and hepatitis-affected dogs. In all the experiments, regardless of the cause of hepatitis, the curve of the specific activity is much below and quite different in shape as compared with normal one. This difference in the shape of the curves of specific activity of blood serum may be due to a disturbance in the phosphorus uptake or its utilization or to both.

This problem has been resolved by a simple experiment. Radioactive phosphorus was administered not *per os* but subcutaneously to healthy dogs and to those affected with hepatitis. Again the curves of specific activity of blood serum were compared

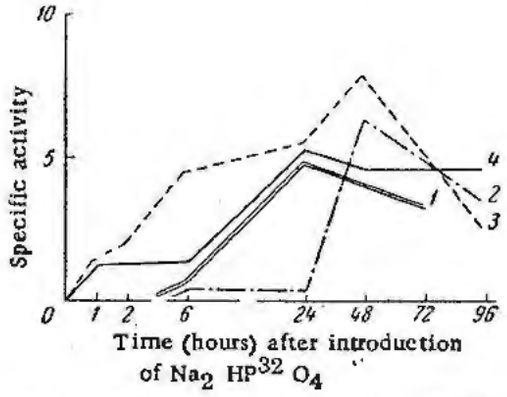

Figure 10. Phosphorus uptake from the intestine in experimental hepatitis of various origins in the absence of jaundice. Specific activity of lipid blood serum phosphorus in normal (curves 1 and 2) and "jaundiced" rabbits (curves 3 and 4)

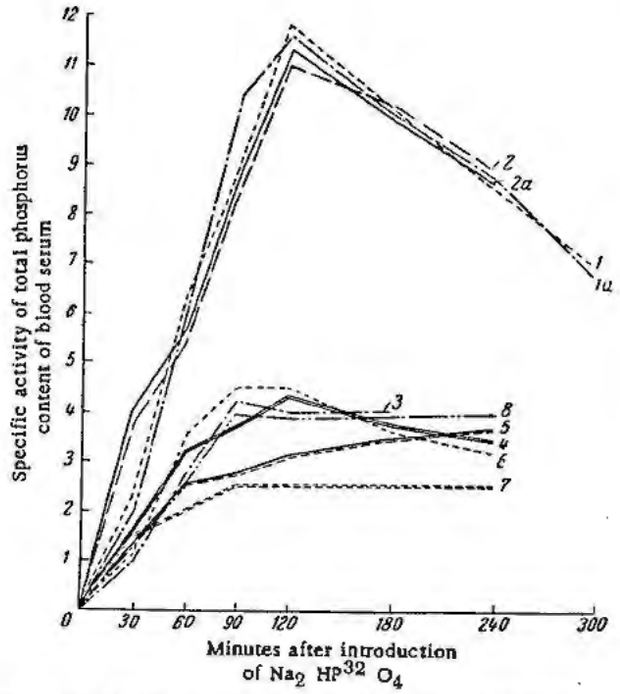

Figure 11. Specific activities of total blood serum phosphorus after per os administration of $Na_2HP^{32}O_4$ to healthy dogs and those affected with hepatitis of various origins. Curves 1, 1a, 2 and 2a, normal. Curves 3 and 4, hepatitis induced by sodium salicylate; 5 and 6, by carbon tetrachloride; 7 and 8, by ascending infection from bile paths

(Fig. 12). The difference in these curves suggests the existence not only of a disturbed phosphorus uptake from the intestines but of its utilization in the organism as well.

It is the isotope method that enabled us to disclose the disturbances in the absorption and utilization of phosphorus in the organism. The answer has not yet been received as to the mechanism of these disturbances. However, it may be expected that the application of the isotope method will be of greatest use in the solution of this problem.

It is quite obvious that the above data on the uptake of radioactive phosphorus in some pathological conditions of the liver suggest a new interpretation of the functional tests of liver disorders, such as the glucose, galactose tests in which the possible disturbances of the uptake of these substances should be considered.

THE EFFECT OF ATROPINE ON INORGANIC PHOSPHATE UPTAKE FROM THE INTESTINE

The effect of atropine on the secretion of the digestive glands and on the motor function of the gastro-intestinal tract has been well studied (I. P. Pavlov and his pupils), while the effect of atropine on absorption from the intestine is still largely in the dark. Such a study is of substantial practical importance in view of the wide clinical application of atropine in gastro-intestinal pathology in particular. The above data show that under the influence of atropine the uptake of inorganic phosphorus from the gall-bladder is greatly reduced.

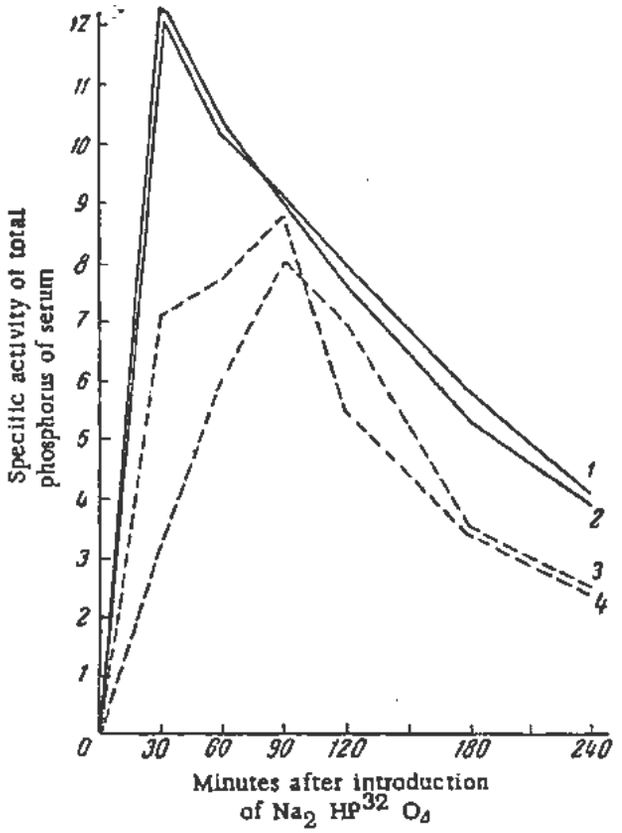

Figure 12. Specific activities of total blood serum phosphorus in healthy dogs and dogs with hepatitis after subcutaneous injection of $Na_2HP^{32}O_4$. Curves 1 and 2, normal; curves 3 and 4, hepatitis induced by carbon tetrachloride

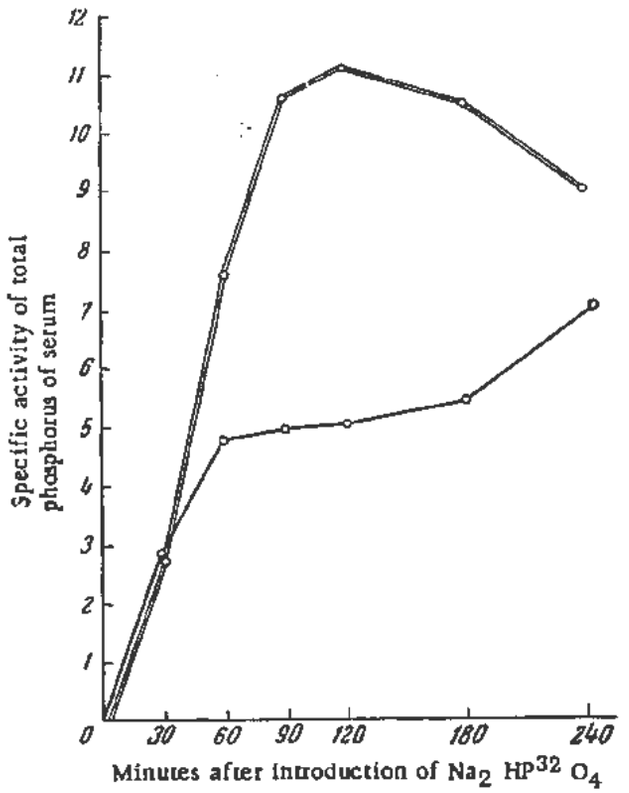

Figure 13. Effect of atropine on inorganic phosphate ($Na_2HP^{32}O_4$) uptake from the digestive tract in dogs. Upper curves, normal; lower curves, after atropine administration

In Fig. 13 the results of experiments are presented in which 2 minutes prior to the *per os* administration of radioactive phosphorus in the form of $Na_2HP^{32}O_4$, a dose of 5 mg/kg body weight of atropine was injected subcutaneously to a dog. A distinct decrease in the specific blood serum activity was observed.

SUMMARY

The above evidence by no means presents a completed idea as to the ways in which the isotope method may be used in physiology, biochemistry and pathology of digestion. But whatever be the physiological significance of the evidence thus far obtained it should be emphasized once again that this could be accomplished only with the aid of the isotope method. Even in those cases where the common classical methods could have yielded the same results, the isotope method made the study more rapid and less cumbersome with more reliable and precise results.

In his Nobel speech[18] of 1904, Pavlov, the great author of physiology of the digestive processes, said: "The exact knowledge of the fate of the food within the organism should be the subject of ideal physiology, physiology of the future". It is owing to the isotope method that the dream of "physiology of the future" is now beginning to turn into reality.

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Use of I^{131} in the Study of the Influence of Climatic Factors on Thyroid Activity and Productivity of Livestock

By C. Blincoe and S. Brody,* USA

Figure 1. *In vivo* determination of thyroid I^{131}

The thyroid is a major regulator of metabolic and productive processes (milk, meat, eggs, wool). Its activity changes adaptatively to environmental conditions, particularly to temperature. The purpose of this research is to study the effects of temperature, air movement, light intensity, and various categories of diurnal temperature rhythms on thyroid activity in relation to productive processes (milk production and growth) in various farm animals, particularly European- and Indian-evolved breeds, and crosses between Indian and European cattle.

METHODS

The radioiodine-uptake method has been used for the study of thyroid activity since 1946 when nuclear reactor-produced I^{131} became available. The amount of I^{131} in the thyroid of the living animal may be measured directly by holding a directionally shielded Geiger-Muller counter over each lobe of the thyroid[1], as illustrated in Fig. 1. The results were compared with similar measurements on a phantom thyroid.

The maximum uptake of the I^{131} is reached in man about 24 hours after its administration, when the readings are usually made clinically in the diagnosis of thyroid pathologies. In cattle the maximum occurs about 72 hours after injection as shown in Fig. 2. The maximum uptake of I^{131} by the thyroid gland is indicative of the uptake, or iodine fixation, phase of thyroid activity. It may not indicate the secretory activity of the thyroid gland, because iodine may be stored and the preformed thyroid hormone released as needed.

Instead of measuring the maximum amount of I^{131} in the thyroid gland, the rate of I^{131} loss by the gland can be measured. This (when corrected for recycling of I^{131}) represents the secretion rate of the thyroid hormone—the rate constant for thyroid hormone secretion, k_4. Similarly, the rate of accumulation of radioiodide in the thyroid may be measured. From this the relative rate of uptake of I^{131} by the thyroid—the rate constant for thyroid uptake, k_1—can be calculated.

Examples of calculations of rate constants are given in Fig. 2. The rate constant for thyroid uptake may also be calculated from the maximum uptake, U, of I^{131} by the thyroid either from the rate of decrease of blood radioiodide concentration or from the rate of excretion of I^{131} (Figs. 2 and 3). A complete theoretical treatment of radioiodine metabolism and derivation of these and other rate constants was given by Oddie[2] and Brownell.[3]

The conversion ratio (ratio of thyroxine-like I^{131} to total I^{131} in the blood plasma) has been used in clinical diagnosis of thyroid pathologies in man.[4,5] This measure, being dimensionless, has the advantage of being independent of the size of the tracer dose of radioiodide employed; but since it is calculated from two non-identical measurements its error is relatively large. In man it has been customary to measure the conversion ratio 24-hours after ingestion of I^{131}. In cattle this measurement was made 24 and 72 hr after injection of I^{131}. The 24-hr measurement in man and the 72-hr one in cattle are made as the thyroxine-like I^{131} is approaching its maximum.

Other methods have been proposed to ascertain the thyroid activity by using radioiodine, but have not been widely used. Pipes, Blincoe, and Hsieh[6] proposed that the minimum amount of exogenous thyroxine necessary to block the thyroid turnover of I^{131} was equal to the normal secretion rate of the gland. This method could not be used in the present study because the administration of thyroxine would alter the metabolic rate.

Data were also obtained on the metabolism of radioiodide and its clearance by various routes—especially milk, urine, and the thyroid gland. The

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rate of transcapillary diffusion of I^{131} has also been studied. Examples of these type data are given in Figs. 3 and 4. These data will appear in forthcoming publications.[7,8]

In the present research an alkaline carrier-free solution of NaI^{131} was injected into the jugular vein. Usually, a polyethylene tube of small diameter was placed in the jugular vein and the injection and bleedings made through it.[9] Blood was collected in vessels containing 10-20 mg heparin (60 Toronto units per mg) per 100 ml blood.

The following dosages were satisfactory for cattle:

Body weight, kg	*Dose, mc I^{131}*
50–100	0.08
100–200	0.1
200–300	0.2
Mature cows	0.3–0.5

These quantities are of the same order of radioiodine per kilogram body weight as used on other species.

Twenty-four hour urine samples were collected with a modified Hansard urinal for cows.[9] Milk samples were taken from each milking during the first 5 days after injection.

The total radioiodine content per unit blood plasma, milk and urine was made by counting 1 to 2 ml samples. The thyroxine-like I^{131} concentration was determined in blood plasma. This was taken as the radioiodine extractable from unhydrolyzed plasma by *n*-butanol and not extractable from the *n*-butanol by Blau's reagent (4*N* NaOH-5% Na_2CO_3).

LITERATURE ON THE EFFECT OF TEMPERATURE ON THYROID ACTIVITY

There is a wealth of literature on the effect of temperature on thyroid activity as measured by heat production.[10,11] Data on heat production, however, include not only the effects of thyroid activity, but also changes in neuromuscular activity, food consumption (hunger and appetite), milk production and activity of other endocrines, particularly the adrenals. Heat production data are therefore ambiguous and attempts have been made to develop other methods for measuring thyroid activity, ranging from levels of blood cholesterol and protein-bound iodine which do not involve the sacrifice of the animal, to the thiouracil-thyroxine method and microscopic structure of thyroid tissue which do involve sacrifice.

Employing the thiouracil method, Dempsey and Astwood[12] reported a decrease of thyroxine secretion rate in white rats, in terms of micrograms/day, from 5.2 at 1°C to 1.7 at 35°C. Others using the same method reported an inverse relation between thyroid activity and temperature in mice[13] and fowls.[14,15]

The results by the protein-bound iodine methods are not as clear cut as by the thiouracil method or radioiodine method. H. G. Turner,[16] in our laboratory, discarded the PBI method on cows, as the results were too erratic.

The radioiodine method was first used to study thyroid activity in rabbits by Hertz, Roberts, and Evans in 1938.[17] Most of the literature since then has

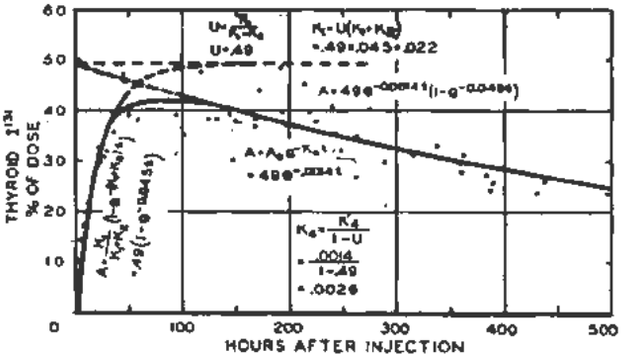

Figure 2. Thyroid I^{131} content. Rising segment shows uptake of I^{131} from blood; declining segment, release of the thyroid hormone containing I^{131}. Equations indicate calculation of rate constants for these processes and correction of the apparent hormone release rate constant, k_4, for re-utilization of I^{131} to give the hormone release rate constant

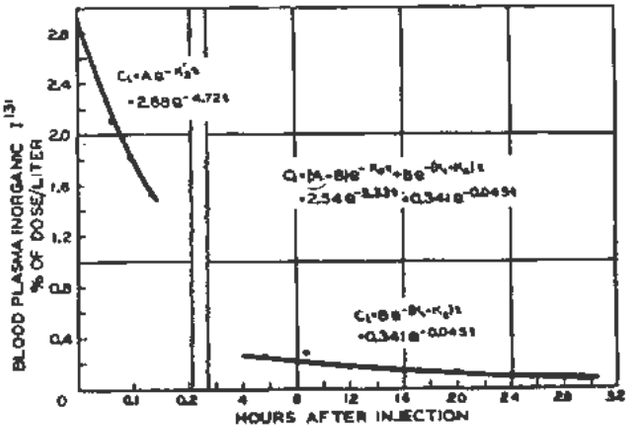

Figure 3. Blood plasma I^{131} concentration. Segment on the left shows the very rapid decrease of blood plasma I^{131} due to transcapillary diffusion; on the right, the slower decrease due to thyroid uptake of I^{131} and excretion of I^{131}. Equations give rate constants for these processes

been concerned with the use of I^{131} in the clinical diagnosis and therapy of thyroid pathologies. Employing the radioiodine method, Werner and co-workers[18,19] reported that they could find no significant seasonal differences between the uptake of I^{131} by the thyroid in normal men and women. Leblond and co-workers,[20] investigating the effect of ambient temperature on the radioiodine metabolism of rats, found that cold (0 to 2°C) increased thyroid activity to a maximum during the first 26 days of exposure. Thyroid activity declined to the pre-exposure level during the subsequent 40 days of exposure to this freezing temperature. Heat (32 to 34°C) depressed thyroid activity during the first day of exposure, and this reduced activity persisted for at least 26 days of exposure. The authors[16] reported that the conversion ratio and the I^{131} uptake by the thyroid at 95°F (35°C) in rabbits and cows were lower than those at 50 to 60°F (10 to 16°C) air temperature.

The rate of biosynthesis of the thyroid hormone is under the control of the thyrotropic hormone of the anterior pituitary gland. Sellers and You[21]

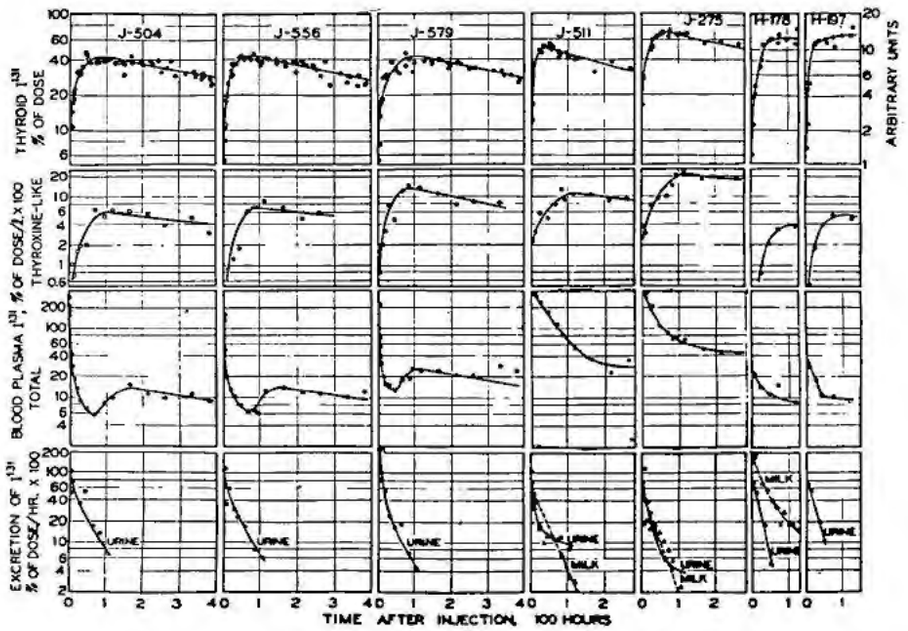

Figure 4. Time relations of metabolism and excretion of injected I^{131}

reported increasing thyrotropic hormone in the pituitary glands of male white rats on reducing the environmental temperature to 1.5°C for 2 to 3 weeks. They assumed that this represented increased production and release of the hormone under the influence of declining temperature.

Relatively little use has been made of the radioiodine method in studies with larger domestic animals. Leblond reported[22] studies on the uptake of radioiodine by the thyroid gland of dogs and Borgman[23] used the uptake of I^{131} to study the effect of exogenous thyroid substances on the thyroid function of immature dogs. Schirmer[24] also used this technique to study the effect of thiouracil administration on dogs.

Radioiodine has been used to demonstrate that the ability of new-born pigs to survive is associated with a high level of thyroid activity.[25] Terry[26] found increased thyroid uptake of I^{131} in sheep exposed to constant darkness and reduced uptake of I^{131} in those exposed to constant light.

Radioiodine was used to study the effect of exogenous thyroxine on the thyroid activity of cattle by Swanson, Monroe, and Comar.[27] Work is now in progress on the relation between dwarfism in cattle and thyroid activity as measured with the aid of I^{131}.

RESULTS

Effect of Constant Temperature, Wind and Radiation (Light)

For these studies, mature cattle (Jersey, Holstein, Brown Swiss, and Brahman) were kept in the Psychroenergetic Laboratory.[28] Temperature, humidity, and air velocity were maintained constant (day and night) for test periods of 2–5 weeks except at temperature above 32°C when shorter periods were necessary. In the studies of light intensity, the radiation level was constant for 12 hours and approximately zero for 12 hours during each day of a 5 week test period. Each set of animals were first subjected to temperatures of 5–16°C and then conditions were changed stepwise to higher or lower temperatures.

Two of the three periods were devoted to a study of the effect of wind at several temperatures and one to the effect of radiation (light) intensity at several temperatures. It will be shown later that wind and light radiation level had no effect on the conversion ratio in the ranges studied. The data may thus be combined as though the only variable was temperature. These data indicate a reduction in thyroid secretory activity in all species studied as the temperature rose above 0–10°C, the zone of thermoneutrality for cattle. At —8°C the thyroid activity of the small Jersey and Brahman cows increased while that of the large Holstein and Brown Swiss cows remained unchanged. The resting heat production of these animals paralleled the thyroid activity with changing ambient temperature. The following table gives the conversion ratios, CR, and resting heat production, RH,[29,30] as a percentage of the values observed in the temperature range 0–10°C, or thermoneutrality. The number of samples at each point is also included.

	−8°C			35°C		
Breed	*No.*	*CR*	*RH*	*No.*	*CR*	*RH*
Jersey	12	+60%	+15%	8	—30%	—15%
Brahman	3	+100%	+60%	—	—	—
Holstein	9	0	0	3	—60%	—30%
Brown Swiss	8	0	0	3	—50%	—20%

Air velocity in the range 0.8 to 17 km/hr did not affect the conversion ratios in Brown Swiss and Holstein cows in the temperature range —8 to 27°C,

and in Jersey and Brahman cows in the temperature range —8 to 35°C. At the higher air velocities at —8°C the Brahmans shivered with consequent increased heat production[29] but not increased thyroid activity.

The addition of 488 kcal/m²/hr radiant energy (largely visible and near infra-red) did not affect the conversion ratio of Jersey, Holstein, and Brahman cattle at 21 or 27°C. This added radiation load did, however, depress the rate of thyroid uptake of I^{131}, k_1, and the maximum thyroid uptake of I^{131}, U, in Jersey and Holstein cows as shown in the following table:

Temp °C	Radiation, kcal/m²/hr	k_1, hr⁻¹		
		Jersey (4 cows)	Holstein (6 cows)	Brahman (1 cow)
21	14	0.019	—	0.004
	488	0.015	—	0.006
27	14	0.017	0.0083	0.007
	488	0.010	0.0077	0.006

As in the two previous studies, the resting heat production[30] and thyroid activity followed similar trends. Details of these studies will appear in a forthcoming publication.[7]

Effect of Diurnally Variable Ambient Temperature

The previous data under constant environmental conditions indicated an effect of constant heat stress on thyroid activity. In this study the ambient temperature was varied diurnally with a period of 24 hours, resembling the climatic diurnal summer and winter rhythms in the Midwest and Imperial Valley. The animals were then not equilibrated but in a continuous state of diurnal thermal adjustment. During these studies air velocity and radiation levels remained constant. Three Jersey and three Holstein cows were used for this study.

The maximum uptake of I^{131} by the thyroid gland, U, the rate constants for thyroid uptake of I^{131}, k_1, and thyroid hormone release, k_4, decreased with increasing ambient temperature. The values of these parameters are given as averages in the following table:

Diurnal Temp range °C	k_1, hr⁻¹	k_4, hr⁻¹	U, % dose
—10 to +5	0.039	0.0107	64.4
4 to 21	0.032	0.0062	57.1
21 to 38	0.022	0.0033	41.3

Details of this study will be published elsewhere.[8]

Comparison of Methods

In collecting all the foregoing data four parameters of thyroid activity were used, which need critical comparison.

In the study of the effect of constant ambient temperature the conversion ratio indicated changes in thyroid activity over wide temperature ranges; whereas in the study of the effect of radiation and of diurnally variable temperature it (the conversion ratio) indicated no change over small temperature ranges. In the latter two studies, the maximum thyroid uptake of I^{131}, U, and the rate constant for thyroid uptake of I^{131}, k_1, indicated changes of thyroid activity. In the study of the effect of diurnally variable temperature the maximum thyroid uptake of I^{131}, the rate constants for thyroid uptake and hormone release all indicated changes of thyroid secretory activity of about equal magnitude. The parameters are determined from a set of several identical measurements and hence have a lower inherent error than the conversion ratio which is determined from two non-identical measurements.

The rate constant for thyroid uptake, k_1, and the maximum thyroid uptake of I^{131}, U, are measures of the uptake phase of thyroid activity whereas the rate constant for thyroid hormone release, k_4, is a measure of the secretory phase of thyroid function. It would appear that the secretory phase of thyroid activity is more significant than the uptake phase from the standpoint of the thyroid gland as a regulator of physiological processes.

The cost of procuring data is often a limiting factor in research. The least costly method was that of measuring the maximum uptake of I^{131}, U, by the thyroid gland (Fig. 2). Once the time after injection at which this maximum occurs is established, one need only make as many measurements as will reduce the standard error to the requisite value. Instrumentation is also relatively inexpensive for the determination of the rate constant for thyroid hormone secretion, k_4, from the rate of change of thyroid I^{131} level. The measurements, however, must be continued over at least a two-week period. Determination of the rate constant for thyroid uptake, k_1, is best made by studying the rate of change of blood radioiodine concentration, but the maximum uptake of I^{131} by the thyroid must also be known, hence more instrumentation is required. The blood samples need, however, be taken over only a four to eight hour period.

Relative Effects of 10 and 27°C Constant Temperature on Tropical-evolved and Temperate-evolved Growing Heifers

The preceding report was on mature lactating cows exposed to a series of ambient temperatures ranging from —12 to 41°C. The following preliminary data are on the relative effects of two ambient temperatures, 10 and 27°C, on three breeds of heifers: (1) Shorthorn, a British breed, (2) Zebu or Brahman, an Indian (tropical-evolved) breed and (3) Santa Gertrudis, a new breed developed at the King Ranch, Texas, during the past 30 years, that combines the excellent beef-producing and heat-tolerance qualities of the original parental stock—Zebu and Shorthorn.

This experiment is currently in progress, and the data for only k_4, the thyroid hormone secretion rate constant (see Fig. 2), during ages 2 to 6 months, were processed. See table at top of first column of page 270.

Breed	Age range, days	10°C		27°C	
		No.	k_4, hr^{-1}	No.	k_4, hr^{-1}
Shorthorn	49–100	2	0.003	—	—
	100–200	5	0.004	8	0.002
Santa Gertrudis	45–100	4	0.004	4	0.002
	100–200	2	0.003	6	0.002
Brahman	44–100	5	0.005	4	0.004
	100–200	2	0.004	6	0.003

The value of k_4 for the Shorthorns at 10°C is seen to be double that at 27°C; for the Santa Gertrudis and Zebu, the difference tends to be somewhat less.

Time did not permit to work up the values for the other constants. But it is hoped that by such systematic studies it may be possible to elucidate the physiological and biochemical (endocrinological) basis of heat and cold tolerance.

SUMMARY

This report includes the following subjects: (1) a historical review of the use of I^{131} in the study of thyroid activity, especially in relation to ambient temperature and (2) a description of our research on the effect of constant ambient temperature, light, wind, and diurnal temperature rhythms on thyroid activity in relation to resting heat production in European-evolved and Indian-evolved lactating cattle and also the metabolism of I^{131} and its clearance by various routes including milk and urine. We used four parameters for measuring thyroid activity: k_1, rate of I^{131} uptake by the thyroid; k_4, rate of release from the thyroid to the blood of thyroid hormone containing I^{131}; U, the maximum uptake of I^{131} by the thyroid; and conversion ratio, the ratio of concentration of blood plasma thyroxine-like I^{131} to the concentration of blood plasma total I^{131}. All of these gave fairly concordant results. Critical discussion led to the conclusion that k_4 is the preferred parameter, followed in the given order by U, k_1, and conversion ratio.

Thyroid activity tends to follow resting heat production, but the rate of decline of thyroid activity, with rising temperature above the comfort zone (10 to 16°C), is approximately double that for resting metabolism. The Indian cattle were less affected by high temperatures and more affected by low temperatures than European-evolved cattle. Increasing ambient temperature to 35°C decreased thyroid activity 30 to 65% below that at the comfort zone; decreasing the ambient temperature to −8°C increased the thyroid activity in the (small) Zebu and Jersey respectively by 60% and 35%, but did not increase it in (large) Holstein or Brown Swiss. A diurnal temperature rhythm 21 to 38°C decreased thyroid activity by about 40% below its value during a 4 to 21°C diurnal rhythm; diurnal rhythm of −10 to 4°C increased the thyroid activity by about 10% below that during a 4 to 21°C diurnal cycle.

The addition of 490 kcal/m²/hr of light energy reduced the thyroid activity in Jerseys at 21 to 27°C, and in Holsteins at 27°C; Zebu cows were apparently not affected.

Preliminary data are also presented on the effects of 10 and 27°C ambient temperature on the thyroid activity of Zebu (Indian), Shorthorn (British), and Santa Gertrudis (evolved from Zebu and Shorthorn) calves. The values of k_4 (thyroid hormone release) for the Shorthorns at 10°C is approximately double that at 27°C. The differences are less for the Zebu and Santa Gertrudis calves.

It is hoped that such systematic studies may elucidate the physiological differences in heat and cold tolerance between European- and Indian-evolved cattle, thus furnishing a basis for developing—by selective breeding—the desired categories of heat and cold tolerance.

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Problems Raised by the Posterior Lobe of the Pituitary—and Studied by Radioactivity

By Albert Jentzer,* Switzerland

We had always thought, until 1951, that the anterior lobe of the hypophysis was the most important part of this organ. Having worked on toxic goiter with iodine-131, and the results of this work having enabled us to show that the goiter had an anatomical base, we wanted to find out whether I^{131} would accumulate exclusively in the thyroid. In order to carry out this experiment, on July 3, 1951, we injected 4 mc of I^{131} into a rabbit weighing 2.500 kg. The animal was killed 14 hours later. The thyroid, pituitary, diencephalon, adrenals, liver, heart, kidneys, muscles and blood were taken out, and the radioactivity of the various organs was compared with the help of Dr. Peter Wenger, Director of the Institut du Radium of Geneva. Here are the principal results obtained:

Thyroid: Activity is so high that it cannot be measured

Pituitary: 12,333 cpm/gm (Geiger counter)

Diencephalon: 42 cpm/gm

Adrenals: 150 cpm/gm

Liver: 78 cpm/gm

Readings from the other organs were not significant. Radioactive iodine is fixed mainly by the thyroid and pituitary. The findings pertaining to the thyroid would not have surprised us, but the 12,333 counts for the pituitary were indeed a surprise. In order to confirm or deny this result, and to find out the exact localization of the radioactive iodine in the pituitary, we made an autoradiograph of one-half of the mass of the gland, in order to have a truly plane-autoradiographic-surface of the two lobes. The result was interesting: the whole of the radioactive iodine was localized in the posterior lobe. This is a test by which the biological functioning of the posterior pituitary can be verified.

In order to arrive at an accurate and objective interpretation of the findings, we placed on the figure, on the one side an autoradiographic section of a pituitary for which the animal had received an injection of 4 mc of I^{131} and, on the other, a hemalum-eosine-stained section of the same gland. Comparison shows that only the posterior lobe is radioactive (Fig. 1).

This test enabled us to prove that the radioactivity of the posterior pituitary had a direct correlation with that of the thyroid. If thyroidectomized rabbits are injected with iodine-131 and killed at different intervals after the injection, their pituitaries show no localization of the radioactivity in the neural lobe; normal rabbits, as already stated, likewise killed at different intervals after the injection of iodine-131, show strong radioactivity in the neural lobe of the pituitary (Fig. 2).

Thus, the presence of the thyroid is necessary for localization in the pituitary, unless one injects, as will be seen, some tagged thyroxine into the thyroidectomized rabbits. Indeed, in such rabbits, we have observed that the injected tagged thyroxine, contrary to ionic iodine, localized, without any thyroid action, in the posterior lobe of the pituitary body (Fig. 3).

Summing up, all these experiments show that the presence of the thyroid is indispensable for the synthesis of thyroxine, which we already knew. When ionic iodine is injected into a non-thyroidectomized rabbit, this iodine is transformed into thyroxine by the thyroid body. What we did not know, however, was the secondary localization of thyroxine in the posterior lobe of the hypophysis.

Once these facts had been well established, we became very much interested, as did a number of our colleagues, on the one hand in the pathogenesis of hibernations which is explained by the "laying off" (the term *"mise en vacance"* is used by Laborit of Paris) of the pituitary (see Fig. 2) and, on the other hand, in Reilly's phenomena, in Selye's pituitary-adrenal axis, the activity of which is reduced to a minimum by hibernation. For this reason we have

Figure 1. Pituitary of a rabbit injected with 4 mc of I^{131} and sacrificed 14 hr later. On the left, a section stained with hemalum-eosine; on the right, a radioautograph

Original language: French.

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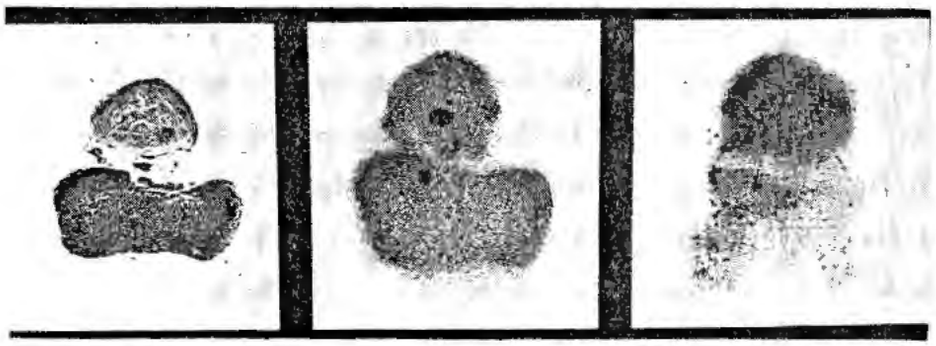

Figure 2. Comparison of neural parts of rabbit pituitaries. Sections on left and centre from a rabbit that had been operated on and hibernated. Section on right from a normal rabbit. Specimen on left stained with hemalum-eosine; specimens in centre and on right, radioautographed. (The small stain in the posterior pituitary is an artefact)

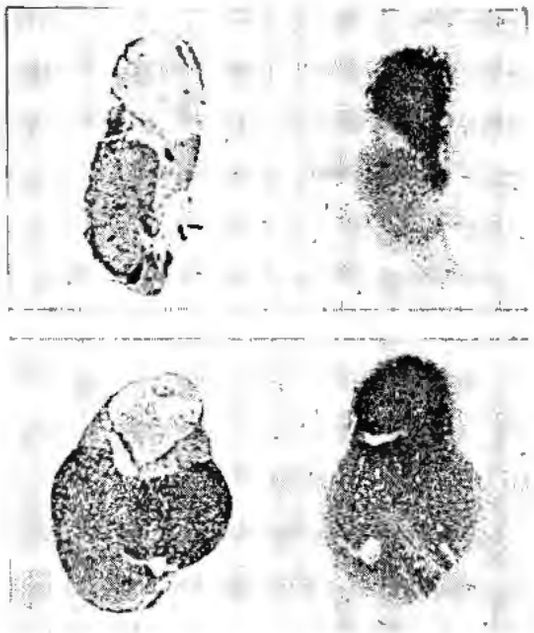

Figure 3. Pituitary of normal rabbits injected with 3 mc of labelled thyroxine and sacrificed one hour later (above), and four hours later (below). Sections on left stained with hemalum-eosine; sections on right, radioautographed

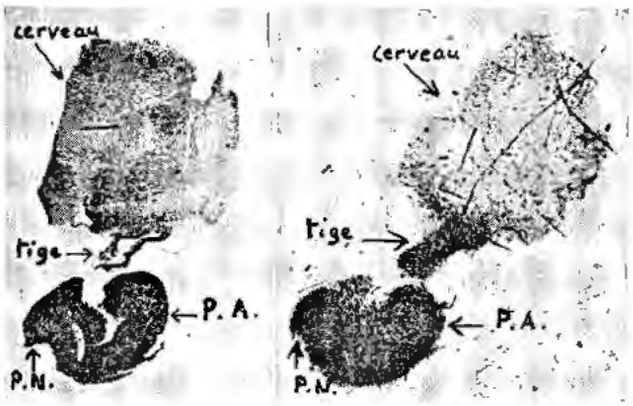

Figure 4. Presence of thyroxine in the pituitary stalk and in the neural part of the pituitary of a thyroidectomized rabbit injected with 2 mc of labelled thyroxine and sacrificed four hours later. Section on left stained with hemalum-eosine; section on right, radioautographed

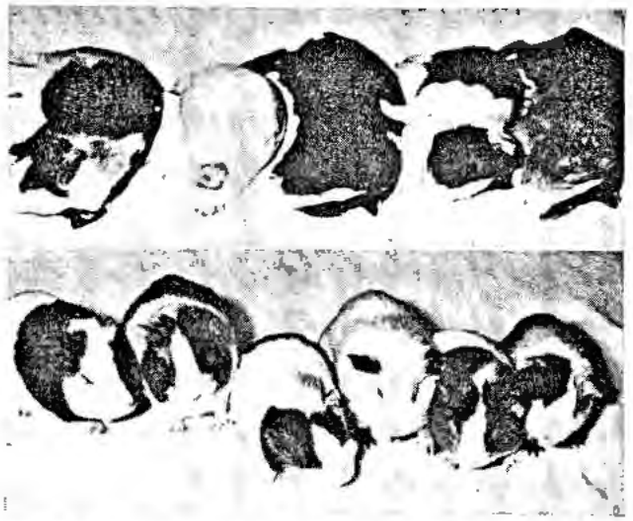

Figure 5. Above: anaphylactic shock without hibernation (all the guinea pigs are dead). Below: anaphylactic shock with hibernation (all the guinea pigs remain alive)

endeavored, in a series of experiments, to catch the passage of thyroxine in the pituitary stalk and in the nuclei of the thalamus and hypothalamus. For the pituitary stalk, we succeeded (Fig. 4); for the sympathetic nuclei, research still is underway. Here are the details of the experiment:

On November 21, 1952, we thyroidectomized three rabbits. On December 12, 1952, three weeks later, the rabbits received 2 mc of tagged thyroxine intravenously. They were sacrificed respectively a half-hour, one hour and four hours after the injection. From the two rabbits sacrificed a half-hour and one hour after injection, the hypophysis and pituitary stalk were taken out. The autoradiographs showed that thyroxine had localized in the posterior lobe of the hypophysis. The pituitary stalk was not to be seen. In the belief that this invisibility of the pituitary stalk was due to its retraction, we took out the hypophysis, pituitary stalk and brain in one single block from the third rabbit. Sagittal sections were made at the level of the hypophysis. They were stained with hemalum-eosine. Other radiographs of the same block show a localization of the thyroxine at the

level of the pituitary stalk and of the neural lobe of the hypophysis.

Thus, thyroxine passes from the posterior hypophysis through the pituitary stalk. It would be surprising if it did not reach the central nuclei. If this physiology or physiopathology were to be confirmed, it might well prove to be the key to a number of pathogenic mechanisms of shock or hibernation.

Figure 5 shows the suppression of anaphylactic shock in guinea pigs through hibernation. This condition inhibits all biological activity on the part of the pituitary and, therefore, prevents localization of radioactivity in it.

Studies on the Metabolism of Calcium and Phosphorus in the Laying Hen

By R. Sasaki,* Japan

Knowledge of the movement of calcium and phosphorus in a hen during the processes of egg-laying and incubation forms a basis for improvement in the production of eggs. Using radioactive Ca^{45} and P^{32} as tracers, a number of experiments have been carried out in Japan on absorption, excretion and retention of calcium and phosphorus in the hen after the administration of feed, the incorporation of these elements into the egg during its formation, and the movement of calcium and phosphorus within the egg at the time of incubation.

The measurement of radioactivity[1] after venous injection of radioactive P^{32} to a cock disclosed the fact that his transparent fluid which corresponds with the secretion of the accessory reproductive organ of mammals is produced from the blood. Further, it also showed that spermatogenesis requires approximately six days.

The absorption, excretion and retention of radioactive Ca^{45} after the administration of $Ca^{45}Cl_2$ containing about 50 μc were investigated in three adult hens, two White Leghorns and one hybrid.[2] Total Ca and Ca^{45} of the eggs produced and urine and feces excreted in five days after the administration were analysed and changes of Ca^{45} activity in the whole blood and excreta during the first 72 hours were estimated.

The activity in the whole blood during the first 72 hours reached a maximum after four hours and decreased after that in all three hens. In one of them which produced two eggs during the time, the decrease was very remarkable until 20 hours later, staying at the low level thereafter, while in the other two hens, which laid no eggs, the decrease was rapid until 8–14 hours, then became gradual until 44 hours, also reaching the low level.

The daily amount of Ca^{45} in excreta, as well as its excretion rate in 72 hours after the administration, was examined. In two hens laying no eggs during the time 30–40% of the dose was excreted during the first four hours; then the rate was retarded, 56–70% being excreted until 24 hours after, while in the hen which produced eggs on the second and third days the rate was very slow, the amount excreted being 1% and 39% in 4 and 24 hours, respectively. Thereafter the rate was also slower in the latter than in the former. Most of Ca^{45} excreted during five days was found on the first day; then the amount decreased suddenly on the second day, and became constant afterwards. The total amount excreted in five days reached 78% in the non-laying hen, to 65% in the hen which produced an egg, while it reached only 40% in the hen which laid three eggs.

As for the three eggs produced by the hen, No. WL-94, the first egg, laid 25 hours after the treatment, contained about 40% of Ca^{45} given, while the successive eggs showed much lower values. An upward tendency of Ca^{45} was recognized in egg white, while in yolk the tendency was to the contrary.

The above results may be summarized as follows: when the hens were laying eggs, most of Ca^{45} absorbed in the body was transferred directly to the eggs and the remaining part thereof which reached also the identical or successive eggs afterwards was deposited in bones or other organs. The amount of Ca^{45} retained in the body after five days was 30% in the non-laying hen, 22% in the hen which laid an egg and 14% in the hen which produced three eggs during the time. The greater part of unabsorbed Ca^{45} was evacuated in the excreta after a few hours, the excretion rate being decreased thereafter.

Further experiments[3] were conducted on the calcium metabolism, in various stages of egg formation. Ten laying hens of White Leghorn breed were divided into five groups, two in each, and they had $Ca^{45}Cl_2$ orally administered to them at different stages of egg formation assumed from previous records; that is, Group I (the ovulation time), Group II (about 5 hours after ovulation), Group III (about 10 hours after ovulation), Group IV (about 15 hours after ovulation) and Group V (the non-ovulation day).

As the transition of Ca^{45} in the fowl-body was most distinguishable four hours after the administration of $Ca^{45}Cl_2$, as clarified in our previous experiment, the activity of Ca^{45} in the egg, blood, excreta and femur as well as in reproductive organs was estimated four hours after the treatment.

As for the amount of Ca^{45} in the egg, about 40% of the dose taken was demonstrated in the egg-shell of Group III, and in the egg-white Group I showed the maximum activity, while in egg-yolk Group I had very little activity, other groups having a negligible amount.

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The activity of the uterus was maximal in Group I and V and minimal in Group II. The isthmus showed the maximum value in Group V and minimum in Group IV. From the albumen secreting portion which showed maximum Ca^{45} in Group I, the activity declined gradually as the egg formation advanced.

In one case where no ovulation occurred, the appearance of Ca^{45} in the blood was more abundant than in other cases where the egg formation was carried on, while in the deposition of Ca^{45} in the largest ovarian follicle and the femur a contrary tendency was ascertained. As the egg formation advanced, the Ca^{45} in the blood or femur had a trend to decrease gradually.

From the amount of Ca^{45} in the excreta, contents of digestive canal and kidney it was considered that the retention of Ca^{45} in the body increased as the egg formation advanced. The retention, however, showed the least value, when no ovulation occurred.

From the results described above, it may be concluded that abundant and urgent requirement of Ca for egg formation can be supplied mainly by Ca absorbed from the feed. As commonly believed, it is proved that the dynamic equilibrium of Ca in the fowl-body is strongly influenced by the egg formation and moreover, it can be asserted that the equilibrium is directly affected by the ovulation itself. Accordingly, the authors consider that the Ca-equilibrium may be controlled by sexual hormones in addition to other hormones already ascertained to be concerned with the Ca-metabolism.

To ascertain the origin of calcium required for the growth of avian embryo, and further to investigate the time of its consumption by each part as well as its course of transfer, another experiment[4] was carried out.

The method adopted was to keep the fertile eggs and infertile eggs injected with Ca^{45} into egg white or yolk during incubation, to take them out at certain intervals, and to measure the Ca^{45} distribution in each part. The results obtained are as follows:

The calcium content and the fresh weight in white decrease as growth advances, and disappear in about 16 days. The yolk increases in volume during the early stage of incubation, but begins to decrease gradually until it becomes about one-half of that of the early stage. The amount of calcium in yolk decreases to less than two-thirds of that of the beginning shortly after the start of incubation, but begins to increase again until it reaches more than that contained in an ordinary egg yolk.

Ca^{45} injected into the white of infertile egg comes to equilibrium within 7 days, in which it stays relatively unchanged, the distribution percentage being, in shell ca 85%, in white ca 10%, in yolk ca 5%. The distribution of Ca^{45} injected into the fertile eggs seems to reach equilibrium, Ca^{45} being, in shell ca 70%, in white ca 25%, in yolk ca 5%.

The transfer of Ca^{45}, however, starts again in about 10 days. Its content in shell and white rapidly decreases, and the content in yolk gradually increases.

Of Ca^{45} injected into the yolk of infertile eggs, about 15% is found in the shell, 5% in white, and 80% in yolk on the seventh day. This distribution percentage does not change much thereafter.

In fertile eggs, Ca^{45} content in white reaches about 25% at one time, but only 5% of it moves to the shell; Ca^{45} thus distributed to both white and shell disappears within 16 days, Ca^{45} content in yolk in the early stage of incubation loses a greater amount than in the yolk of infertile eggs, and is reduced to ca 65%. At some later time, it regains as much as 20%, but returns to 65% at the last stage of incubation.

The accumulation curve of calcium in embryo roughly agrees with the growth curve. Ca^{45} accumulation curve in embryo injected with Ca^{45} into white shows also a similar variation to the calcium accumulation curve. When injected into yolk, the accumulation of calcium is greater in the first half of incubation, but in the latter half it is considerably less than that in the case of white.

The amount of Ca^{45} distributed in amniotic and allantoic fluids, when injected into white, reaches a considerable volume at the middle stage of incubation, and then, after a marked drop, returns to the former amount at the last stage. When injected into yolk, it reaches the largest amount at the early growth stage of liquid portion.

Another experiment[5] was made to clarify further the utilization of shell calcium by chick embryo for its development.

The first and second eggs laid by the six hens within three days after oral administration of Ca^{45} were incubated, and the change in Ca^{45} amount distributed in different parts of the egg were measured on the 5th, 12th and 18th days of incubation and at hatching. Various kinds of calculation were conducted on the transfer of Ca based upon the specific activities in the sectioned parts of the eggs. Comparison of these results with those results of the authors' previous experiment where Ca^{45} was injected into yolk and white disclosed the following facts.

The egg shell loses about 150 mg of Ca during incubation, which is about three times the amount in yolk and white. The Ca amount utilized for the development of embryo in the early stage of growth was mainly derived from yolk and a little from white. Ca in amniotic and allantoic fluids during the middle stage of incubation was mostly derived from yolk. During the latter half of incubation, more than half of the total amount of Ca collected by the embryo was from the shell, about ¼ from yolk and the rest from white and others. During the later stage, yolk was a main source of Ca supply to the embryo, while yolk itself was receiving a considerable amount of Ca from the shell. The amount of Ca flowing into yolk during incubation was approximately the amount contained in the yolk at egg laying.

A further experiment[6] was intended to determine the metabolism of phosphorus in hens from the change

of the distribution of P^{32} in several P-containing fraction in eggs which were produced after the oral administration of radioactive P^{32} in connection with the lapse of time. In the first experiment, two White Leghorn laying hens with high laying records were given *per os* $P^{32}O_4$ (about 75 μc per head) and then the eggs laid in 10 days were brought into analysis. In the second experiment, two White Leghorn hens were treated in the same manner as in the above experiment; and their eggs were analyzed seven days later. The results obtained are briefly summarized as follows:

EXPERIMENT I

The radioactivity was detected early in the first eggs, produced in 24 and 26 hours after the administration to a considerable extent, the greater part being in the shell. In the second eggs, laid after 72 and 76 hours, the total activity increased remarkably and the greater part was found in the yolk. The activity in the shell was less brisk than before, and reverse is the case with the white. After the third eggs, the yolk demonstrated the greater part of the activity, which increased to the maximum in the fourth eggs (126 hours and 143 hours after the treatment), then decreased gradually. Even in the seventh eggs the activity was detected in a considerable degree.

The total activity accumulated in the seven eggs produced in 10 days following the administration amounted to 7.8% and 6.3%, respectively, of the total intake *per os* and more than 90% of the accumulated P^{32} was found in the yolk. The simple phosphorous compounds and phosphorus-containing proteins were fractionated from the egg-white. P^{32} in the former reached the maximum in the second eggs, and P^{32} in the latter was the maximum in the second and the third among the eggs.

It may be considered that the phosphorus-containing proteins are formed in the hen's body at a slower rate than the simple phosphorous compounds.

In the yolk, a large amount of P^{32} was accumulated in the protein or fat fractions. P^{32} in the former showed the largest value in the second eggs (71.5 hours and 75.5 hours after P^{32} feeding), while in the latter the strongest activity was found in the fourth eggs (126 hours and 143 hours after the administration). From the above fact, it is presumable that the lipoproteins are made in the egg earlier than the phosphatides from phosphorus compounds in the feed.

EXPERIMENT II

As stated above, the greater portion of P^{32} was found in the yolk. Accordingly, more detailed analysis was conducted in several fractions of the yolk. As in the case of the result in the former experiment, the maximum activity in the yolk was found in the fourth eggs and P^{32} appeared in the protein earlier than in the fat, although the time in which the maximum in activity reached was retarded considerably in the lipoproteins as well as in the phosphatides as compared with that in the former experiment, probably due to the individuality of the hens, environmental conditions, etc.

The change in the ratio of P^{32} distributed in free, combined phosphatides and lipoproteins in the yolk was the most interesting aspect of this experiment. In the percentage of P^{32} in the yolk, the free phosphatides were in inverse relation to the combined phosphatides and lipoproteins; the former increased gradually in percentage, while the latter two decreased in general.

It might be considered that the above fact was due to the difference of the former from the latter two in producing organs of the hen or in the transportation speed from the hen's body to the egg. Further studies on these matters will be needed to clarify the results.

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A Method of Recording the Pulmonary Circulation Times in the Cat

By A. S. Paintal,* India

The importance of knowing the pulmonary circulation time in experimental animals is now realised since certain drugs produce reflex effects with distinctive injection-reflex times depending on the location of the receptors in the thoracic viscera.[1,2] Stewart[3] determined the pulmonary circulation time in the rabbit by a conductivity method which enabled him to determine only the shortest pathway of the lesser circulation as is indeed the case with most methods in use clinically. On the other hand Gray and Paton[4] using a more elaborate arrangement of his method obtained records of time-concentration curves from which detailed analysis of circulation times was made possible, but unfortunately they did not study the pulmonary circulation time. This has now been done using radioactive phosphorus combined with an unconventional technique of recording the scintillation pulses directly without the use of counters. As will become evident this method promises to yield fruitful results.

METHOD

Experiments were performed on adult cats anaesthetized with chloralose. The chest was opened in the midline in each case taking care that bleeding was minimal. Experiments in which the circulation was not satisfactory were discarded.

Injection of radiophosphorus (carrier-free solution of NaH_2PO_4 mixed with 0.9% NaCl (w/v)) were made through a No. 6 USA cardiac catheter 19 cm in length inserted through the right external jugular vein such that its tip lay in or very near the right atrium.

The ventilation was signalled by a capacitance or mirror membrane manometer. The ecg lead II was recorded in every experiment.

Recording of Pulmonary Circulation Time (PCT)

This was done by injecting radiophosphorous into the right atrium and recording its arrival in the left atrium with a scintillation type β-ray detector. This consisted of an anthracene phosphor fixed at the end of a "perspex" light guide the other end of which was stuck to an EMI 6260 photomultiplier tube. The output of this was fed to an amplifier through a cathode follower and the output of the amplifier was connected to one beam of a double beam cathode ray tube. The ecg was displayed on the second beam. The scintillation pulses were thus directly viewed on the cathode ray tube and permanent photographic records taken with a camera and recording paper moving at a constant speed.

To facilitate detailed analysis of the pulses, the latter were displayed simultaneously on a second cathode ray tube in which the sweep moved in a diagonal direction thus yielding the sweeps seen in Fig. 1. Each sweep occurred immediately after the flyback of the previous one so that pulses were not lost. By this means even the smallest pulses could be taken into account and the frequency of scintillation pulses with the course of time determined, to yield the curve of Fig. 2. This is, in fact, what is done automatically by a counting rate meter. However, the available rate meters cannot respond faithfully to sudden fluctuation in the frequency of pulses[5] and since it was felt that there may be rapid cardiac fluctuations in the scintillation rate, the rate meter was discarded in favour of direct recording of pulses.

In order to avoid recording β-rays from sources other than the left atrium, the anthracene crystal used in the first series of experiments was 5 mm in diameter and 1 mm thick. A "perspex" rod 5 cm in length and 1.25 cm in diameter was turned down to 5 mm diameter over 1 cm of its length and at the end of this the phosphor was stuck. The phosphor could thus be inserted directly into the left atrium through its auricular appendage. This was easily carried out as the phosphor and "perspex" rod formed a separate piece held in a suitable clamp. Good optical contact with the photomultiplier tube was obtained with transparent grease. The second and better arrangement used consisted of a 1.25-cm-diameter $\times$ 1-cm-thick anthracene crystal stuck to the end of a "perspex" rod. The phosphor was shielded all round with lead (400 mg cm^{-2}) with the exception of a small segment which formed about a fifth of its cylindrical surface. This part of the crystal (covered with cellophane) was applied against the wall of the left atrium in such a way that it received practically no radiation from the pulmonary artery or its branches. Since the bottom was also shielded, radiation from the pulmonary veins or lung tissue was also excluded. Suitable positioning of the phosphor was facilitated by turning the cat a little on its right side.

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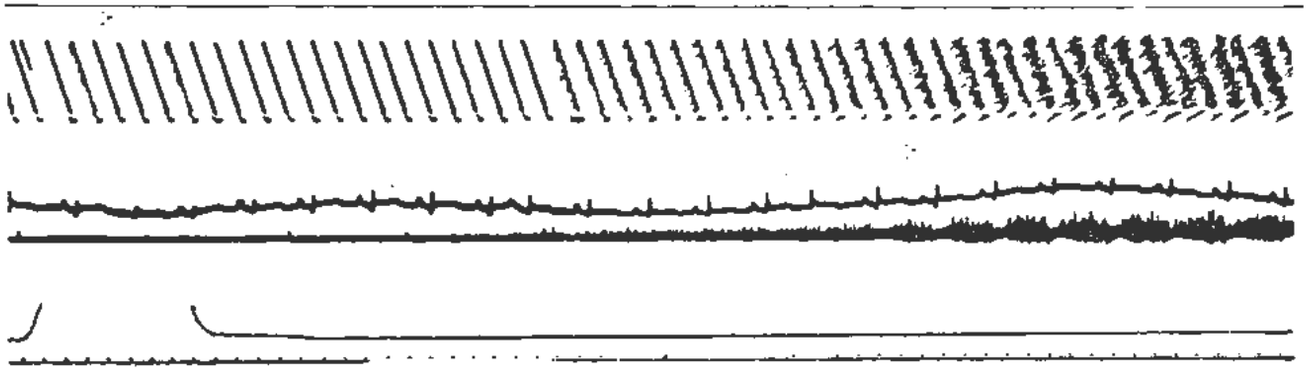

Figure 1. Record of scintillation pulses following injection of radiophosphorus at signal. From above downward: diagonal sweeps, ecg, scintillation pulses, record of artificial ventilation, time in 1/10 sec, and injection signal. Note the marked and characteristic cardiac rhythm in the frequency of pulses

Injection Signal

The metallic portion (plunger, neck and nozzle) of a 2.5-ml syringe formed part of an electrical circuit consisting of a 2.5-v accumulator and a bulb which was placed in vertical alignment with the spots of the cathode ray tube. With this arrangement the beginning and end of the injection was accurately signalled. The duration of injection in the great majority of cases was 1.0 ± 0.2 sec; this corresponds to the rapid injection of Gray and Paton.[4]

In estimating the amount of radiophosphorous injected, the dead space of the catheter which was 0.2 ml was taken into account. Doses from 1.3 mc to 50 μg per injection were given.

RESULT

Satisfactory records of the interval between the beginning of injection of radiophosphorus into the right atrium and its appearance in the left atrium here referred to as the PCT where obtained in eight experiments. In five of these the phosphor was inserted into left atrium and in the remaining three it was placed next to the left atrium as described above. However, the results obtained under the two conditions did not reveal significant differences and have, therefore, been lumped together. Since the circulation times are affected by the state of the circulation, which may vary considerably in an animal with open chest, the lowest PCT obtained in any one cat with repeated injections was taken as that most likely to approach values obtained under normal conditions.

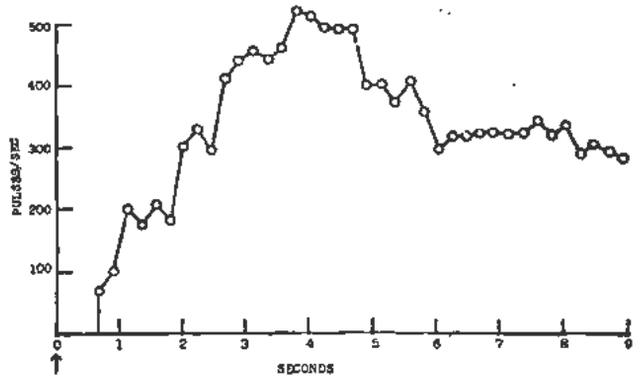

Figure 2. Graph of frequency of scintillation pulses following injection of radiophosphorus at arrow. Start to start time is 0.7 sec, start to peak time 3.8 sec and start to end time 6.0 sec

Pattern of Responses

Figure 1 shows a typical record obtained after injecting 180 μc into the right atrium. Pulses appeared suddenly after the injection, reached a peak frequency of impulses to fall off gradually to a new level of activity (Figs. 1, 2). The pulses observed varied considerably in height. This is to be expected because of the broad energy spectrum of P^{32} (max. 1.69 Mev), and loss of light energy in the "perspex" which has a transmission factor of 90-92%.

The pattern of pulses in different cats varied considerably. In some the peak frequency was attained rapidly and in others gradually. Some records showed two peaks of activity. This is suggestive of recirculation which can be confirmed by recording the activity in the right and left atria simultaneously.

An interesting feature observed in most records was the unmistakable appearance of a cardiac rhythm which showed a definite pattern of pulses. During ventricular systole these rose to a peak frequency which fell considerably during atrial systole. The reason for this becomes evident when it is realised (*a*) that the frequency of pulses is a measure of the quantity of radiophosphorus in the left atrium and therefore of the volume of blood in that chamber and (*b*) that atrial filling gradually rises to a peak during ventricular systole and falls with the opening of the a-v valves, and auricular systole. The pattern of pulses can, therefore, be taken to represent the phasic changes in left atrial volume during each cardiac cycle. The great similarity of the sequence of scintillation pulses to the impulses from vagal afferent fibres arising from atrial type B receptors[6] is therefore very interesting since much evidence has been presented to show that these receptors signal changes in atrial volume.

Pulmonary Circulation Time

The PCT was studied from three aspects: (*a*) the interval from the beginning of the injection of radio-

phosphorus to the moment it appeared in the left atrium, viz., the start to start time; (*b*) the start to peak time, i.e., the moment when peak frequency of pulses appeared in the left atrium and (*c*) the start to end time. The relevant statistics pertaining to these PCTs are given in Table I.

The start to start time could be determined accurately since the scintillation pulses appeared suddenly (Figs. 1, 2) whereas there were no (in the case of the first injection) or few impulses (in the case of subsequent injection in the same animal) prior to the injection. The following observations sufficed to show that the earliest pulses from which the start to start time was reckoned did not arise from the right atrium, ventricle, or pulmonary arteries but from the left atrium itself:

(*a*) No pulses appeared during the early part of the injection at which time the right atrium and ventricle would be highly "active". Similarly, the pulmonary artery should be "active" by the first or second cardiac cycle after the beginning of the injection but no pulses were seen at this time either (Fig. 1).

(*b*) Injection of P^{32} into the right atrium at the end of the experiment after the heart had stopped beating and the pulmonary artery was ligatured did not yield any increase in the pulses. Similarly injection of P^{32} into the pulmonary artery also did not produce an increase in pulses. These facts show that the anthracene phosphor was effectively shielded from β-rays arising from parts of the lesser circulation other than the left atrium; and, therefore, the small start to start time (Table I) are genuine and so are probably near the true pulmonary circulation time.

In two cats injection of radiophosphorus into the femoral vein yielded a start to start time of 1.9 and 1.6 sec respectively with the corresponding start to peak time of 3.5 and 4 sec. These values are less than what would be expected from the results of earlier investigators,[3,4] but further work is required to establish this difference.

In some experiments injecting a larger quantity of P^{32} gave shorter start to start PCTs. This is what one would expect from theoretical considerations which have been dealt with before.[7] However, since a continuous record of the state of the circulation was not maintained in these experiments, it is not possible to arrive at definite conclusions.

DISCUSSION

The results of these experiments have revealed unexpectedly short start to start PCT. Since measurements of circulation times by any method will tend to vary inversely with the quantity of the tracer substance injected, such as dyes, ether[8] decholin, histamine, radioactive substances and with the sensitivity of the detecting device, it is likely that the great sensitivity of the experimental arrangement used in this investigation has been responsible for yielding such small values as 1.0 sec (mean) start to start time.

Table I. Pulmonary Circulation Times in the Cat

	No. of experiments	*Range, sec*	*Mean, sec*	*SD, sec*
Start to start time	8	0.6–1.5	1.0	0.28
Start to peak time	8	1.2–3.1	2.3	0.66
Start to end time	8	2.0–6.0	4.6	1.32

The results have also shown that it is possible to record phasic changes in atrial volume using radioactive substances. Experiments to measure these changes quantatively are now in progress (there is no reliable method of measuring atrial volume at present). In this connection it may be mentioned that existing rate meters would not be able to follow faithfully the rapid changes in scintillation pulse frequency occasioned by the events of the cardiac cycle. This is clearly supported by Fig. 9 in the paper by Andrew and Roberts.[5] Perhaps their circuit for a pulse-interval may overcome this defect.

Direct record of the scintillation pulses would thus appear to offer certain advantages in addition to simplicity of equipment—only a scintillation detector and an amplifier being required.

SUMMARY

Pulmonary circulation times have been determined in cats with open chest using radiophosphorus and a technique of recording the scintillation pulses directly on a cathode ray tube. This method has also revealed certain phasic changes in atrial volume and may provide the means of studying quantitatively the dynamic changes in atrial volume.

ACKNOWLEDGEMENTS

I am grateful to the Indian Atomic Energy Commission for a grant which made this investigation possible.

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Respiratory Carbon-14 Patterns and Physiological State

By B. M. Tolbert, J. H. Lawrence and M. Calvin,* USA

Carbon-14, the most useful radioactive isotope of carbon, has been very important in chemical and biochemical research since *circa* 1945. The radioactivity from this weak beta emitter (maximum energy of the β-particle is 155 kev) is difficult to detect and it is not possible to measure the radioactivity or determine its location from outside the body. Thus, animal studies using this isotope usually require sacrifice of the animal, unless measurements are restricted to excretion products and blood. Partially because of difficulties of this nature, carbon-14 has not yet become a useful tool in clinical medicine.

Although carbon-14 cannot be detected in animals *in situ*, it can be measured as it is excreted. Of the several catabolic end fates of carbon in animal systems, quantitatively the most important is breath excretion as carbon dioxide. In addition, carbon dioxide excretion responds rapidly to changes in the metabolic level of the body—the only carbon excretion product to do so. The breath excretion of carbon-14 following administration of a labeled organic compound can therefore be used in intact animals to study the metabolism of specific compounds and of the particular labeled carbon atom in the compound.

Furthermore, if one has established the respiratory pattern of a given labeled compound in an animal system, variations in this pattern can potentially be interpreted in terms of the physiological state of the animal on a biochemical level. Since an almost unlimited number of compounds labeled in various positions is presently available or can be synthesized, the excretion of labeled carbon dioxide from specific substrates is potentially one of the most important nondestructive tests that can be made in metabolic studies in intact animals.

Many experiments have been made on respiratory carbon-14 excretion in animals,[1,2,3] but the procedures for measuring this excretion have been slow or have required large amounts of radioactivity. In this paper we describe some of our measurement techniques which overcome these difficulties, and present typical experimental data for a number of animal systems.

The effects of physiological state are shown in some cases.

EXPERIMENTAL TECHNIQUES

The respiratory excretion of $C^{14}O_2$ is usually measured as follows: After injection with a labeled substance, the animal is placed in a closed cage. The air from the cage is passed through a dilute sodium hydroxide or barium hydroxide solution. These solutions are changed periodically and the collected CO_2 is precipitated, usually as the insoluble barium carbonate, filtered, dried, weighed, and analyzed for radioactivity.[4] This method gives results in terms of the specific activity of the $C^{14}O_2$, and if quantitative measurements are made on the CO_2 excretion, cumulative excretion of C^{14} can also be calculated.

This method of analysis is used by the authors for long-term respiration studies because it may be made very sensitive if special radioactive assay procedures are used.[5,6] It suffers the disadvantage of being slow, or requiring lots of work, and/or permitting only a limited number of samples.

As an alternate procedure we have devised an apparatus that continuously measures the $C^{14}O_2$ excreted by an animal (Fig. 1).[7] In this system an animal is placed in a small cage. Air is pulled through the cage at a constant rate and is passed through a drying tube and through an ionization chamber. The radioactivity produces a charge on the collecting electrode of the ion chamber; this charge is directly proportional to the radioactivity in the chamber. A vibrating-reed electrometer and potentiometer recorder make a continuous tracing of these radioactivity levels.

For mice, rats, and rabbits 100 to 1000 cm^3 ionization chambers are used. Air flow rates are from 100 to 400 cm^3/minute. The sensitivity of this equipment is such that a normal dose of 40 μcuries C^{14}/kg body weight is used for 24-hour studies using simple body metabolites such as acetate or glycine. The electrical and gas-flow time constants for 50% response are about one minute.

Most of the acetate-coenzyme A (CoA) experiments described in this paper were made using such equipment. This instrument measures the rate at which carbon-14 dioxide is excreted; the cumulative excretion curve may be obtained by integrating this rate curve. However, one does not get the specific activity of the $C^{14}O_2$, such as is obtained by the barium carbonate measurements and which is probably a better measure of the radioactivity in the body bicarbonate pool.

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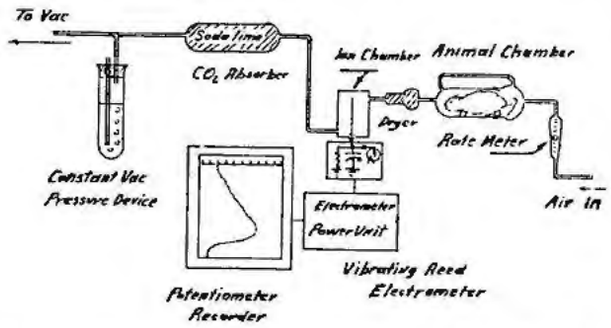

Figure 1. Apparatus for continuous measurement of breath $C^{14}O_2$ radioactivity

In order to automatically and continuously determine breath specific activity we have constructed a second type of instrument.[8,9] This improved apparatus continuously measures and records (*a*) the CO_2 excretion by infrared absorption, (*b*) the C^{14} excretion by an ionization chamber and (*c*) the specific activity of the $C^{14}O_2$ by a ratio analyzer.

As before, the animal to be studied is injected with a labeled compound and is placed in a cage no larger than necessary for the particular animal's size (Fig. 2). For humans a transparent helmet is used together with a slightly modified flow system and safe tracer doses are used[10] (Fig. 3). Air is provided to the subject from a tank of compressed air which has been aged to allow decay of short-lived naturally occurring α-radioactive gases—mostly radon and thoron.

The air is passed through a sulfuric acid bubbler to remove water and through an ionization chamber. As described above the radioactivity is determined by a vibrating-reed electrometer and potentiometer recorder. The air then passes through a variable-volume chamber and an infrared CO_2 analyzer. The variable-volume chamber adjusts the response time of the CO_2 analyzer to match that of the radioactivity-measuring unit.

The electrical signal corresponding to the % CO_2 in the breath is recorded. This recorder also determines the ratio of the radioactivity signal to the % CO_2 signal and plots this figure. This ratio is directly proportional to the specific activity of the

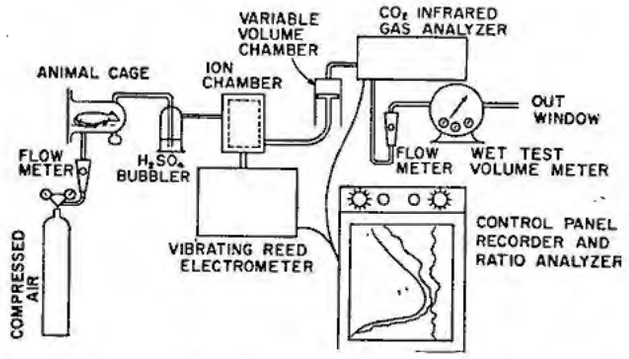

Figure 2. Schematic diagram of respiratory $C^{14}O_2$ analyzer for small laboratory animals

excreted $C^{14}O_2$. An example of the original data is shown in Fig. 4.

For small laboratory animals and 100 to 1000 cm^3 ion chambers, the radioactivity dose was about 40 μc C^{14}/kg body weight. For humans a normal dose was 10 μc of simple organic metabolites, such as acetate or glycine. This corresponds to a dose to weight ratio of only 0.14 μc/kg body weight which is well within the safe tracer dose. The lower figure is made possible by use of larger ionization chambers —from 10 to 20 liters in size.

The time for 50% response of this instrument as used is about one minute. This time constant can be decreased by increasing air flow rates and decreasing the ion chamber sensitivity. A more rapid response does not seem necessary, however, if one considers the biological system. The carbon dioxide produced in a cell becomes part of the body bicarbonate pool. Using known figures for plasma bicarbonate levels, total body water and CO_2 excretion rate, we calculate that the half-time for excretion of CO_2 from the human body is 10 minutes, assuming complete mixing at all times. This agrees well with an early

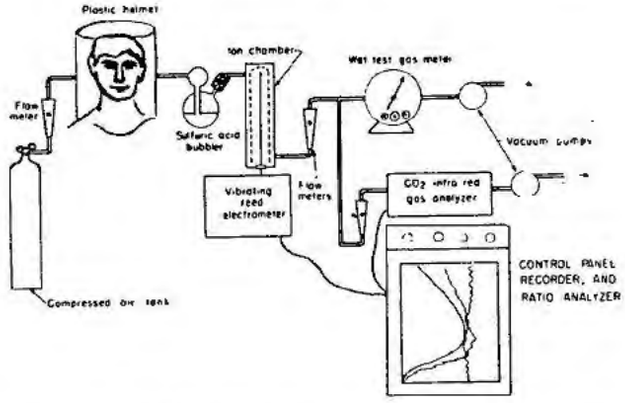

Figure 3. Schematic diagram of respiratory $C^{14}O_2$ analyzer for humans

experimental value of 15 minutes for the CO_2 turn over time in rats which can be calculated from the work of Gould *et al.*[2]

ACETATE METABOLISM AND COENZYME A

The acetate fragment has been one of the most extensively studied compounds in biochemistry.[11] A schematic diagram of this compound is shown in Fig. 5, which emphasizes the role of CoA. Acetate is presumed mostly metabolized through the intermediate, acetyl CoA. Oxidation of the acetyl CoA to CO_2 may procede via the citric acid cycle, or the acetyl CoA may be synthesized into fats, using more acetyl CoA for each two-carbon addition. Acetyl CoA can also be formed from sugars via pyruvic acid oxidation.

This dynamic equilibrium may be conveniently studied in the intact animal by the equipment described. CoA and labeled acetate are readily available and rats may be made deficient in CoA by feeding them on a pantothenic acid deficient diet

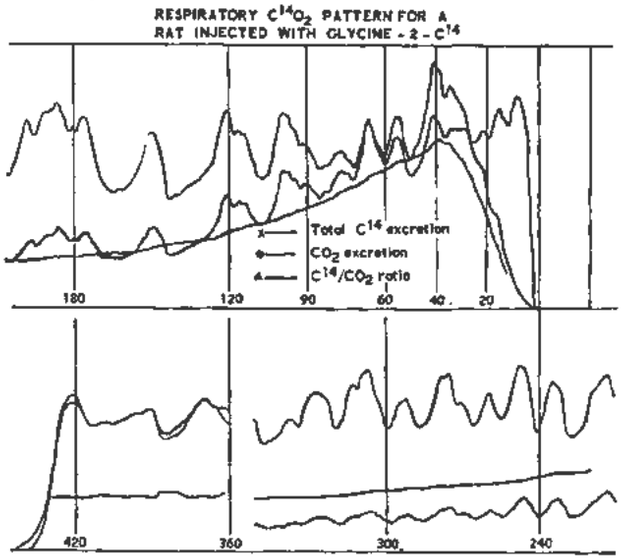

Figure 4. An example of a respiratory $C^{14}O_2$ pattern as made by the instrument described

(PAD rats).[12] The pattern of excretion of $C^{14}O_2$ can therefore be determined as a function of body CoA levels and the nutritional state induced by pantothenic acid deficiency.

A number of variables that could influence the respiration patterns were briefly investigated. Intraperitoneal (ip) injection of the CoA gave more consistent results than intravenous (iv) or subcutaneous injection. No differences in patterns were found with air flow rates that gave cage CO_2 concentrations from one to four per cent. The effective body pool size of acetate in rats should be quite large since 0.9 to 1.2 gm of acetic acid are formed per day per 100 gm body weight.[13,14] In agreement with this assumption no significant differences were observed with doses of labeled acetate varying from 0.2 to 20 mg; 2 mg doses were used. Individual animal variations, especially of the PAD rats, were such that 6 to 20 separate curves were measured for each nutritional state.

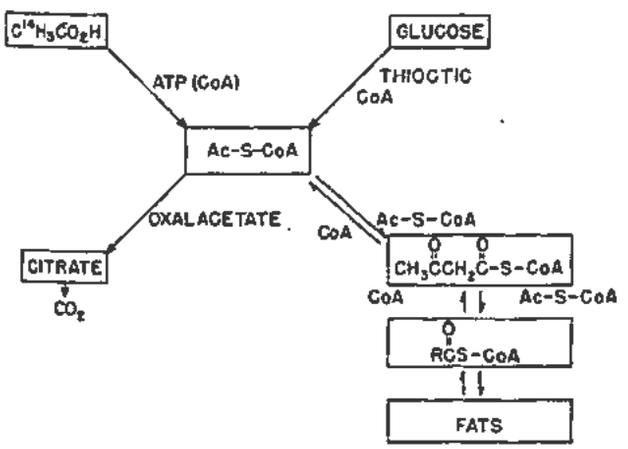

Figure 5. Schematic diagram of acetate metabolism

Figure 6 shows the rate of excretion of $C^{14}O_2$ after injection of sodium acetate-2-C^{14} in rats of four different physiological states. Figure 7 shows the cumulative excretion curves for this same series. From these patterns one sees that labeled acetate is more rapidly and more completely oxidized to CO_2 in PAD rats than in normal rats. One also sees that CoA depresses $C^{14}O_2$ excretion in both normal and PAD rats, and by comparable amounts.

These data are consistent with the acetate metabolism scheme presented. Since PAD rats are deficient in CoA, they should have a smaller acetyl CoA pool than normal rats. Therefore, in PAD rats, after administration of acetate-2-C^{14}, a higher C^{14} specific activity should be observed in products directly derived from this intermediate, such as $C^{14}O_2$ formed via the citric acid cycle. This should be a short-term effect and is indeed seen only in the early part of the rate curves.

Conversely, injected CoA should increase the acetyl CoA pool, and this should decrease the peak CO_2 specific activity, as observed. But this change in pool size should not change the cumulative C^{14} excretion as shown in Fig. 7, unless alternate

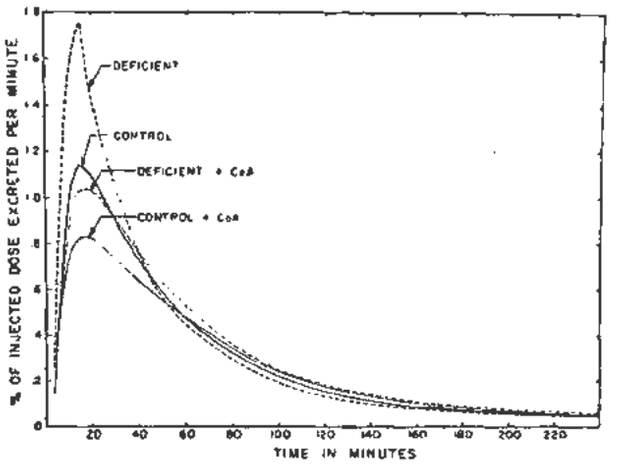

Figure 6. The effect of pantothenic acid deficiency and CoA on the rate of excretion of $C^{14}O_2$ after injection of sodium acetate-2-C^{14}

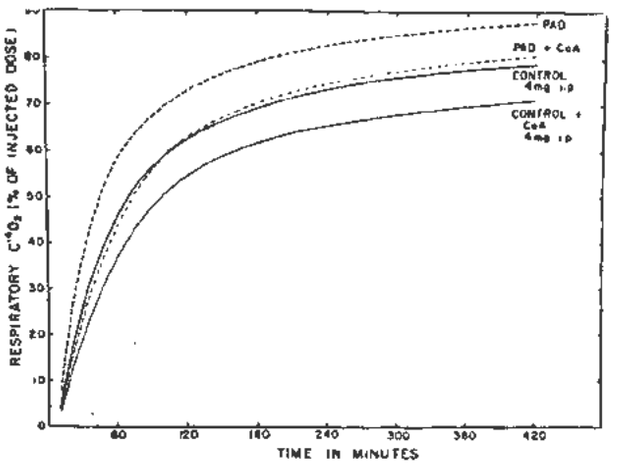

Figure 7. The effect of pantothenic acid deficiency and CoA on the cumulative excretion of $C^{14}O_2$ after sodium acetate-2-C^{14} injection

metabolic fates for the acetate fragment are enhanced by the CoA. A pathway that is probably enhanced is fat synthesis, in which each major step requires an acetyl CoA molecule. Furthermore, an animal deficient in CoA will have a fat deficiency, which should be selectively alleviated when more acetyl CoA is provided.

This hypothesis was further confirmed by making measurements of liver fat radioactivity. PAD rats (average of 4) that were given labeled acetate and 6 mg of CoA incorporated 5.2% of the injected label into liver lipids in one hour; PAD rats (average of 2) given labeled acetate only, incorporated 1.2% of the injected radioactivity into liver lipids in the same time.

RESPIRATORY PATTERNS OF SIMPLE METABOLITES

Preliminary animal studies have been made using the instrument that gives CO_2, C^{14}, and C^{14}/CO_2 analysis of the breath.[8,9] The respiratory patterns from rats for radioactivity from sodium acetate-2-C^{14}, glucose-C^{14}_6, DL-leucine-3-C^{14}, glycine-2-C^{14}, and tripalmitin-carbonyl-C^{14}_3 are presented as a series of curves in Figs. 8 and 9.

The acetate and glucose are both about 60% oxidized to CO_2 in 7 hours. The glycine and leucine have similar peak excretion specific activities, but the leucine is oxidized at a much higher rate than the glycine in the later part of the curve. This is probably so because half the leucine is the abnormal D-metabolite, which cannot be directly used[15] and is slowly de-aminated[16] and oxidized to CO_2. The later part of the leucine rate curve is nearly a straight line when plotted on semi-log paper—a good confirmation of the above suggestion. The tripalmitin is so slowly oxidized to CO_2 that one can postulate that most of it is stored in body tissues as a fat, perhaps without change.

These respiratory data establish a rapid method for accurate measurement of breath $C^{14}O_2$ excretion, both in terms of specific activity and cumulative excretion. Certain other interesting observations are also possible. At the start of our work an important question was whether specific activity curves were continuously decreasing functions, once a peak was reached. Within the limit of our instrumentation this is true for the glycine, acetate, leucine, and tripalmitin. It is not true for glucose; sharp reversals of $C^{14}O_2$ specific activity were observed in every rat studied and corresponded with periods of marked physical activity. These reversals probably correspond to mobilization of recently deposited glycogen in the liver—a selective phenomenon observed by Stetten and Stetten.[17] The specific activity curves for adenine-2-C^{14}, adenine-4,6-C^{14} and adenine-8-C^{14} in mice[18] have also been determined on this unit and are not continuously decreasing functions.

As mentioned earlier the half-time for response of the $C^{14}O_2$ analysis unit is about one minute. After ip or iv injection of such simple metabolites as labeled acetate or glucose in animals and man, detectable amounts of radioactivity were observed in the breath in 1 to 2 minutes. This, then, represents an upper limit for the minimum respiratory excretion time for these compounds. For all five compounds maximum excretion rates occurred within the first hour, provided the animal was not in an abnormal physiological state.

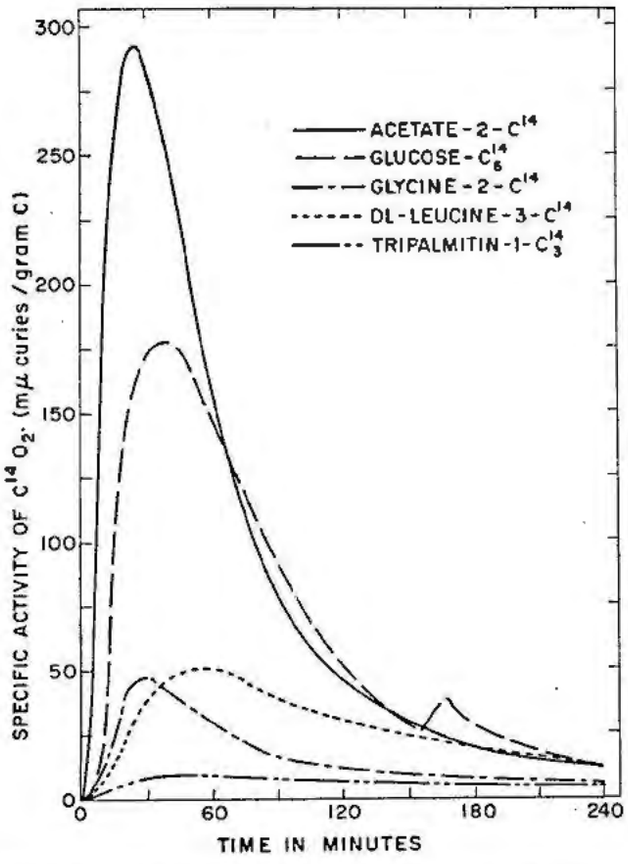

Figure 8. Specific activity of $C^{14}O_2$ from rats injected with various labeled compounds. The data are normalized to a 250 gm rat and 10 μ injected dose

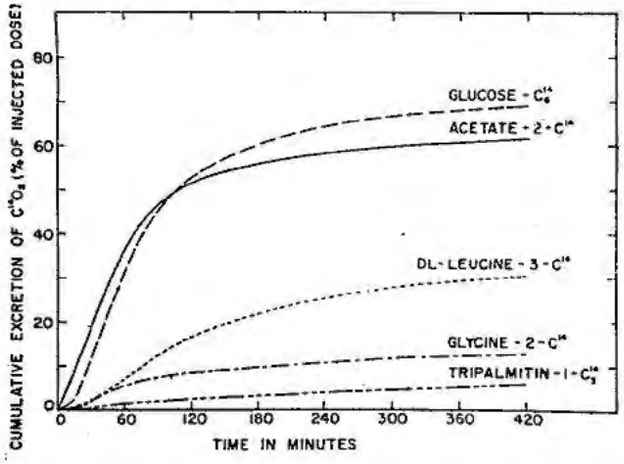

Figure 9. Cumulative $C^{14}O_2$ excretion patterns from rats injected with various labeled compounds

GLYCINE-2-C^{14} IN MAN

Respiratory $C^{14}O_2$ excretion studies have been made by use of glycine-2-C^{14} [5,6] and the barium carbonate radioactivity analysis method. We have now repeated the short term respiration studies for a number of patients with advanced cancer or polycythemia vera. Figure 10 shows the specific activity rate curve for Mr. F., a polycythemia patient in remission. This curve is typical for patients in an apparent normal condition, but it appears to be drastically modified by advanced cancer.

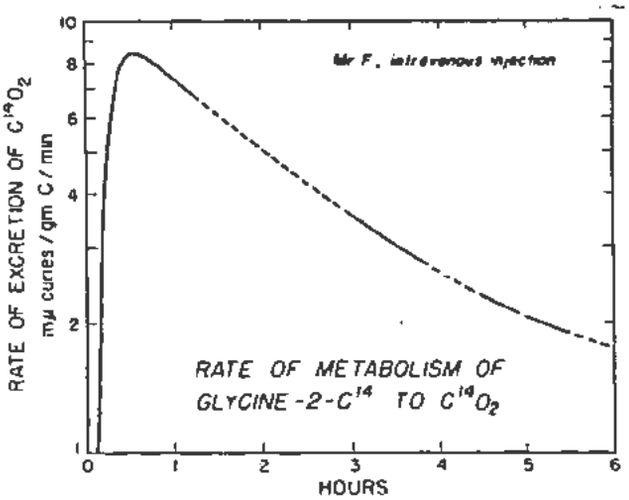

Figure 10. Respiratory $C^{14}O_2$ excretion of glycine in a patient

The similarities between human and rat glycine-2-C^{14} curves (Fig. 8) are very striking. Both the general shape and the time of peak $C^{14}O_2$ specific activity are almost identical. This would indicate that the metabolic pathways are very similar, and are the limiting rate factors, rather than circulation and mixing times.

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The Metabolism of Mucopolysaccharides*

By Albert Dorfman and Sara Schiller,† USA

Mammalian connective tissues are composed of several types of fibers imbedded in an amorphous ground substance. Although the chemical composition of the ground substance has not been fully elucidated, there seems little question that the mucopolysaccharides are important constituents of connective tissue and play a role in governing the physiological functions and pathological behavior of connective tissue. This tissue represents a phase which separates parenchymal cells from the circulation and, as such, probably takes part in governing the nutrition of such cells. The acid mucopolysaccharides of the ground substance may well be critical factors that control the metabolism of inorganic ions and water since these compounds are flexible chain polymers of high negative charge and with a high affinity for cations and water.[1,2] Changes in concentration or molecular size of such substances may affect the capacity of connective tissue to bind water and inorganic ions, or modify the characteristics of this barrier between blood stream and cells.

It has been emphasized repeatedly that the mucopolysaccharides are altered as a result of physiological or pathological transformations. For the most part these speculations have depended upon results obtained by the use of imperfectly understood histochemical reactions. Until recently, almost no information was available regarding the metabolism of the mucopolysaccharides, due largely to the absence of adequate methods for such investigations. It is the purpose of this communication to describe a series of experiments concerned with measurement of the rates of synthesis and breakdown of mucopolysaccharides of the skin of rats and rabbits.

The term acid mucopolysaccharides has been widely used to denote a group of compounds that are high molecular-weight polymers composed of the hexosamines, glucosamine and/or galactosamine, glucuronic acid, acetate, and in some cases esterified sulfate. A possible exception appears to be keratosulfate, a substance recently isolated from cornea which contains no uronic acid. Of the connective tissue mucopolysaccharides, hyaluronic acid (HA) and chondroitinsulfuric acid (CSA) have been most thoroughly characterized and the occurrence of either one or both substances has been demonstrated in a variety of tissues. Compounds that are identical in composition with CSA of cartilage, but which differ in other properties have been found. Evidence to show that a mixture of such compounds is present in skin of rats and rabbits will be discussed below.

The choice of appropriate experimental material for studying the mechanism of biosynthesis of components of connective tissue poses serious problems. Although connective tissue is widely dispersed throughout the body it is difficult to find a source of metabolically active material which is adequate for appropriate biochemical techniques. Therefore, in this laboratory, the mechanism of HA biosynthesis was first studied in a strain of Group A streptococcus (AIII). It had been previously demonstrated that the capsular polysaccharide of this microorganism is very similar or identical to that isolated from mammalian tissues.[3] Studies employing 1-C^{14}-glucose and 6-C^{14}-glucose, established that both the hexosamine and uronic acid moieties of HA arise from glucose without previous scission of the carbon chain.[4,5,6] The labeling of the acetyl group of HA when the organisms were grown on carboxyl-labeled acetate established the fact that acetate could be directly incorporated into the HA molecule. If glucosamine labeled with both C^{14} and N^{15} was included in the medium in which the streptococci were grown, the glucosamine of the HA produced showed a C^{14}/N^{15} ratio which was identical with that added to the medium, thus indicating that glucosamine could be incorporated into HA without previous deamination.[7]

These data on the incorporation of various isotopic precursors into the HA molecule suggested that similar methods could be applied to a study of the rates of synthesis and degradation of acid mucopolysaccharides of mammalian connective tissue. An investigation was undertaken, therefore, on skin since this tissue contains mucopolysaccharides in sufficient quantity for isolation and degradation.

The first experiments which have been detailed elsewhere[8] were concerned with the development of methods for the isolation and identification of the mucopolysaccharides from rabbit skin. The previously published method of Meyer and Chaffee[9] was found to be inadequate for our purposes.

In all experiments the mucopolysaccharides were extracted in the following manner: Acetone-dried ground skin was extracted at room temperature with a solution of 2 per cent NaOH. After dialysis, the

* These investigations were aided by grants from the Chicago Heart Association, the Lasdon Foundation and the Variety Club of Illinois.

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extract was digested with trypsin and precipitated with trichloracetic acid. Following further dialysis and concentration, a mixture of polysaccharides was precipitated with 4 volumes of 95 per cent ethyl alcohol. This crude mixture was subjected to zone electrophoresis on a slab of Celite with a resultant clear-cut separation of sulfated and non-sulfated mucopolysaccharide fractions. Analysis of the more slowly migrating fraction gave values which conformed with those expected for HA, while the more rapidly migrating fraction was analytically identical in most respects with CSA isolated from cartilage, but behaved differently than the latter in several ways. The skin CSA showed a lower color equivalent by the carbazole reaction, a different optical rotation, and a partial resistance to hydrolysis by testicular hyaluronidase as measured by the turbidity reduction method. These differences led us to believe that the CSA isolated from skin is a mixture of CSA similar to that found in cartilage (chondroitinsulfuric acid *A* in the designation of Meyer) and another compound which has been called chondroitinsulfuric acid *B* by Meyer.[10] This may be identical to β-heparin of beef lung described by Marbet and Winterstein[11] and polysaccharide B isolated from pig gastric mucosa by Smith and Gallop.[12] Recently this mixture has been resolved in this laboratory.

With methods available for the isolation of the mucopolysaccharides at a high level of purity and in sufficient quantities to permit metabolic studies, our first experiments were designed to estimate the rate of synthesis and breakdown of these components under normal conditions. While these studies were in progress, several reports appeared concerning the metabolism of CSA in cartilage and skin utilizing $S^{35}O_4^{=}$.[13] It is impossible to study the metabolism of HA or the metabolism of portions of the CSA molecule other than the ester sulfate with this tracer. For these reasons we utilized carboxyl-labeled acetate, uniformly-labeled glucose and $S^{35}O_4$. These precursors, singly or in various combinations, permitted a comparison of the turnover rates of the various parts of the mucopolysaccharide molecules, since it had been previously shown that glucose serves as a precursor for the glucosamine and glucuronic acid portions of the molecules, acetate serves as a precursor for the acetyl portions of the molecules and sulfate as a precursor for the ester sulfate portion of the CSA molecule. Experiments have been carried out on both rats and rabbits. In the latter case the skin of individual animals was studied; while with rats, the skins from groups of animals were pooled.

In the first experiments, which have been published elsewhere in detail,[14] three pairs of rabbits were injected subcutaneously with 150 μc of C^{14}-carboxyl-labeled sodium acetate daily for 3, 5 and 8 days respectively. Determination of the C^{14} of the isolated polysaccharides indicated a more rapid incorporation of radioactivity into HA than CSA, suggesting a more rapid rate of synthesis of the former compound. Since the experiment was not conducted for a sufficiently long period to obtain maximal labeling, it was not possible to estimate the rate of turnover.

In subsequent experiments, labeled compounds were given over a period of one day and the animals were sacrified at various intervals after the last injection. Under these conditions it was possible to obtain a measure of the "half-life time" of the compounds under consideration. In one such experiment 750 μc of C^{14}-carboxyl-labeled sodium acetate was administered as a 0.15*M* solution in three doses over an eight-hour period to each of eight rabbits. Pairs of rabbits were sacrificed at 1, 4, 8 and 12 days after the first injection. The extent of labeling of the isolated polysaccharides is illustrated in Fig. 1. It will be noted that the HA was more highly labeled than the CSA in the case of the first animals sacrificed (18 hours after the last dose of acetate), thus confirming the results of the previous experiment. It was furthermore demonstrated that the decay rate of the HA was greater than that of the CSA, adding further confirmation to the previous conclusion that HA is more rapidly metabolized than is CSA. The lack of linearity of the decay curve makes accurate estimation of the half-life time difficult, but graphic approximation suggests that the half-life time for HA is approximately 1.9 days, while that for CSA is 7 days. The latter figure is consistent with the half-life time of 8–9 days reported by Boström and Gardell[15] for CSA of rat skin utilizing S^{35} as a label.

In order to locate the C^{14}, the respective mucopolysaccharide fractions from each pair of rabbits were pooled and the N-acetyl moiety was isolated as the 2-methylbenzimidazole derivative of a chromic acid oxidized aliquot of each fraction. Upon degradation of the methylbenzimidazole derivative, the radioactivity was found to be confined to the benzimidazole

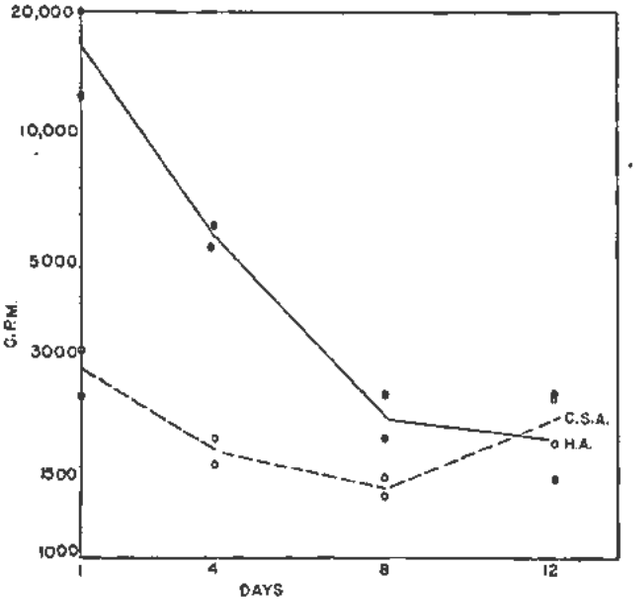

Figure 1. Log of the average radioactivity of the HA and CSA fractions from rabbit skin (corrected for body weight) plotted against time in days after the first injection of $CH_3C^{14}OONa$

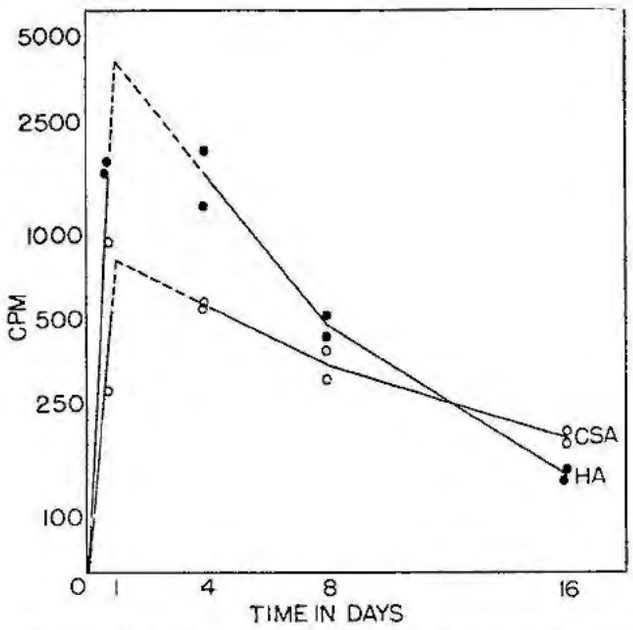

Figure 2. Log of the average C^{14} of the HA and CSA of the skin of rabbits (corrected for body weight) injected with C^{14}-glucose and $Na_2S^{35}O_4$. Zero time indicates last injection

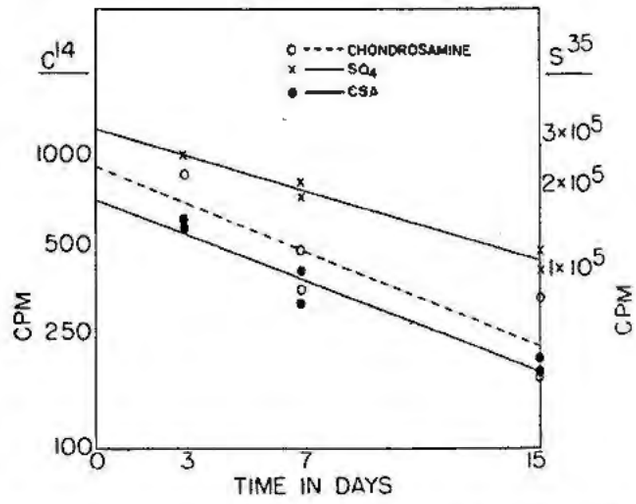

Figure 3. A semi-logarithmic comparison of the decay curves for the C^{14} of chondrosamine and CSA and for the S^{35} of the CSA isolated from the skin of rabbits injected with C^{14}-glucose and $Na_2S^{35}O_4$. The straight lines were drawn by the method of least squares

nucleus which represents the acetyl carboxyl carbon atom. When the total radioactivity of the HA and the CSA fractions was compared with the radioactivity of the respective benzimidazole fractions, the bulk of the radioactivity of the two mucopolysaccharides was accounted for in the N-acetyl moiety.

As has been noted above, the data obtained in this experiment do not follow apparent first order kinetics. Although some of the inconsistency may result from animal variation and experimental error, the observed discrepancies are somewhat greater than might be expected from these errors. Since the amount of radioactive acetate administered was relatively large, it is possible that release of acetate from the metabolism of highly labeled substances such as lipids may have occurred, thus accounting for the significant increase in radioactivity of the CSA and the failure of the radioactivity of the HA to decline between the 8th and 12th day. A similar explanation has been offered by Pihl, Bloch and Anker[16] for the failure to attain constant radioactivity in acetate excreted upon the continued feeding of labeled acetate.

The use of carboxyl-labeled acetate results in specific labeling of the N-acetyl portion of the molecule, permitting the objection that the observed rates are a function of exchange reactions of the acetyl portion of the molecule and therefore do not adequately reflect the metabolism of the polysaccharide molecule.

For this reason, a subsequent experiment was performed utilizing C^{14}-uniformly-labeled glucose and $Na_2S^{35}O_4$. Each of 8 rabbits received 48 μc of C^{14}-labeled glucose and 1.2 mc of $Na_2S^{35}O_4$ as an isotonic mixture of the two substances. One pair of rabbits was sacrified 16 hours after the last injection and the remaining six rabbits were killed in pairs, 4, 8 and 16 days after the first injection. The polysaccharides were isolated as in previous experiments. The radioactivity of the respective fractions is illustrated in Fig. 2. It is apparent that the 16-hour interval permitted before sacrifice of the first animals was not sufficient to obtain maximal labeling. This interval was adequate in previous experiments following acetate administration. The difference is probably due to slower absorption of subcutaneously administered glucose. The activity at zero time, therefore, was obtained from the intersection of extrapolations of the uptake and decay curves. Despite the departure of linearity, the data depicted in Fig. 2 demonstrate that the C^{14} is lost from HA more rapidly than from the CSA. The half-life times, as calculated from the line of best fit, are 3.7 days for HA and 7.7 days for CSA.

The hexosamines isolated from hydrolysates of the HA and CSA contained approximately the same C^{14} concentrations as the whole molecule, and showed the same decay rate.

A close parallelism was found between the decay rate of CSA as determined by S^{35} and by C^{14}. This is illustrated in Fig. 3. The half-life time of the CSA calculated from the curves was found to be 7.7 days and 10.0 days, respectively, on the basis of C^{14} and S^{35} disappearance rates.

In order to determine the rates of metabolism of these substances in another species and to further relate the results obtained with acetate to those obtained with $SO_4^=$, an additional experiment was performed utilizing carboxyl-labeled acetate and $Na_2S^{35}O_4$ simultaneously. For this purpose sixty adult male rats of the Sprague-Dawley strain were each injected with 57 μc of C^{14}-carboxyl-labeled acetate and 15 μc of $Na_2S^{35}O_4$ as an isotonic mixture. The animals were sacrified in groups of 12 at 1, 3, 5, 9 and 17 days after the first injection. The results in rats were similar to those in rabbits. Furthermore,

Table I. A Comparison of the Half-life Times of the Mucopolysaccharides and Some of its Components in the Skin of Animals Injected with C^{14}-acetate, C^{14}-glucose, and $Na_2S^{35}O_4$

Species	*Hyaluronic acid (HA)*			*Chondroitinsulfuric acid (CSA)*			
	C^{14}–HA days	*N–acetyl days*	*Glucosamine days*	*C^{14}–CSA days*	*N–acetyl days*	*Chondrosamine days*	*S^{35} days*
Rabbit *	2.4	2.7		7.6	5.3 (?)		
Rat *	4.5			8.1			10.7
Rabbit †	3.7		3.4	7.7		7.4	10.0

* After C^{14}-acetate and NA_2 $S^{35}O_4$. † After C^{14}-glucose and NA_2 $S^{35}O_4$.

no essential difference was demonstrable in the half-life times of the skin HA and CSA of rats after simultaneous administration of C^{14}-acetate and Na_2 $S^{35}O_4$ as compared with the skin mucopolysaccharides of rabbits after C^{14}-labeled glucose and $Na_2S^{35}O_4$ administration.

Table I presents a summary of the data which have so far been obtained with respect to the normal rate of metabolism of the mucopolysaccharides of rat and rabbit skins. It is quite clear that a striking difference exists in both species with respect to the rate of metabolism of HA and CSA. That the rates measured reflect synthesis of the entire mucopolysaccharide molecules is apparent since similar rates were found in studies of different moieties (acetyl, hexosamine and sulfate) of the polysaccharide molecules. No direct measure of the turnover rate of the uronic acid portions of the molecule has been obtained, since in the experiments with C^{14}-uniformly-labeled glucose the radioactivity of the uronic acid portion of the mucopolysaccharide molecule was too low for accurate accounting. However, since the C^{14} concentration of the whole molecule and that of the hexosamines was identical, it can be inferred that the C^{14} of the uronic acid moiety is likewise identical.

The interpretation of these data with respect to the CSA fraction is proposed with some reservation since the CSA as isolated appears to be a mixture of sulfated polysaccharides, each of which may have different rates of synthesis. The two components of rabbit skin have been separated by alcohol fractionation, but insufficient material has so far been available for separation of the individual fractions from isotope experiments.

Having established methods for measuring the rate of metabolism of the mucopolysaccharides, and having determined their normal rate in two different species, we studied the influence of various factors on the rate of mucopolysaccharide synthesis.

Of particular interest is the effect of insulin. Since it has previously been shown that both the glucose and glucuronic acid portions of HA derive from glucose, and since the role of insulin in the utilization of glucose is now well established, it seemed reasonable to postulate that insulin might play a role in the biosynthesis of the acid mucopolysaccharides. To test this hypothesis an experiment was conducted on sixty male Sprague-Dawley rats maintained on a diet of Rockland chow and water *ad libitum*. Alloxan diabetes was induced in 20 rats by a single subcutaneous injection of 150 mg of alloxan monohydrate per kg as a 5 per cent solution. Isotopic compounds were administered 3 weeks after diabetes was established. A second group of 20 untreated rats served as controls while a third group of 20 normal animals was maintained on half the average daily food intake for 3 weeks prior to and during the experiment. The weight loss in the latter group was similar to the diabetic animals over the three-week period. Each animal was injected once subcutaneously with 1.2 ml of a solution containing 80 μc of C^{14}-carboxyl-labeled sodium acetate and 2.7 μc of $Na_2S^{35}O_4$, as an isotonic mixture. Ten rats in each group were sacrificed at intervals of one and five days after the injection. The polysaccharides were isolated by the previously described methods, the $S^{35}O_4$= being isolated following acid hydrolysis. Figure 4 illustrates the result of this experiment. In the diabetic animal a striking decrease was found in the uptake of C^{14} in both the HA and CSA fractions as well as a decrease in S^{35} in CSA. No such change was observed in fasted animals, although weight loss was approximately the same as that observed in the diabetic animals. The average body weights for the normal, fasted and diabetic rats were 355, 212 and 210 gm, respectively. The decrease in the rate of metabolism of the mucopolysaccharides was manifested not only by the decrease in the rate of uptake of isotope, but also by a decrease in the rate of decay. Although half-life times calculated on the basis of two points are subject to error, values calculated from these data are shown in Fig. 4 for comparative purposes.

In order to verify this finding a second experiment was performed utilizing as precursors uniformly-labeled glucose and $S^{35}O_4$=. In addition, some of the diabetic animals were treated with insulin and a group of normal animals treated with insulin were included. Figure 5 illustrates the fact that insulin administration restores to normal the rate of turnover of the mucopolysaccharides in diabetic animals. The administration of insulin to normal animals had no apparent effect. This may be due to the limited amounts of insulin that could be employed without producing fatal hypoglycemia.

It seems reasonable to conclude from these data that insulin plays a role in the biosynthesis of the acid mucopolysaccharides of the ground substance of connective tissue. It is not possible to define the

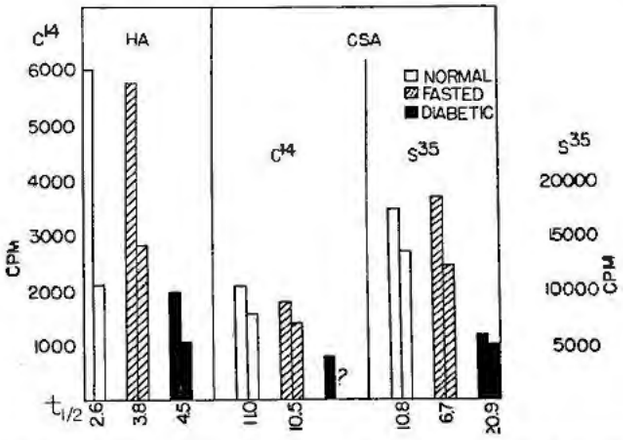

Figure 4. A comparison of radioactivity of various fractions following the administration of $CH_3C^{14}OONa$ and $Na_2S^{35}O_4$. The left hand bar in each case represents the value at 1 day while the right hand bar represents that value at 5 days

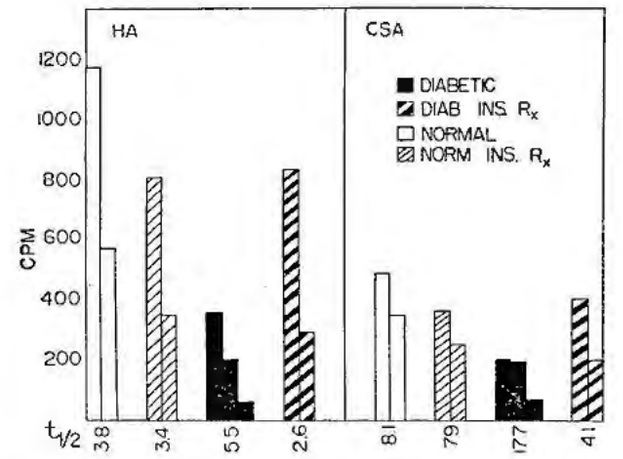

Figure 5. A comparison of the radioactivity of various fractions following the administration of C^{14}-glucose and $Na_2S^{35}O_4$. The bars in each case reading from left to right represent 1, 5 and 17 day samples

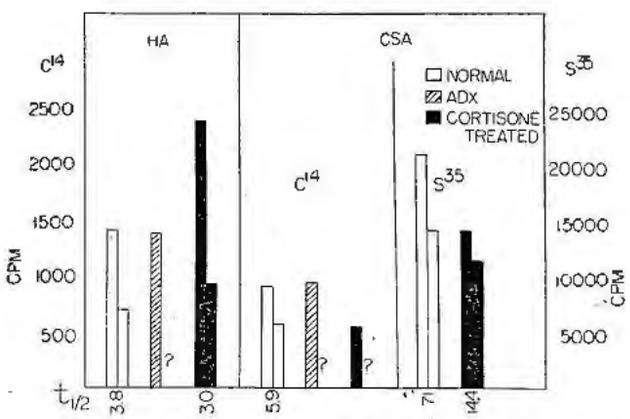

Figure 6. A comparison of the radioactivity of various fractions following the administration of $CH_3C^{14}OONa$ and $Na_2S^{35}O_4$. The bars in each case, reading from left to right, represent the 1 and 5 day samples

mechanism of this effect since definitive information regarding the detailed mechanism of synthesis of the acid mucopolysaccharides is lacking, and conclusive proof as to the locus of action of insulin is not available. It is, however, apparent that synthesis of mucopolysaccharides involves the utilization of glucose by way of phosphorylated intermediates which are common to the utilization of glucose for other metabolic purposes.

The role of insulin in the metabolism of mucopolysaccharides is of considerable biological interest. Failure of wound healing and increase of susceptibility to local infection have long been known to be characteristic of diabetes mellitus. These may well be a reflection of a decreased ability to synthesize the acid mucopolysaccharides. Of even greater interest is the possibility that this observation may throw some light on the accelerated vascular degeneration which is characteristic of this disease.

In view of the striking effects of adrenocorticotropic hormone and cortisone on diseases of connective tissue, it has been postulated that benefit is due, in part, to the fact that these substances affect connective tissue. This has been supported by the observation that under certain conditions these hormones cause a delay in wound healing.

A preliminary experiment was performed, utilizing the technique previously described, to compare the rates of metabolism of the mucopolysaccharides in the skin of cortisone-treated rats, adrenalectomized rats, and normal rats. The results of this experiment are depicted in Fig. 6. These results indicate no decrease in the rate of HA synthesis in contrast to a decreased rate of CSA synthesis. Although the explanation of these results is not clear, it is quite apparent that they open up a new realm of possibilities for the exploration of the mechanism of action of adrenal hormones on connective tissue.

SUMMARY

1. Methods have been devised for the isolation and degradation of the mucopolysaccharides, hyaluronic acid and chondroitinsulfuric acid, from the skin of rats and rabbits.

2. A study of the rate of metabolism of these two compounds has revealed that hyaluronic acid is metabolized at a considerably more rapid rate than is chondroitinsulfuric acid.

3. Evidence has been obtained which indicates that the rates measured reflect total degradation and resynthesis of the respective molecules.

4. There is a marked decrease in the rate of synthesis of both polysaccharides in the skin of alloxan-diabetic rats. The rate is restored to normal by the administration of insulin.

5. Preliminary results indicate a depression of synthesis of chondroitinsulfuric acid following administration of cortisone. No such depression was observed in the metabolism of hyaluronic acid.

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Isotopes in Research on Animal Nutrition and Metabolism

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The present paper deals mainly with investigations of the "Davis Tracer Team" for metabolic research on farm animals with isotopes as tracers. The work is carried out as a cooperative effort in which chemists, biochemists, veterinarians and animal husbandmen participate.

The team has specialized in work with intact animals, particularly dairy cows (technique and results nf perfusion experiments with isolated udders have been excellently reviewed by Marian Silver [1]). The major part of such metabolic work on intact animals has only become possible since isotopes of major elements such as Ca, P and C became available as biochemical tracers. The question of the relative importance of biochemical work on intact animals and that on isolated systems is irrelevant. Progress in biochemistry depends on both approaches and is best served by integration of the results of all the specialized work.

For this report we have selected from research in animal nutrition and metabolism a few problems for whose solution isotopes as tracers are especially important.

ENDOGENOUS CONSTITUENTS IN FECES AND "TRUE" DIGESTIBILITY

Only a part of the fecal material constitutes undigested residues from the food—the other part is made up of substances from the animal body such as sluffed off tissues of the intestinal tract or secretions. The presence of these endogenous constituents in the feces has been known for a long time. Fecal nitrogen excreted in excess of the intake of food nitrogen was obviously of endogenous origin. Methods for determining this endogenous N in feces were develnped already during the last century.[2] One such method was based on the postulate that fecal N, whicb could he digested by pepsin and trypsin preparations *in vitro,* was of endogenous origin; the true digestibility was thus defined as the quotient: (N in feed — non-digestible N in feces)/N in feed.

No such methods for estimating endogenous fecal P are available. One may feed a low P ration so that the fecal P might exceed the P in feed, but such a regime produces unphysiological conditions and the excess of fecal P excretion over intake of feed P in such a case cannot be taken as an index for the normal rate of excretion of the endogenous fecal P.

The use of P^{32} as a tracer made the measurement of endogenous P in feces possible. Hevesy *et al.* used this method for man.[3] Kleiber *et al.*[4] measured the endogenous P in cow's feces. They injected radiophosphorus into the cow's blood twice daily during a month. They thus established nearly constant levels of the tracer in the blood and in the feces. The ratio of these two levels indicated the fraction of endogenous to total fecal phosphorus as the ratio of the secretion and the excretion rates. This calculation is illustrated by Fig. 1.

Subtraction of the endogenous from the total fecal P ($f - s$) led to the non-digested fecal P excretion. The difference of P intake and fecal excretion of undigested P was taken as "truly digested" P. The apparent digestibility of food P in one case was only 12%, the true digestibility, however, 50%. Visek *et al.*[5] used the same method to determine the fecal excretion of endogenous calcium.

After injection of P^{32} into the bloodstream of a cow, the casein of the milk becomes radioactive. Lofgreen *et al.*[6] could feed this radioactive casein to calves and thus measure their excretion of endogenous P. The calculation of the endogenous P in this case is not as rigorous as that after injection of P^{32} into the blood. A part of the P^{32} injected will be absorbed and then re-excreted into the intestinal tract. Lofgreen *et al.*, however, could estimate the extent of this re-excretion. Feeding the tracer has also been used fnr the measurement of endogenous fecal Ca by Comar *et al.*[7]

Multiple injections are a cumbersome procedure. Attempts have, therefore, been made to measure the endogenous P in feces by a single injection. Instead of using a ratio of constant tracer concentrations in plasma and feces, one might use the ratio of the time integrals of these concentrations when they vary, a procedure which will be discussed for C^{14} later. To decrease the variations in tracer concentration J. Luick tried subcutaneous and intrapentoneal injections of $CO_3(P^{14}O_4)_2$. Both procedures led to rather uniform levels of P^{32} in plasma of sheep for several days and offer the possibility of being used routinely.

MINERAL EXCHANGES IN THE ANIMAL BODY

Comar *et al.*[8] studied calcium exchange between bones and blood with Ca^{45} as a tracer. Black *et al.*[9] used P^{32} for similar research. They measured the

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specific P^{32} activity of the skeletal P in a cow sacrificed 72 hours after intravenous injection of P^{32}. The mean for the specific activity of the skeletal P amounted to 1% of the specific activity in the plasma. This result indicated that the pool designated as labile bone phosphorus in the cow (P in equilibrium with plasma P at 72 hours after injection) amounted to 29 grams of P or 1% of the total bone P. Radioautograms indicated that the labile P is concentrated in discrete spots of the bone.

J. Luick (unpublished) has used Ca^{45} as a tracer in the study of "milk fever," a disease of high-producing dairy cows who seem unable to mobilize Ca at a rate sufficient for providing the necessary Ca in the milk.

PRECURSOR-PRODUCT RELATIONSHIP IN MILK FORMATION

Advantages of Cows for Biochemical Research

A major difficulty in studies on intermediary metabolism is the low concentration of important intermediary metabolites with high turnover rates. In a lactating animal some of those metabolites are continuously dumped into the cystern of the mammary gland where they seem to be protected against further change and thus accumulate to concentrations suitable for their isolation.[10] One of these substances is citric acid caught in the udder as if by Neuberg's "Abfangmethode" but by a physiological process rather than the addition of "catching" reagents or the poisoning of enzyme systems. Lactation is thus a process par excellence for metabolic research.

Among lactating animals, the dairy cow offers particular advantages. She produces a lot of milk sufficient for the preparation of minor components, such as citric acid, and allows seven blood samples to be taken within three hours, each sample sufficient for the preparation of enough volatile acids, beside glucose, for combustion and determination of the specific C^{14} activity in the blood at a given time. The cow furthermore in comparison to small animals acts like a time magnifier. The time to metabolize a certain percentage of a body constituent in a cow is about ten times as long as that in a mouse.

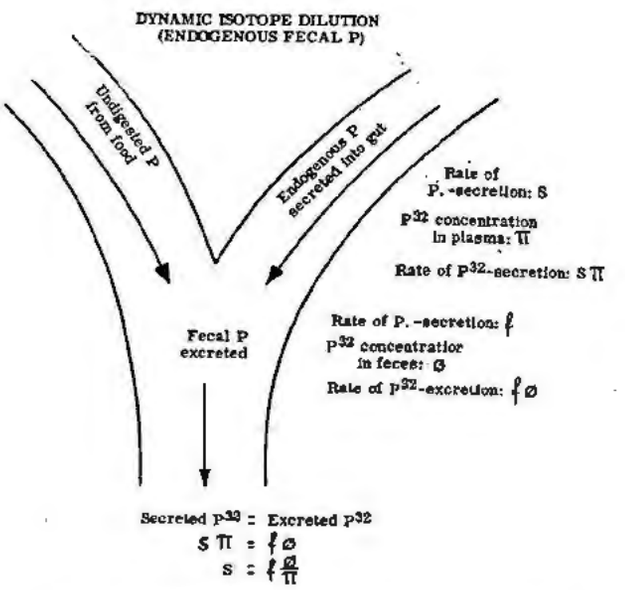

Figure 1. Dynamic isotope dilution. (Endogenous fecal P)

Since the metabolic rate of homeotherms is nearly proportional to the ¾ power of body weight [11] and since a similar relation exists for other rates such as food intake [12] one may postulate that generally the rate of a change of body substances, Δ/t, is proportional to $W^{-¼}$ where W stands for body weight. Thus:

$$\frac{\Delta/t}{W} = kW^{-¼} \text{ or } \frac{\Delta}{W} = ktW^{-¼} \qquad (1)$$

Accordingly for a given period of time the relative change, Δ/W, in cow and mouse is in the following ratio:

$$\frac{\frac{\Delta}{W}(c)}{\frac{\Delta}{W}(m)} = \left(\frac{W(c)}{W(m)}\right)^{¼} \qquad (2)$$

For the same relative change, $\frac{\Delta}{W}(c) = \frac{\Delta}{W}(m)$ the cow requires more time than the mouse so that

$$kt(c)W(c)^{-¼} = kt(m)W(m)^{-¼} \qquad (3)$$

Consequently,

$$\frac{t(c)}{t(m)} = \left(\frac{W(c)}{W(m)}\right)^{¼} \qquad (4)$$

For a cow of 500 kg and a mouse of 50 gm body weight the metabolically equivalent times are in the ratio $10{,}000^{¼} = 10$.

Figure 2 illustrates this effect of body size on oxidation rates of fatty acids in cows and mice. For the results on mice we are indebted to H. B. Jones of the Donner Laboratory at Berkeley.

Precursors

Various authors have attempted to study the precursor-product relationship in milk formation by measuring the concentration of metabolites in the blood entering and the blood leaving the mammary gland.[14–17]

The results were conflicting which is not surprising in view of the probability that the udder itself may produce some of the metabolites tested as precursors such as lactic acid.

The introduction of tracers led to more satisfactory results. Soon after P^{32} became available Aten and Hevesy [18] injected labeled phosphate into a goat and could conclude that the P atoms used in the synthesis of casein are drawn mainly from the inorganic P of the plasma, not the plasma lipids.

The use of isotopes as tracers led to a change and clarification of the concept precursor.[19,20]

A precursor now is regarded as any substance which can contribute components to the synthesis of a product.

As precursors we have chosen for our study: CO_2, the lower fatty acids from formate to caproate, an amino acid (norleucine) and glucose. The labeling

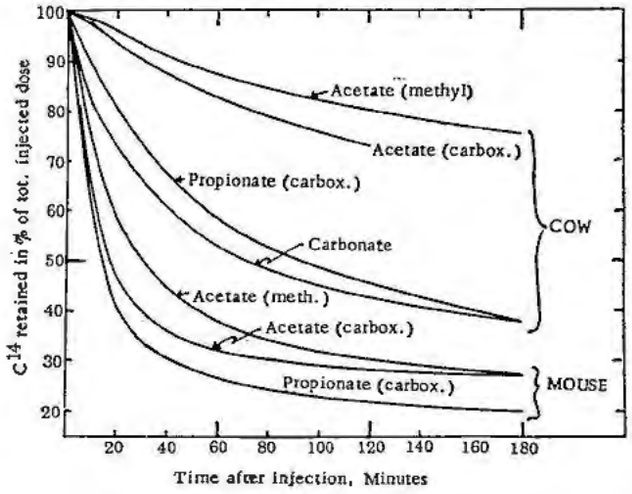

Figure 2. Relative C^{14} retention

of the fatty acids and norleucine was performed by the bioorganic group at Berkeley (M. Calvin) under the direction of Dr. Bert Tolbert by Mrs. Patricia Adams. A uniformly labeled glucose sample was prepared for us by Dr. Stadtman, Department of Food Technology at Davis and another sample by Mr. Terry Rogers, graduate student in the Department of Animal Husbandry at Davis. The C^{14} labeled precursors were injected into the jugular veins of the cows through canulas of polystyrene which permitted painless injection and taking of blood samples. Recently Mr. Kaneko of the School of Veterinary Medicine succeeded in inserting such a canula also into the mammary vein. In some cases labeled precursors were injected also into one of the four cysterns of the udder.

Products

Among the products studied is the respiratory CO_2 (a product as well as precursor). The apparatus for measuring the rate of the cow's CO_2 production and obtaining samples for the determination of the radioactivity in that CO_2 is shown in Fig. 3.[21]

The injection of the labeled compound is as a rule followed by a 3-hour respiration trial at the end of which the cow is milked. The second milking is performed 9 hours after injection. Later the cow is milked at the regular 12-hour intervals. Milk fat, casein and lactose are prepared from the milk. The casein is hydrolyzed and the amino acids are separated by column chromatography and checked for purity by paper chromatography.[22] Lactose has been degraded to study the labeling pattern [23] and milk fat has been separated to glycerol and various fractions of the fatty acids.[24]

Transfer Quotient

In the study of the precursor-product relationship, one may ask what fraction of a given precursor appears in a given product. This fraction is usually called the efficiency of the utilization of the precursor for the formation of the product. Later sections will deal with this efficiency. One may ask the reverse question: How much of a given product originated in a given precursor pool? The answer to this question may be expressed as the transfer quotient. The ratio endogenous fecal P/total fecal P discussed above is a transfer quotient calculated for the special and simple condition of constant specific activities.

Now the more general case of variable specific activities in precursor and product and the passage through two (as an example for several) intermediary pools will be discussed to show that also under those conditions the calculation of the transfer quotient is justified. The calculation is explained with the help of a scheme (Fig. 4).

A stands for the pool of precursor originally labeled (for example carbonate in plasma, labeled by injection of $C^{14}O_2$). *L* indicates the pool of the product (such as lactose in milk). Labeled material from *A* may travel to *L* via intermediary pools *B*

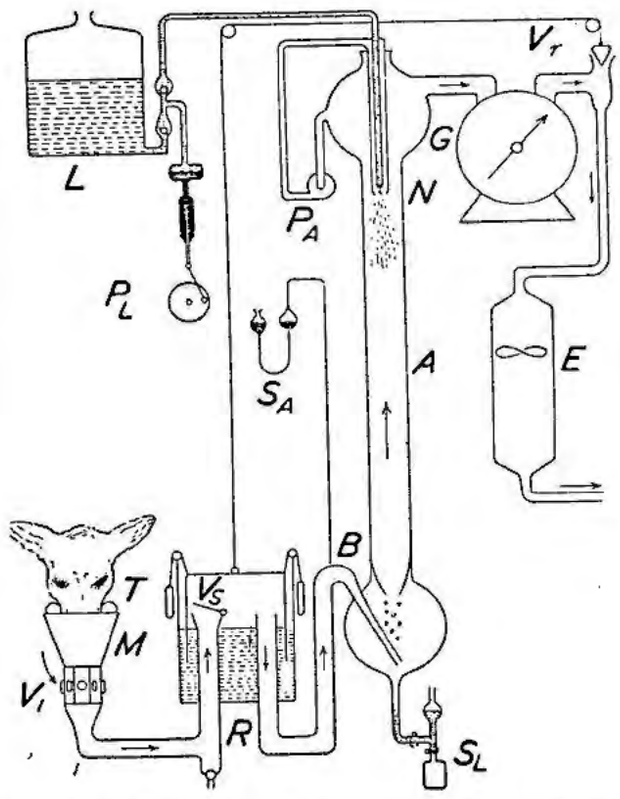

Figure 3. Respiration apparatus for measurement of CO_2 and $C^{14}O_2$. A, absorbing tower; B, outlet for gas sample; E, vacuum cleaner; G, gas meter; L, storage tank with normal NaOH solution; M, edick mask; N, spray gun for NaOH solution; P_A, air pump for spray gun; P_L, pump for NaOH solution; R, spirometer; S_A, gas sampler; S_L, collector for NaOH; T, inner tube; V_1, air inlet valves; V_r, valve to regulate ventilation by vacuum cleaner; V_s, air inlet valve of spirometer

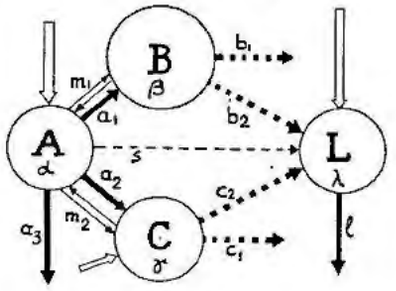

Figure 4. Transfer diagram

and C. Each of these pools may obtain material from other sources than A and give off material not only to L with the rates b_2 and c_2 but also to other pools with rates b_1 and c_1. Pools c and b may also transfer labeled material back to A (m_1, m_2), but it is assumed that no labeled material flows back from L to B or C. Not all the tracer that leaves pool A reaches pool L and not all the precursor for L comes from A. But all the tracer that appears in L or leaves L must have come originally from A since A is the pool into which the tracer has been injected. We can think of a hypothetical fraction of the total flow out of pool A as the rate, s, of that part of the precursor leaving A which ultimately reaches L. When the tracer concentration in A is α then $s\alpha\, dt$ is the amount of tracer that leaves A during the time interval dt bound for L. During the same time element the amount $l\lambda\, dt$ leaves pool L. These two amounts, $s\alpha\, dt$ and $l\lambda\, dt$ are not equal, but if we add up all the small amounts of the tracer that leave A for L until the tracer concentration in A becomes negligible, then we have summed up all the tracer that goes from A to L. We can similarly add all the amounts of tracer that leave L till the tracer concentration λ becomes insignificantly small. That sum then expresses all the tracer that has gone through pool L. This sum must be equal to that which has left A on the way to L, or, in mathematical formulation:

$$s\int_0^\infty \alpha\, dt = l\int_0^\infty \lambda\, dt \qquad (5)$$

The integration to infinite time means here simply to integrate long enough that further additions do no longer change the integral. The quotient:

$$\frac{s}{l} = \frac{\int_0^\infty \lambda\, dt}{\int_0^\infty \alpha\, dt} \qquad (6)$$

gives the ratio of the rate of which precursor comes to L from pool A to the rate at which the product is formed. This ratio is called the *transfer quotient.* Its determination required the measurement of the specific activities in the precursor pool and the product pool at various times after injection. When l expresses the rate of the only process by which the labeled material leaves pool L or the sum of the rates of all these processes, then the transfer quotient expresses the transfer between the original precursor and the product. It is independent of number and size of intermediary pools and number and rates of intermediary paths.

Transfer of C from Precursor to CO_2

Since the exchange of CO_2 from alveolar blood to alveolar air is a rapid process, the rate at which C^{14} is given off in respiratory CO_2 is an index for the rate at which C from a C^{14} labeled organic compound is transferred to carbonate.

Table I shows what fraction of the injected C^{14} is excreted as CO_2 in three hours. The rate at which the carboxyl carbon appears in respiratory CO_2 seems to increase with increasing size of the molecule of the fatty acid.

Table I. Fraction of Carbon from Various Precursors Appearing in Respiratory CO_2 in Three Hours

Labeled precursor injected intravenously	*Per cent of injected C^{14} excreted in respiratory CO_2 in three hours*
Carbonate	62, 78, 67
Formate	27, 27
Acetate 1-C^{14}	32, 48, 48
Propionate 1-C^{14}	62, 62
Butyrate 1-C^{14}	59, 60
Valerate 1-C^{14}	39, 51
Caproate 1-C^{14}	69
Acetate 2-C^{14}	24, 30
Propionate 2-C^{14}	31, 30
Butyrate 2-C^{14}	34, 30
Valerate 2-C^{14}	28, 22
Caproate 2-C^{14}	21, 20
Glucose, all labeled	23

The transfer rate of the α carbon of the fatty acids to CO_2 however, depends very little on the nature of the fatty acid.

CO_2 As Precursor, Carbonate Fixation in Milk Formation

Soon after isotopes of carbon became available as tracers, in 1950, CO_2 fixation by animal tissue was discovered by research workers at California, Chicago, and Columbia universities and a year later a Harvard team published the evidence for carbon transfer from CO_2 to glycogen in the intact rat.

When sufficient C^{14} could be obtained, Kleiber *et al.*[25] studied the carbon transfer from CO_2 to organic constituents of the milk in the intact cow. They calculated the transfer quotients as discussed above and concluded that about 10% of the carbon in lactose, 4% of the carbon in casein and 2% of the carbon in butterfat originate from the plasma carbonate pool.

The role which carbonate fixation plays in the synthesis of the major milk components from fatty acids is shown in Table II.

The transfer of carbon from other than the carboxyl position of the molecule of fatty acids involves

the carbonate pool to a smaller extent and only 1% of the carbon transferred from glucose (all labeled) goes via carbonate.

Efficiency of Fatty Acids for Milk Formation

Among the precursors of milk components tested so far in our trials glucose was the most efficient. No less than 56% of the C^{14} labeled glucose injected intravenously into a cow appeared in organic milk constituents within 48 hours, as much as 46% of the injected C^{14} was incorporated in lactose alone.[26] The general efficiency for milk formation of carbon atom 3 in norleucine and of carbon atom 2 in caproate was about ½ of that of the average carbon atom in glucose. This efficiency was about 1% per kg milk for the C-2 atom of acetate and decreased in the order C-2 propionate, C-2 butyrate, C-1 acetate, C-1 propionate, formate, C-1 butyrate and C-1 valerate to 0.1% per kg milk for carbonate as precursor. The latter result means that about 2% of the total C^{14} injected as carbonate was found in the organic compounds of the milk in two days following the injection.

Among the 3 major components of milk, lactose received a particularly high share of carbon from glucose, casein from formate, and butterfat from acetate.

Gluconeogenic vs Lipogenic Behavior of Precursors

The ratio of the amount of C^{14} from a given precursor that appeared in lactose and the corresponding amount that appeared in milk fat may be used as an expression for the degree to which a precursor specializes in carbohydrate formation.

Such a quotient would, however, depend on the composition of the milk, especially the fat content.

Table II. Contribution of Carbonate Fixation to Transfer of C from Metabolites to Milk Constituents *

Metabolites	*Milk constituents*		
	Lactose %	*Casein* %	*Fat* %
Formate	18	3	20
Acetate 1-C^{14}	74	40	5
Propionate 1-C^{14}	31	36	14
Butyrate 1-C^{14}	27	18	8
Valerate 1-C^{14}	20	18	9
Caproate 1-C^{14}	23	11	6
Acetate 2-C^{14}	17	8	2
Propionate 2-C^{14}	6	8	12
Butyrate 2-C^{14}	15	6	6
Glucose, all labeled	1	4	1

$$*\% = 100\,q \int_0^{70} \rho_s\, dt \Big/ \sum_0^{70} \lambda_s \Delta t$$

where q = carbonate transfer quotient, ρ_s = spec. activity of C^{14} in respiratory CO_2, λ_s = spec. activity of C^{14} in milk constituent, t in hours.

Table III. Gluconeogenic vs Lipogenic Effect of Metabolites in Milk Formation

Metabolite	*C position of label in test*	$\sum_0^{48} \lambda_s \Delta t$ *in lactose* / $\sum_0^{48} \lambda_s \Delta t$ *in milk fat*
Glucose	all	15.3
Caproate	2	9.0
Propionate	2	8.3
(Formate		8.0)*
Carbonate		6.1
Valerate	2	4.8
Norleucine	3	4.4
Butyrate	2	3.8
Valerate	1	3.5
Propionate	1	2.5
Butyrate	1	2.5
Caproate	1	1.8
Acetate	2	0.7
Acetate	1	0.3

* Mean for formate is unreliable; quotient varied from 2.5 to 13.4.

A criterion for gluconeogenic vs lipogenic behavior of precursors, independent of the amount of product and thus independent of the composition of milk, is the ratio of the mean specific activities of C^{14} in lactose and milk fat or the ratio of the time integrals of the specific activities. Since the milk samples are taken at the end of considerable periods of time, the specific activity of each sample represents a mean for the period and the time integral is the sum of the products of the specific activity in, and the duration of, each period.

Table III shows the ratios of these sums and ranks the precursor according to their "gluconeogenicity".

The carboxyl carbon of the fatty acids behaves less gluconeogenic than does the α carbon even though the greater involvement of carbonate fixation in 1-C transfer (Table II) would favor gluconeogenesis.

Acetate is distinguished from all other precursors tested as the only one that is more lipo-than gluconeogenic. We do not know yet whether this observation indicates a special condition in a lactating cow or is an example illustrating a more general biochemical rule. Non-lactating animals or even lactating cows whose milk fat content differs from that of our Jersey cows, might conceivably show different ratios between gluconeogenesis and lipogenesis.

In line with observations by other scientists, our work shows that glucose is the precursor par excellence for lactose formation. Since in the ruminant most of this important sugar has to be synthesized from fatty acids, it is understandable that a high producing dairy cow might encounter difficulties in maintaining the necessary rate of glucose supply and then develop a disease known as ketosis,

which Mr. Macleod in our laboratory is now investigating by the use of C^{14} as tracer.

CONCLUSION

An adequate appraisal of the literature and a more thorough discussion of the biochemical implications of our own results is beyond the scope of this paper. We have attempted to indicate with a few examples the nature of our own investigations. We hope that our contribution to this Conference together with those from other laboratories may integrate to a picture which will demonstrate the importance of isotopes as biochemical tracers.

The production of these isotopes is unquestionably outstanding among the peaceful applications of atomic energy. The use of these isotopes as tracers has inaugurated a new epoch for biochemistry, greatly expanded knowledge and clarification of new problems, and more rewarding approaches to old problems.

Isotopes as tracers have stimulated all branches of biochemistry. They brought especially radical changes in experimental methods, and offer particularly great promise for future development, in the field of metabolic research on intact animals.

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Studies on Physiology of Lactation

By R. Sasaki,* Japan

Knowledge of the mechanism under which milk is produced in the mammary gland forms the fundamental basis for the production of milk. The application of the latest scientific achievements is most needed in Japan where it is necessary to produce more milk from the limited area of land.

Milk contains a considerable amount of radioactivity,[1, 2, 3] almost the whole of which is, however, the natural radioactive isotope K^{40}. As there is a wide range of difference in quantity of K in milk according to individual cows, lactation and time of production as well as feeding, there exists considerable difference in the amount of K^{40} in milk.

In order to clarify the mechanism of synthesis of milk casein,[4] the radioactive isotopes CA^{45} and P^{32} were venously injected into a goat to observe their appearance in the blood, urine and feces. Ca^{45} begins to appear in the milk 30 minutes after it is injected. In 2 hours it suddenly starts increasing and reaches the maximum in 5 hours. Rapid decrease of Ca^{45} begins thereafter and it completely disappears 27 hours after the injection. On the other hand, Ca^{45} appears in the mammary vein blood in an hour. However, because it easily moves to milk while in the mammary gland, evident increase of Ca^{45} is not observed.

The acid-casein separated from the above milk contains hardly any Ca^{45}, while rennet-casein, separated from the same, includes a fairly large amount. The milk-serum separated from the acid-casein contains more Ca^{45} than the milk-serum separated from the rennet-casein. Most of the Ca^{45} precipitates when the rennet-whey, after the protein has been removed, is neutralized by adding sodium hydroxide. The protein, separated by adding ammonium sulfate to skimmed milk, contains hardly any Ca^{45}. However, when the same is separated by the way of half saturating ammonium sulfate, little Ca^{45} remains. Ca^{45} in this protein completely disappears when dialysed. No Ca^{45} was detected in the goat's feces or urine excreted, and analyzed, 5 hours and 7 hours after the injection of $Ca^{45}Cl_2$.

After the injection of P^{32} to a goat's neck vein, milk samples were taken after 2-, 4- and 7- hour periods. The sample taken 4 hours after the injection showed the highest radioactivity. The acid-casein separated from such milk contained less radioactivity than the separated rennet-casein. The precipitate, which was formed when $Ca(OH)_2$ was added to proteinless milk-serum, contained considerable P^{32}. According to this experiment, the phosphorus in the milk-serum seems to be in the form of phosphate. The goat's neck vein, 2 hours after the injection of P^{32}, contained approximately 1/10 the concentration of P^{32} in the milk. At the same time, feces and urine contained but little P^{32}. Six hours after the injection, the feces showed remarkable increase of P^{32}, while it hardly increased in the urine. The result of this experiment is shown in Table I.

In another experiment a mixture of P^{32} and Ca^{45} was injected into a goat, and the P^{32} and Ca^{45} in the milk, taken 2 hours and 12 hours respectively thereafter, were estimated. The ratio of P^{32} to Ca^{45} in the milk is higher than that of P^{32} to Ca^{45} injected as mixture. It therefore seems clear that Ca is more rapidly translocated to milk than P. The acid-casein separated from the above milk contained little Ca^{45}. Comparing the milk drawn 2 hours and 12 hours after the injection, the latter showed decrease of Ca^{45}, while the content of P^{32} was doubled. This fact seems to show that the P^{32} had become a constituent element of casein. It was also disclosed that P^{32} appeared in the casein molecule later than its appearance in the milk as a whole.

Radioactive calcium chloride and sodium phosphate were added to the solution of calcium caseinate and skimmed milk, and filtered by the ultrafiltration method in order to measure the radioactivity of protein fraction and filtrate fraction. By this experiment, the state of the combination and substitution of Ca^{45} and P^{32} with reference to casein was presumed.

It was disclosed, as the result of this experiment, that both calcium and phosphorus combined with casein, but that phosphorus showed better combination with casein when it was with the calcium. Calcium triphosphate also combines with casein but slightly. The phosphorus combined with casein is easy to separate from casein by ultrafiltration, while calcium combined with casein is fairly stable. Therefore, it seems that calcium is chemically combined with casein while the combination of casein and most of the phosphorus is loose.

Another experiment[5] was tried in order to confirm the effects of administration of radioactive material on milk. The ashes of "Haenawa" rope on the deck of the 5th Fukuryumaru (after the nuclear detonation in the Bikini Islands) were orally administered to

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Table I. Tracing Experiment of Radioisotopes in Goat's Milk after Injection to Neck Vein

cpm/ml or/0.1 gm	Ca^{45}			P^{32}		
Hours after injection	*2*	*4*	*7*	*2*	*4*	*7*
Milk	80	109	85	1616	2080	1280
Rennet-casein	557	876	656	6080	7776	5056
Acid-casein	42	41	42	1600	2304	1618
Rennet milk-serum	65	71	62	784	592	448
Acid milk-serum	—	—	—	768	1680	1536

Table II. Radioactivity of Milk

Sampling period, hr	*No. 1*				*No. 2*			
	Total ash, gm	*Ash measured, gm*	*cpm*	*Total cpm, excluding K*	*Total ash, gm*	*Ash measured, gm*	*cpm*	*Total cpm, excluding K*
Before administration								
0–6	7.2	0.5	26	0	6.4	0.5	27	0
6–12	4.7	0.5	21	0	3.3	0.5	18	0
12–24	5.1	0.5	25	0	4.0	0.5	27	0
After administration								
0–6	1.4	0.5	35	22	0.8	0.5	24	2
6–12	1.2	0.5	38	36	0.9	0.5	28	4
12–24	2.5	0.5	41	50	2.0	0.5	29	16
0–24	5.1	—	—	108	3.7	—	—	22
24–48	7.4	0.5	31	59	3.6	0.5	30	42
48–72	5.1	0.5	23	20	3.6	0.5	36	14
72–96	4.2	0.5	21	8	3.8	0.5	36	30
96–120	4.2	0.5	17	0	3.1	0.5	27	6
120–144	4.0	0.5	21	0	4.0	0.5	28	0
144–168	4.3	0.5	21	0	3.0	0.5	31	6

Table III. Radioactivity of Feces

Sampling period, hr	*No. 1*				*No. 2*			
	Total ash, gm	*Ash measured, gm*	*cpm*	*Total cpm, excluding K*	*Total ash, gm*	*Ash measured, gm*	*cpm*	*Total cpm, excluding K*
Before administration								
72–48	63.7	0.5	5	0	66.8	0.5	4	0
48–24	43.5	0.5	2	0	75.0	0.5	3	0
24–0	57.9	0.5	3	0	59.7	0.5	4	0
After administration								
0–6	4.5	0.5	10	230	8.2	0.5	10	0
6–12	5.7	0.5	8	40	11.2	0.5	7	250
12–24	25.4	0.5	2112	74400	22.7	0.5	52	1400
0–24	35.6	—	—	74670	42.1	—	—	1650
24–48	35.1	0.5	1725	111300	54.8	0.5	1120	61350
48–72	48.8	0.5	299	19300	51.0	0.5	680	58200
72–96	50.0	0.5	113	5050	47.4	0.5	295	19000
96–120	46.4	0.5	28	3300	47.6	0.5	125	6750
120–144	48.7	0.5	12	1500	56.0	0.5	40	2400
144–168	29.4	0.5	8	0	48.2	0.5	20	2000

two goats. Daily measurement was made of radioactivity in their milk, urine and feces.

The radioactive analysis of the sample rope was done in the isotope laboratory in the National Institute of Agricultural Science. It was fractionated with ion-exchanger, and the radioactive energy of β-ray in every fraction was sought by the absorption curve of aluminium plate, and the decay curve was sought. The radioactive elements were determined referring to literature. The results disclosed that

Table IV. Radioactivity of Urine

Sampling period, hr	No. 1				No. 2		
	Total ash, gm	Ash measured	cpm	cpm/500 mg ash in urine, excluding K	Total ash, gm	Ash measured	cpm
Before administration		mg/cm²				mg/cm²	
72–48	26.7	106	39	0	29.4	205	61
48–24	40.3	464	59	0	31.6	354	64
24–0	20.6	320	51	0	25.2	392	53
After administration							
0–6	4.0	155	71	4	5.5	176	43
6–12 }							
12–24 }	29.8	238	44	2	12.4	250	72
0–24	33.8				17.9		
24–48	25.6	286	48	1	30.2	338	53
48–72	45.6	446	63	2	20.7	331	72
72–96	38.6	229	49	1	18.9	336	82
96–120	25.5	237	54	1	27.7	366	67
120–144	14.6	288	59	3	15.2	149	47
144–168	14.3	133	45	2	32.2	298	75

there was more radioactivity due to Ru^{106}, Rh^{106}, Y^{90}, Y^{91}, Ce^{144}, Sr^{89}, Sr^{90}, Zr^{95}, Nb^{95}, etc. This rope ash was fed to the goat, and the radioactivity in her milk excluding that attributed to K was estimated and is shown in Table II. The radioactivity of the feces in shown in Table III. It is difficult to correct the sedimentation of the ash of feces against self-absorption excluding K, so it was calculated as against the total amount of feces, and is presented as total cpm, excluding K. The decay curve, is not corrected either since it is too slight. As it is of qualitative analytic nature, the amount of radioactivity in reality must he far more than this.

The radioactivity of urine is shown in Table IV. The result of the measurement of 500 mg of ash of urine, excluding K, shows that there exists only a very slight amount of radioactivity, if any.

The results obtained are summarized as follows: Less than 0.1% of radioactivity in the ash administered to the goats appeared in their milk during 5-day period. The radioactive elements in milk were assumed to be Sr^{89} and Ca^{45} hy the method of Al-absorption of β-emission. Most of the fission products were excreted very speedily in the feces. The radioactivity of urine was negligible and that of the organs was also only slight.

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Studies of Brain Potassium in Relation to the Adrenal Cortex

By John R. Bergen, David Stone and Hudson Hoagland,* USA

Using radioactive potassium (K^{42}) as a tracer, Hoagland and Stone[1] investigated the concentration of brain and muscle potassium of rats under a variety of conditions. They found that uncompensated adrenalectomy increased the concentration of K^{42} in brain by 24% and in muscle by 35%. On the other hand Bergen and Hoagland[2] have determined the total K content of brains and muscle using flame photometry and have shown that there is no difference in the K content of brains from normal and adrenalectomized animals. Further, the increase in total muscle K as a result of adrenalectomy was only 6.2% ($P = 0.01$). Davenport[3] also found no difference in total brain K between normal and adrenalectomized rats. Leiderman and Katzman[4] have confirmed both of these observations and offer evidence that these seemingly contradictory results may be explained on the basis that in normal adult rats 20% of the brain K is non-exchangeable and following adrenalectomy all of the brain K becomes exchangeable. Stone and Shapiro[5] determined the percentage of diffusible potassium occurring in normal rat brain and muscle by means of an ultrafiltration apparatus where Visking cellulose sausage casing served as a semipermeable membrane. They found that 25% of brain K and 28% of muscle K were non-filterable. We have further investigated the possible relationship between non-exchangeable K and the non-filterable K fraction in normal and adrenalectomized rats. In addition we have studied the K^{42} uptake by brain and muscle and the urinary K excretion in normal and adrenalectomized rats.

METHODS

In all experiments Charles River strain of Sprague-Dawley male albino rats were used. In most cases the rats weighed 150–170 gm but in two experiments where older animals were used the animal weights averaged 320 gm and 400 gm respectively. Approximately half of the rats served as normal controls and the remainder were adrenalectomized by the lumbar approach four days prior to the start of the experiment and maintained until 6 hr before the experiment with physiological saline. Except when stated, the rats were fed a diet of standard laboratory chow *ad lib.*

The normal and adrenalectomized rats were injected at the same time with a solution containing radioactive KCl. In the ultrafiltration experiments isotonic radioactive KCl was administered in three equal injections spaced twenty minutes apart. The quantity injected amounted to 3 ml 150 gm body weight. In uptake studies the solution contained 1/2 volume of isotonic radioactive KCl and 1/2 volume of isotonic NaCl so as to offset disturbances in salt and water balances at zero time. Blood samples were obtained in heparinized syringes by cardiac puncture at the time of sacrifice. The blood was centrifuged immediately after collection and aliquots of the plasma were obtained for K and K^{42} determinations.

In one experiment (urine excretion study) the normal and adrenalectomized rats were placed in metabolism cages, three per group, and urines were collected from five normal and 5 adrenalectomized rat groups. None of these animals were fed during the experiment although all normal groups of rats received tap water and the adrenalectomized rats were given physiological saline *ad lib.* From these rats urine samples were collected 5, 17, 24, and 41 hours after injection of the isotope.

All K^{42} counts were made using an end window counter. Samples were counted in duplicate and the theoretical counting error for each planchet amounted to less than 2%. To eliminate geometrical errors planchets were rotated after each count and the values of duplicate counts averaged. All counts were corrected for half-life decay.

Ultrafiltration Experiments

At the time of sacrifice (35–38 hr post injection), a group of normal rats alternated with adrenalectomized rats in the sequence of etherization and decapitation. Immediately afterward the cerebrum and left gastrocnemius muscle were removed from each animal and, in groups of three tissue samples each, weighed and homogenized in a Waring Blender in 100 ml of iced, distilled water. Aliquots were removed for total potassium analyses and K^{42} determinations. The remainder of each homogenate was transferred to an ultrafiltration apparatus[5] and filtered under negative pressure at 3–4°C for 12 hours. Aliquots of the ultrafiltrates were then analyzed for K and K^{42}.

Total K analyses were carried out on aliquots acidified with HCl, pressure cooked at 20 lb pressure for 20 min and then cooled and neutralized with NH_4OH. A Perkin-Elmer flame photometer (Model 52-C) with an internal standard was used to perform the analyses.

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Uptake Experiments

At specified intervals following the radioisotope injection the animals were sacrified in the manner described above. The cerebrum and left gastrocnemius muscle were immediately removed, weighed, and transferred to separate 50 ml volumetric flasks containing 20 ml of distilled water acidified with HCl and processed for K analysis and K^{42} estimation. The specific activities of the samples have beeu plotted as a function of time. The curves drawn are the theoretical curves obtained by integration of the data using a Philbrick analog computer.

RESULTS

Blood Plasma

Blood samples were taken in heparinized syringes from normal and adrenalectomized rats at the time of sacrifice and the plasma analyzed for K and K^{42}. The mean value obtained from 30 groups of normal animals is 4.07±0.07 mg K/l and for 38 groups of adrenalectomized rats 6.37±0.29 mg K/l. The adrenalectomized rats show an increase in blood plasma K over normal rats of 52% which is highly significant. Specific activity measurements (cpm/mg K) on these plasma samples yielded a value of 8.3×10^5 for normal rats and 10.6×10^5 for adrenalectomized rats (Table I).

Table I. Comparison of Specific Activity of K^{42} in Normal and Adrx. Rat Brain and Muscle. Data on 3 Experiments, Average of 30 Rats/Experiment

Samples	*Normal sp. activity* $\times 10^5$	*Adrx. sp. activity* $\times 10^5$	*% increase above normal*
Plasma	8.3	10.6	28
Brain total	6.8	8.8	29
Brain ultrafiltrate	6.5	8.5	31
Muscle total	8.6	10.9	27
Muscle ultrafiltrate	7.9	10.1	28

Table II presents the data from one of our experiments. The values for total brain K for normal and adrenalectomized rat brains are not significantly different from each other and are in agreement with previously reported values.[2,3,4] Ultrafiltration of homogenates of brain show that approximately 30% of the K is not filterable and there is no significant difference between normal and adrenalectomized rat groups. The K^{42} content of the brain of the adrenalectomized rat groups shows an increase over the normal animals but this increase is not reflected in the per cent of non-filterable K^{42}.

The K content of gastrocnemius muscles of adrenalectomized rats shows a slight, non-significant increase over the normal value. The lack of a larger difference was surprising because sodium determinations made on some of these muscle preparations showed a mean decrease in sodium content of 15%.

Ultrafiltration of muscle homogenates did not reveal any significant difference of amounts of filterable K in the two groups. The K^{42} content of muscle from adrenalectomized animals is elevated to a highly significant degree but no striking difference in the per cent non-filterable K^{42} is observable.

A comparison of the specific activities of the various samples is given in Table I. The specific activity (SA) of all samples obtained from adrenalectomized animals is approximately 30% greater than the values obtained for similar samples from normal rats. Within the normal group the plasma and muscle SA are almost identical. The brain SA is decreased by 18%. The adrenalectomized animals show similar results. The plasma and muscle SA are identical but the brain SA is approximately 17% lower.

The constancy of the difference between the SA values of brain, and of plasma and muscle suggested the desirability of investigating the dynamic relations of the potassium distribution as a function of time between these three tissues.

Potassium Uptake Studies

Figure 1 shows the changes in plasma and brain SA over 70 hours. Each point on the curve expresses the average value obtained from samples of no less than two normal fed young adult rats. The muscle values are identical with the plasma values indicating the rapid exchange of K between these two compartments. The brain, however, shows a slow uptake of

Table II. Comparison of K and K^{42} Values in Brain and Muscle of Normal and Adrx. Rats Fed Before and During Experiments (without Saline)

	Total K ng/gm wet wt.	*Ultrafiltered K per gm wet tissue*	*Total* K^{42} $C\times10^4$*/gm wet wt.*	*Ultrafiltered* K^{42} $C\times10^4$*/gm wet wt.*	*% K Bound*	*% K^{42} Bound*
Normal brain	3.99 ± 0.10 n = 4	2.81 ± 0.06 n = 4	281 ± 9.0 n = 4	190 ± 4.3 n = 4	29.6	32.3
Adrex. brain	3.89 ± 0.05 n = 5	2.72 ± 0.10 n = 5	326 ± 7.9 n = 5	220 ± 10.3 n = 5	30.0	32.5
	P = n.s.	P = n.s.	P = < 0.01	P = 0.02		
Normal muscle	4.12 ± 0.07 n = 4	3.31 ± 0.07 n = 4	350 ± 6.5 n = 4	260 ± 10.6 n = 4	19.7	25.7
Adrex. muscle	4.30 ± 0.05 n = 5	3.34 ± 0.07 n = 5	453 ± 17.3 n = 5	321 ± 14.5	22.3	29.2
P = n.s.		P = n.s.	P = < 0.001	P = < 0.02		

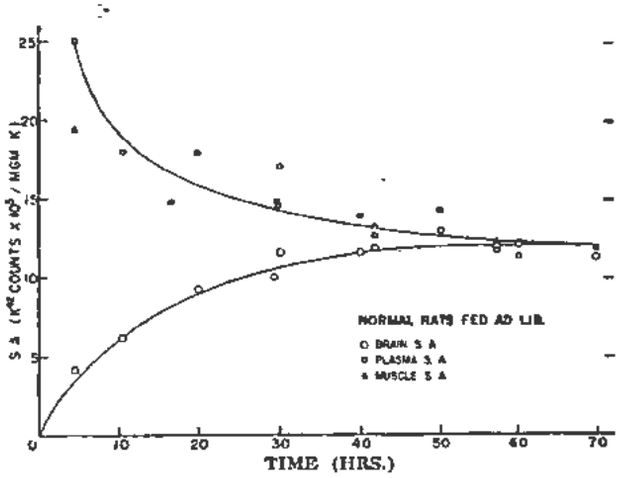

Figure 1. K^{42} uptake by brain in normal fed *ad lib.* young adult rats

K^{42} and the establishment of a steady state at approximately 55 hours. At this time the plasma and brain SA values become asymptotic to the same line indicating equilibration of the isotope with brain K.

A similar plot of data obtained from adrenalectomized rats fed *ad lib.* is shown in Figure 2. The plasma SA values are constant for 72 hours in contrast to the rapid fall noted for the normal fed rats. The brain SA rises more sharply and approaches the SA of the plasma. Mathematical analysis of the curve indicates that it is asymptotic to the plasma steady state level.

A comparison of the brain SA of the normal and adrenalectomized fed rats is made in Figure 3. The higher SA of the brains from adrenalectomized rats is related to the higher plasma SA. The lack of dilution of plasma SA as compared to the normal animals suggests a diminished intake of exogenous K.

This hypothesis was tested by determining the uptake curves for fasted normal and adrenalectomized rats. Figure 4 shows that under this condition the uptake curves are identical. Thus, if the same relative quantity of radioactive isotope is injected into normal and adrenalectomized rats and the possibility of dilution of the isotope with sources other than body K is removed the plasma SA value of the normal rat

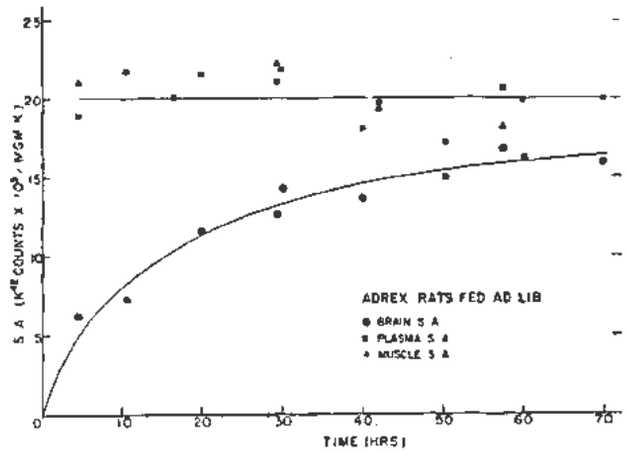

Figure 2. K^{42} uptake by brain in adrenalectomized fed *ad lib.* young adult rats

should equal the SA value of the adrenalectomized rat and remain constant with time.

The excretion of K by normal and adrenalectomized fasted rats is shown in Fig. 5. Each point on the curve represents the K value of the urine pool from a group of 3 rats in a single metabolism cage. In this study the adrenalectomized rats excreted approximately 25% less K than normal rats and this decrease in the ability to excrete K is probably related to the increase in the K level of the plasma noted in adrenalectomized rats.

Since mathematical treatment of the data indicated a two compartment K system, the possibility that the young adult rats (ca 2.5 mo) had not yet developed a third, slowly exchanging compartment was investigated.

An experiment was performed with 5 normal fed rats 5–6 mo of age and having an average weight of 320 grams. The brain and plasma SA were compared 65 hours post injection of the isotope, i.e., after the steady state has been established. The SA difference amounted to 10.6% and was statistically significant at less than the 0.01 level.

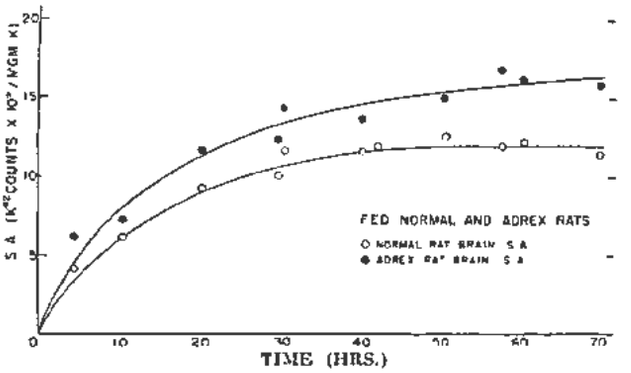

Figure 3. K^{42} uptake by brain in fed normal and adrenalectomized rats

Repetition of this experiment with older normal fed rats (8 mo of age, average weight 400 gm) confirmed this result. The SA difference between brain and plasma was 11.8% ($P = 0.001$).

The inability of the brain to equilibrate with the blood plasma in a time interval sufficient for complete mixing of brain K with plasma K indicates a slowly exchanging K compartment in these older animals.

DISCUSSION

A search for the basis of the apparent discrepancy between the content of non-radioactive K and the radioactive isotope of K in brains and muscles of normal and adrenalectomized rats has led to the variety of experiments described above.

Potassium has been reported to be bound[6,7] and Folch[8] claims that binding by complex lipids can amount to 27% of the potassium occurring in the brain. Using an ultrafiltration apparatus, Stone and Shapiro[5] found that approximately 30% of the brain and muscle potassium of normal rats is non-filterable. The differences in tissue content of K^{42} between

normal and adrenalectomized rats are of this same order of magnitude. If the 30% increase in brain K^{42} following adrenalectomy was due to an unbinding of the 30% non-filterable K, then the ultrafiltration technique would demonstrate this relationship. However, while our present ultrafiltration studies confirm the fact that 30% of the brain and muscle K of normal rats is non-filterable, they show that adrenalectomy does not alter the size of this non-filterable fraction (Table I). Therefore, the possibility was considered that the amount of bound, or non-filterable K remains the same in normal and adrenalectomized rat brains but that in the normal animal non-filterable K is also non-exchangeable whereas after adrenalectomy it becomes freely exchangeable with K^{42}. The results in Table I, however, show that in the normal and adrenalectomized rat brains, 32.3% and 32.5% respectively of the total K^{42} is bound. Thus, it follows that adrenalectomy does not alter the distribution of K^{42} between the non-filterable and filterable compartments but that in both normal and adrenalectomized rat brain K^{42} is homogeneously distributed.

Leiderman and Katzman[4] consider that the consider that the contradictory results between experiments measuring total potassium and K^{42} with normal and adrenalectomized rats are due to differences in brain potassium exchange rates produced by hormonal deficit. Using adult rats up to 18 mo of age, they determined from timed K^{42} uptake studies that at the steady state, the brain specific activity stabilized at a level 20% below the plasma specific activity. However, after adrenalectomy, the brain and plasma had identical SA values after the development of the steady state. Application of a general exchange equation developed by Solomon[9,10] enabled them to calculate the brain potassium influx and outflux values. For intact control rats the calculated potassium influx was 2.89 meq/kg/hr and that of the outflux 3.64 meq/kg/hr. The ratio of influx to outflux is 0.80 and since influx must equal outflux when the steady state obtains they considered that only 80% of the brain K was able to exchange with K^{42}.

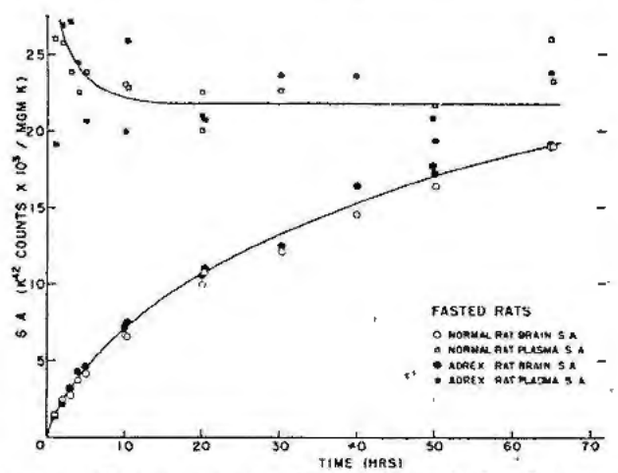

Figure 4. K^{42} uptake by brain in fasted normal and adrenalectomized rats

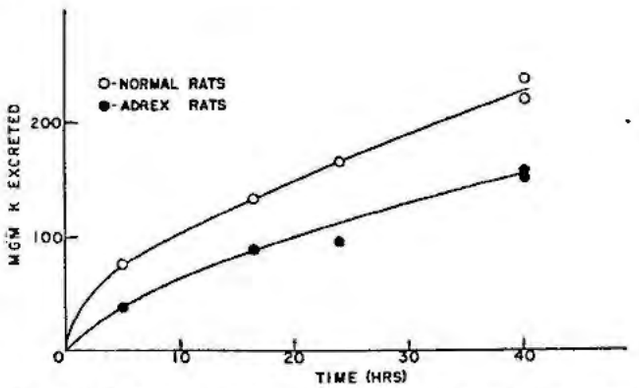

Figure 5. Total mg K excreted in urine over 40-hr period by normal and adrenalectomized rats

Adrenalectomy decreased the outflux rate so that the influx-outflux ratio became unity indicating 100% exchangeability of brain K.

The calculated K influx rate as determined by Solomon for the normal fed young adult rats in our uptake study is 2.90 meq/kg/hr and the outflux rate is the same. Thus the influx-outflux ratio of unity indicates complete brain K exchangeability as may be seen from Fig. 1.

These data indicate that in our experiments the increase in total brain K^{42} following adrenalectomy, is not related to a slowly exchangeable K compartment, but must be related to factors other than those suggested by Leiderman and Katzman. Our uptake studies with normal and adrenalectomized rats fed *ad lib.* show that the normal animal ingests food which contains potassium, resulting in a progressive fall of plasma SA. The adrenalectomized rat, on the other hand, eats little or nothing and consequently the ratio of K^{42} to K remains constant over the period of observation and no change in plasma SA is found. Hence with the progressive fall of the plasma SA in the normal fed animal the brain equilibrates with a lower K SA than does the adrenalectomized animal. The only role that adrenalectomy appears to play in relation to brain K uptake as described in these experiments is to produce a loss of appetite.

This consideration is substantiated by the results obtained in uptake studies using fasted normal and adrenalectomized rats (Fig. 3). Here, neither group has the opportunity to dilute body K and K^{42} with K gained from ingested food and the SA levels of plasma and brain are identical between the two groups throughout the experiment.

The high plasma K levels observed in the adrenalectomized rats, as compared with normal rats, appear to be related to the diminished urinary excretion of K following adrenalectomy and bear no relation to K content of the brain.

The data of Katzman and Leiderman[11] in conjunction with those reported here suggest that with increasing age a continually increasing amount of brain K becomes non-exchangeable or more slowly exchangeable. The nature of the non-ex-

changeable compartment and its possible geriatric significance are under consideration at the present juncture.

SUMMARY

1. Using ultrafiltration techniques we find that 30% of brain potassium of normal rats is non-filterable. This is not altered by the performance of adrenalectomy.

2. In normal and adrenalectomized rat brains K^{42} distributes homogeneously between the filterable and non-filterable potassium fractions.

3. The data from K^{42} uptake studies suggest that the increased amount of brain K^{42} in adrenalectomized, young adult rats as compared with normal rats is not due to an increased rate of exchange of a potassium compartment.

4. Our data indicate that the lower K^{42} content of normal rat brain as compared to adrenalectomized rat brain is due to the greater dilution of K^{42} by potassium ingested in the food. Adrenalectomized rats display anorexia.

5. In fasted normal and adrenalectomized rats there is no difference in the rate of K^{42} uptake by the brain.

6. The excretion of potassium by the adrenalectomized rats is 25 per cent less than that observed with normal animals. This appears to be related to the higher plasma potassium values observed after adrenalectomy.

7. Our results, together with those previously reported by others, indicate that potassium is freely exchangeable in young rats. They show, furthermore, that with age a slowly exchanging compartment develops.

ACKNOWLEDGEMENT

We wish to express our indebtedness to Dr. A. K. Solomon who with the use of a Philbrick analog computer analyzed and interpreted our data from the potassium uptake studies.

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Use of Radioactive Iodine (I^{131}) and Thyroxine to Determine the Thyroid Hormone Secretion Rate of Intact Animals

By E. P. Reineke and H. A. Henneman,* USA

The remarkable ability of the thyroid gland to collect iodine, together with the ease of measuring thyroidal radioiodine in living animals by external gamma counting, renders this gland peculiarly adaptable to research with isotopic tracer methods. Both the rate of fixation of radioiodine by the thyroid and its rate of release to the general circulation have been used widely as comparative measures of thyroid function.

By determining the effect of suitable doses of exogenous thyroxine upon thyroidal I^{131} output it is now possible to estimate indirectly the actual thyroid secretion rate. The administration of thyroxine will reduce both the thyroidal I^{131} uptake[1] and output.[2] Perry[3] reported that in groups of rats given a tracer dose of I^{131} followed by graded doses of thyroxine the inhibition of thyroidal I^{131} output in the respective groups was proportional to the thyroxine dosage. In an extension of these techniques developed in our laboratories, a quantitative estimate of the daily thyroid secretion rate of individual animals can now be made.

PRINCIPLE OF DETERMINING THYROID SECRETION RATE

The principle of the method is best shown by our recently published data on rats.[4] In each experiment rats of only one sex and of similar age and size were used. Each rat was injected intraperitoneally with 30–40 μc of carrier-free I^{131} as NaI. Forty-eight to 72 hours were allowed for I^{131} accumulation in the thyroid to pass its maximal level and enter the output stage. External thyroid counts were taken at this time and at suitable intervals thereafter by means of either a bismuth cathode gamma counting tube or a scintillation counter connected to a count rate meter. Prior to each count the rats were immobilized by sodium pentobarbital anesthesia, supplemented with ether when necessary. The thyroid counts were corrected by one-half the body background plus the general background as well as for physical decay.[2]

Rats receiving thiouracil to block thyroid hormone formation had a greatly accelerated thyroidal I^{131} output (Fig. 1). The output half-time for these rats was 1.5 days, compared to 4 days for the normal control group. Rats receiving 2 μg of L-thyroxine daily showed a slight accumulation of I^{131} during the first three days. At lower dose levels the slope of the output curves was reduced in proportion to the thyroxine dosage. This effect was evident within 24 hours and was maintained consistently during the four days of the experiment.

When the individual thyroid counts from this experiment were expressed as per cent of the initial count and plotted against the thyroxine dose (Fig. 2) a highly significant correlation (r=0.979) between per cent of initial count and thyroxine dose was found. The thyroxine secretion rate was estimated by computing the thyroxine dosage required to maintain the thyroid count at 100 per cent of the initial level. The data, when fitted by the least squares method, yielded the regression equation: $y = 43.81 + 25.96x$. Substituting in the equation y for per cent initial count and x for thyroxine dosage, the average thyroid secretion rate for male rats in this experiment was estimated as 2.15 μg L-thyroxine per 100 gm body weight.

The rapid and consistent reduction of thyroidal I^{131} output in proportion to graded doses of thyroxine given to different groups of rats raises the question whether the thyroid mechanism will respond in stepwise and quantitative fashion when the thyroxine dosage is increased progressively in individual animals. The results of an experiment designed to test this question are shown in Fig. 3. Seventy-two hours after injecting 40 μc of I^{131} per rat in a group of 4 females weighing an average of 240 gm an initial thyroid count was taken. Each rat was then injected with 1 μg of L-thyroxine for 2 consecutive days, and a second thyroid count was taken on the third day. Thyroxine dosage was increased progressively at 2-day intervals, a thyroid count being taken before each increase in dosage.

The retention of I^{131} by the thyroids increased regularly with increasing increments of thyroxine to the point where output was almost completely inhibited, and then leveled off. When the successive counts on the ascending portion of the curve were expressed as per cent of the preceding count and equated against thyroxine dosage a highly significant correlation ($r = 0.843$) was found between thyroxine dose and per cent of preceding count. From the regression equation: $y = 78.364 + 8.435x$ the

* Michigan State College. Including work by S. A. Griffin and O. N. Singh, Michigan State College.

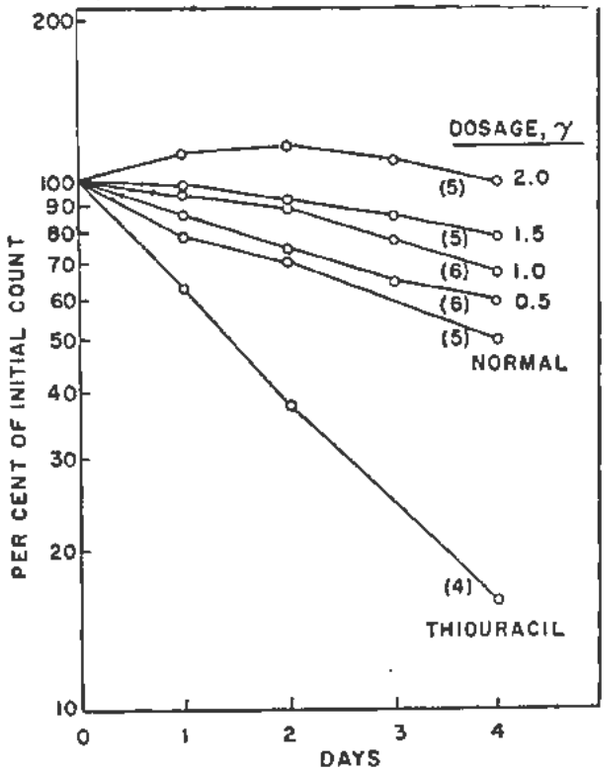

Figure 1. Effect of varying dosage levels of thyroxine given continuously to groups of rats on the thyroidal I^{131} output curve. Number of rats averaged per group is shown in parentheses. Taken from the report of Reineke and Singh[4]

thyroid secretion rate was computed as 2.56 μg/100 gm body weight. Highly significant correlation coefficients of 0.970, 0.937, 0.951 and 0.919 were obtained when the data for the 4 rats were treated separately.

Estimated thyroid secretion rates for the individual rats were 2.51, 2.41, 2.29 and 3.05 μg/100 gm body weight.

The principles demonstrated in these experiments in rats have been applied to the measurement of thyroid secretion rate in sheep, with some modifications in procedure. The output half-time for thyroidal I^{131} of sheep under our conditions is approximately 6–15 days. The counting and thyroxine dosage procedures have been started 7–10 days after injecting a tracer dose of I^{131} to permit the I^{131} turnover curve to reach the output side. Also, because of the slower output rate compared to rats a given dosage of thyroxine has been continued longer (3 to 4 days) to permit a greater net change in thyroid count between changes in thyroxine dosage. When using an end-window GM tube for external thyroid counting 1.0 μc of I^{131} has been given per pound of body weight. For scintillation counting 0.1 μc per pound gives a satisfactory counting rate. Counts are taken by placing the counting tube firmly against the neck in the thyroid region and shifting it about slightly until a maximal counting rate is attained. In other respects the procedure is comparable to that described for rats.

RELATION OF THYROID SECRETION IN SHEEP TO SEX, AGE, LACTATION AND SEASON

Under our climatic conditions the domestic sheep shows the usual seasonal rhythm in metabolic and reproductive functions. For this reason it was of interest to determine whether significant seasonal variations in thyroid function occur in this species. In a previous report[5] we published values obtained for ewes of the Shropshire and Hampshire breeds taken over a twelve-month period. Both breeds showed a pronounced decline in thyroid secretion during the summer as compared to the winter months. A decline of thyroid function with age is indicated by the fact that 2-year-old Shropshire ewes had a consistently higher thyroid secretion rate than 4-year-olds during all months for which determinations were made. It is of interest that the thyroid secretion rate of pregnant ewes did not differ from the values for non-pregnant ewes taken at the same age and season. However, the thyroid secretion of lactating ewes was significantly higher ($P = 0.05 - 0.01$) than in either pregnant or non-pregnant ewes.

In Fig. 4 we present for the first time a comparison between the thyroid secretion rate of 2-year-old male and female sheep of the Shropshire breed at various periods of the year. Each bar in the lower one-half of the graph represents the average thyroid

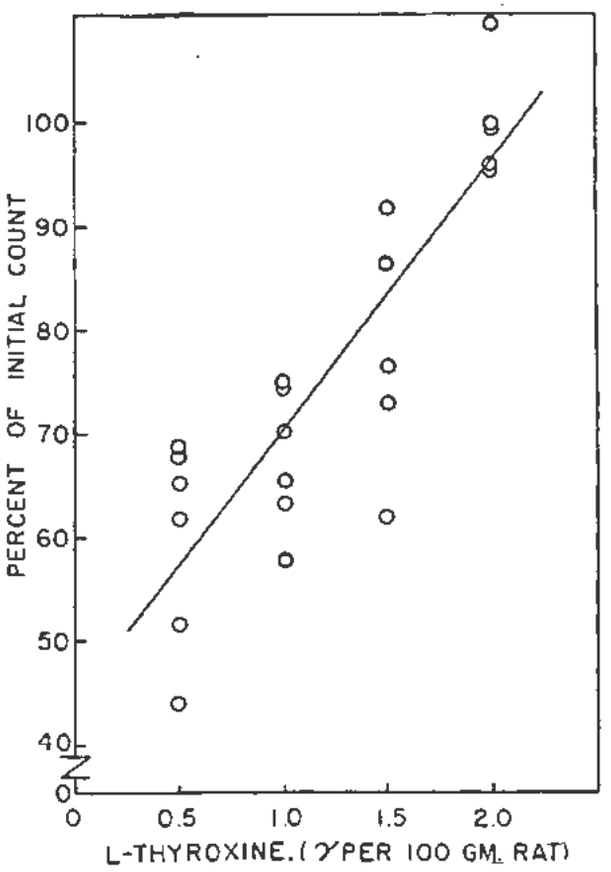

Figure 2. Effect of progressively increasing thyroxine dosage between groups on I^{131} retention in their thyroids

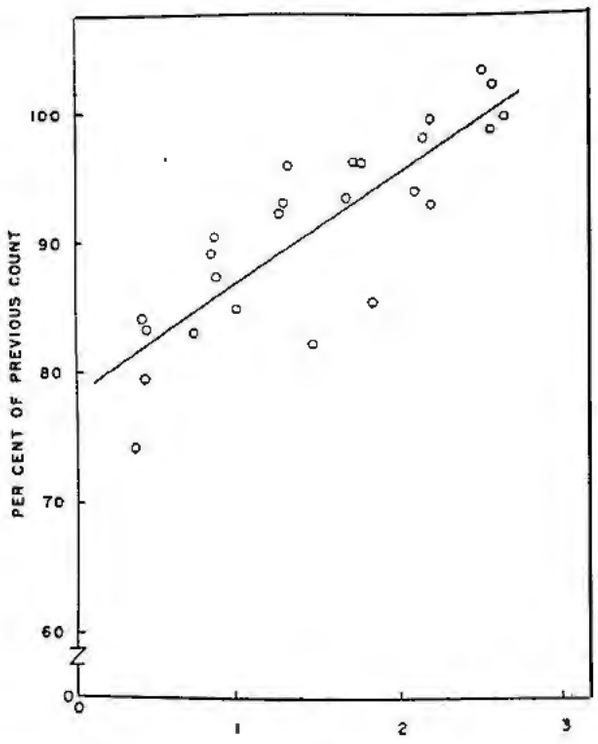

Figure 3. Effect of progressively increasing doses of thyroxine in individual rats on retention of I^{131} in their thyroids. Taken from the report of Reineke and Singh[4]

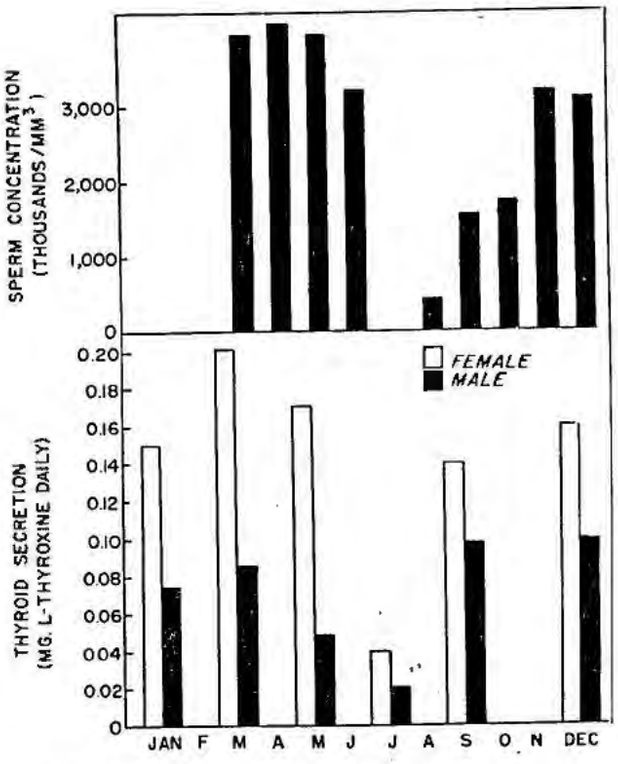

Figure 4. Comparison of seasonal changes in thyroid secretion of male and female sheep and sperm concentration of the males

secretion of 4 or 5 animals except in January and March when values were obtained for only 2 and 3 females, respectively, and in May when values were obtained on only 2 males. We are indebted to S. A. Griffin for permission to present the data on males. Seasonal trends in sperm concentration of semen collected from the males are shown in the upper one-half of the figure.

As in the data on rats presented earlier, the thyroid secretion rate of female sheep is higher than in males. In both sexes there is a pronounced seasonal variation in thyroid secretion. The decline in females from the winter levels of 0.16–0.20 mg thyroxine daily during December to May down to the minimum of 0.04 mg in August represents a 4–5 fold decrease. In males the December value (0.104 mg) is approximately 5 times the August minimum (0.021 mg)

A similar decline during the summer of the sperm concentration of semen collected from the males was observed. It is of particular interest that the summer decline in thyroid secretion rate precedes the decline in sperm concentration, and the rise in thyroid secretion in autumn also precedes the autumn rise in sperm concentration. Values on percentage motility of the sperm showed very similar trends. We also have unpublished data on thyroid secretion rate and semen quality of rams of the Hampshire breed that parallel very closely the values which are reported here.

RELATION BETWEEN THYROID SECRETION AND GROWTH RATE OF LAMBS

Although thyroid hormone is known to be essential for normal growth, data have not heretofore been available on possible relationships between the thyroid secretion rate and rate of growth in normal animals.

Data on some recent experiments in this regard have been provided to us by O. N. Singh. In one experiment the body weight gains over a 30-day period of 12 Shropshire ewe lambs approximately 120 days old that were dry-fed in a barn under uniform conditions were determined. During the same period the thyroid secretion rate was determined by a procedure similar to that already described for sheep. In Fig. 5 the pounds of gain of each lamb are plotted against its thyroid secretion rate. The equation to the line as determined by the method of least squares is: $y = -0.59 + 97.25x$, where y equals pounds gain and x equals mg L-thyroxine secreted daily. A correlation coefficient of 0.792 was obtained between the thyroxine secretion rate and growth rate of ewe lambs. This is statistically significant at the one per cent level of probability.

DISCUSSION

The method presented here makes possible the quantitative estimation of thyroid secretion rate of living intact animals. Thyroid secretion values, expressed as μg L-thyroxine daily per 100 gm body weight have ranged from 2.21 to 2.56 for female and 2.15 for adult male rats. The most extensive

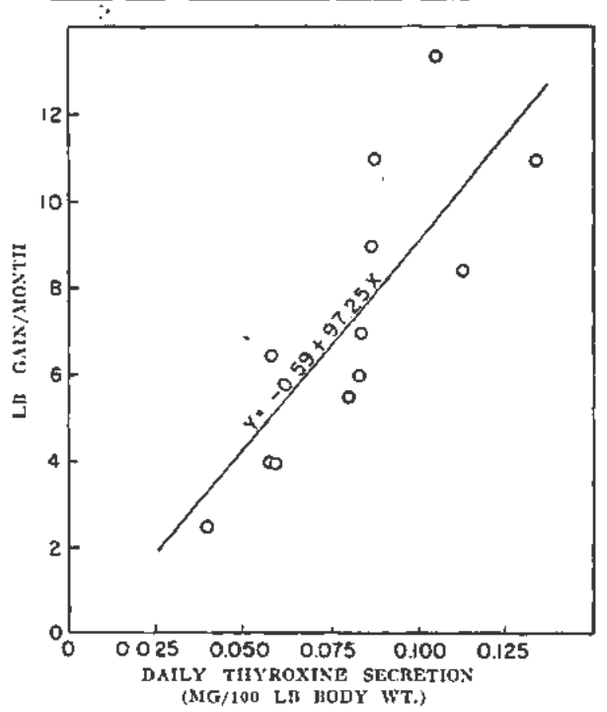

Figure 5. Relation between daily thyroxine secretion of ewe lambs and their gain in body weight during a 30-day period

determinations of thyroid secretion rate by the goitrogen technique[6] have yielded values of 2.8–3.5 μg D,L-thyroxine per 100 gm body weight daily in adult rats. If it is accepted that the L-isomer has twice the potency of the racemic mixture[7], this is the equivalent of 1.4–1.75 μg of L-thyroxine. Thus the values obtained by the tracer method appear to be somewhat higher than by the goitrogen technique, but they are quite repeatable. No independent observations are available with which to compare the determinations on sheep.

The inhibition of thyroidal I^{131} output by administered thyroxine upon which the method is based is explained by its suppression of thyrotrophic hormone output by the anterior lobe of the pituitary. Thyroxine administration at high levels suppresses I^{131} output by the thyroid to about the same extent as hypophysectomy.[2] However, there seems to be a small intrinsic turnover of iodine by the thyroid that is not under pituitary control. A small iodine turnover occurs even in animals receiving thyroxine equivalent to their daily secretion rate.[3] In normal rats, I^{131} output is approximately balanced by uptake so there is no appreciable change in thyroid radioactivity when a critical thyroxine dosage is given, despite a small amount of recycling of I^{131}.

The seasonal decline in thyroid secretion observed in sheep during the summer is in harmony with earlier observations in chickens,[8] and also with the report that high temperatures depress thyroid function in rats.[9] The increase in thyroid secretion during lactation is of particular interest in view of the fact that thyroidectomy depresses and thyroprotein increases milk secretion.[10]

Numerous investigations indicate that the thyroid hormone is implicated in some manner in the reproductive processes of both the female and the male.[11,12] It is of special interest that the thyroid secretion rate declines shortly before the onset of the anestrous period in ewes and summer sterility in rams.

There is also much evidence to indicate that growth of young animals is affected adversely by hypothyroidism and stimulated slightly in some species by properly regulated thyroid therapy.[10] The significant positive correlation between thyroid secretion level and growth rate of lambs disclosed in the present report provides direct evidence that differences in growth rate of normal animals may be due, at least in part, to variations in thyroid function.

Research reported thus far gives a small indication of some of the results to be obtained in the field of animal physiology by use of radioisotopes. The application of isotopic methods to problems of animal production appears to hold unlimited possibilities for progress in the future.

SUMMARY

A procedure is described for the determination of the thyroid secretion rate in individual animals. The method is based on the stepwise inhibition of thyroidal radioiodine output by progressively increasing doses of thyroxine.

As determined by this method in both rats and sheep, females have a consistently higher thyroid secretion rate than males. Thyroid secretion is increased significantly during lactation. It is significantly higher in 2-year-old than in 4-year-old ewes.

The thyroid secretion rate of both rams and ewes declines 4–5 fold from the winter to the summer months. The minimum values are found during the non-breeding season.

In growing ewe lambs a significant positive correlation was found between thyroid secretion level and growth rate.

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Kinetics of the Distribution of Radiosodium in Rabbits in Hypothermia

By F. Morel and A. Combrisson,* France

Experimental hypothermia, with its profound alterations of circulatory hemodynamics and its considerable reduction in oxidative processes, represents a peculiar physiological condition which should make it possible to determine experimentally the physical-chemical mechanisms which control the exchanges taking place in the walls of the blood capillaries, especially the exchanges of electrolytes. It is known, in fact, that three types of mechanisms can insure passage of one sodium ion through a living permeable membrane: simple diffusion, convection (if there is ultrafiltration through this membrane), and finally active transfer.

EXPERIMENTAL TECHNIQUE

The kinetics of distribution of radiosodium in the intravascular compartment, and between the intravascular and interstitial compartments have been observed following intravenous injection of a small volume of isotonic $Na^{24}Cl$ to the plasma of thirty rabbits, either at a normal temperature (9 animals) or in various stages of hypothermia (21 animals). Hypothermia was induced by placing the animals for one to two hours at a temperature of 5°C in a hermetically sealed twelve-liter container, according to the technique described by Giaja and Andjus[1] for the rat. The experiment was carried out at once under nembutal anesthesia. In addition to rectal temperature, cardiac rhythm and hematocrit readings were noted, the pressure in the femoral artery was recorded, and in some cases, the plasma concentration of sodium was measured with a flame spectrophotometer, and the volume of the circulating mass of blood was determined by means of P^{32} tagged red cells. The curve showing the decrease in plasma radiosodium was recorded directly, by a previously described method.[2]

RESULTS

Circulatory Hemodynamics

The cardiac rhythm goes down progressively with the temperature, from 240 per minute at 37°C to 70 per minute at 20°C; at the same time, the circulation time from the ear to the carotid increases, going up from 6 seconds at 37°C to 20 seconds at 20°C. On the contrary, the femoral pressure remains high until comparatively low temperatures are reached (5 to 8 cm of mercury at 20°C). Finally the hematocrit readings reveal a slight hemoconcentration during hypothermia; measured by the tagged red cell technique, the circulating blood mass is reduced by some 15 to 20%.

Sodium Interchange in the Capillaries

The reduction in plasma concentration, which expresses a distribution of the injected radiosodium between circulating plasma and interstitial liquids can be described, in the rabbit, by a single exponential[3]

$$C = (C_0 - C_{equ})\, e^{-xt} + C_{equ}$$

in which C is the concentration of radiosodium in the plasma at any given time (in per thousand of the dose injected per kg found in each ml), C_{equ} represents the equilibrium concentration toward which the exponential decrease tends; this figure makes it possible to measure the volume of the extracellular compartment in which the radiosodium diffuses, and x represents the exponential decrease rate per minute.

At a normal temperature (9 rabbits) we find:

$$C = 8.9e^{-0.836t} + 5.14$$
$$x = -0.836\ (\pm \sigma = 0.14)$$
$$C_{equ} = 5.14\ (\pm \sigma = 0.35)$$

For a body temperature between 25 and 28°C (7 rabbits) we find:

$$C = 14.9e^{-0.74t} + 7.54$$

For a body temperature between 21 and 24.5°C (9 rabbits) we find:

$$C = 15.3e^{-0.64t} + 9.12$$

For a body temperature between 16 and 20°C (5 rabbits) we find:

$$C = 12.7e^{-0.29t} + 7.77$$

At a normal temperature, the average value of C_{equ} corresponds to the distribution volume, for radiosodium, equal to 19.5% of the weight, i.e., very close to the volume of extracellular liquids.

In all hypothermic rabbits, the value of C_{equ} decreases markedly; therefore, in all of them, the

Original language: French.
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volume of distribution on equilibrium is reduced, falling, on an average, from 19.5 to 12% of the weight. On the other hand, the exponential decrease rate is not modified, in any rabbit, between 25 and 28°C. It is reduced significantly in two rabbits at 21°C, and in all rabbits between 16 and 20°C. During hypothermia, the plasma concentration of the total sodium is not modified.

DISCUSSION

The distribution volume of radiosodium at equilibrium appears to be reduced in a very consistent and considerable measure during hypothermia, which indicates an average decrease of 38% in the accessible extracellular liquids of all of the hypothermic rabbits. Rodbard and his co-workers[4] have already drawn attention to a 30% decrease in the distribution volume of thiocyanate in hypothermic rabbits. It is difficult to judge whether this decrease is due to the fact that some circulatory territories and the corresponding interstitial liquids are shut out of the circuit (as suggested by Rodbard[4]) or to the passage, during the hypothermia of a part of the extracellular liquids into the intracellular compartment.

The joint analysis of the other results indicates the existence of characteristic modifications of the circulatory hemodynamics during hypothermia: the circulating blood mass and the arterial blood pressure are but slightly reduced, while the cardiac rhythm and the speed of circulation suffer a very substantial modification. Such a picture suggests the existence of an increase in the resistance of the peripheral vessels rather than the fact that important vascular territories are placed out of the circuit. In fact, this second mechanism would call for a more marked reduction of the volume of the circulating blood and would not cause so marked a slowing down of the circulatory flow as that which we have observed.

The capillary sodium exchanges remain unmodified until a body temperature of about 20°C is reached. Below 20°C, their apparent intensity decreases markedly; but, at such temperatures, the arterial pressure has collapsed, and there is a grave circulatory failure. It is possible that the exponential decrease rate then observed is no longer determined by the rate of sodium exchange through the capillary walls, but rather that the peripheral circulatory output itself acts as a limiting factor.

Finally, the coexistence (above 20°C) of deep circulatory modifications and of a normal rate of capillary exchange of the sodium indicates that the passage of sodium ions throngh the capillary walls takes place principally by diffusion and not by ultrafiltration or active transport.

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The Mechanism of Gastric Acid Secretion as Revealed by Radioisotopes

By C. Adrian and M. Hogben,* USA

In elucidating the manner in which living cells regulate the movement of ions, isotopes yield unique information. Isotopes permit precise analysis of the two opposing unidirectional rates of ionic movement without demanding provisional assumptions concerning the nature of the intervening membrane. The unidirectional rates of movement are called fluxes. A systematic description of ionic movement through living membranes can be developed from analysis of ionic flux.[1] Passive diffusion of an ion can be unambiguously defined by the ratio of its two fluxes, the ratio of its chemical activity on either side of a membrane and the electrical potential difference across the membrane.[2] Passive ionic flux can be expressed as partial ionic conductance. The sum of ionic conductances can be related to the total membrane conductance. Active transport, defined as the uphill movement of an ion through a membrane, gives rise to an electrical current. The algebraic sum of all active ionic transport between two identical solutions is equal to the electrical current drawn from a short-circuited membrane. In addition to the movement of ions by passive diffusion or active transport there may be isotopic exchange independent of net ionic movement.

The application of these principles to ionic movement through the isolated frog gastric mucosa provides a basis for investigating the mechanism of hydrochloric acid secretion. The frog gastric epithelium can be easily separated from the muscular coat as a thin sheet about 1-mm thick. The epithelium, one cell layer thick, is involuted to form the gastric glands. It consists of two cell types, a surface epithelial or mucous cell and the tubular secretory surface cell which is thought to be analogous to the mammalian parietal cell. In addition to the epithelium proper there is the thin band of the muscularis mucosae and some loose submucosal connective tissue. When the mucosa is bathed by a saline solution containing glucose and saturated with 100% oxygen, it spontaneously secretes acid and develops a distinctive electrical potential. The mucosal potential is about 35 mv with the serosal surface positive to the lumen surface.

Ionic movement through the mucosa can be studied by placing the epithelium between two chambers so that each surface is exposed to a stirred solution of known composition. A radioisotope of an ion placed in one chamber, after a short delay, will appear in the other chamber at a constant rate. The unidirectional flux is the product of this rate of isotope appearance and the ratio of carrier ion and isotope in the first chamber. The difference between the two unidirectional fluxes of an ion is equal to the net ionic movement. If the volumes of the solutions are sufficiently large there will be essentially steady state concentration gradients of both carrier and isotope for the duration of the experiment. The two directions of flux through the gastric mucosa will be spoken of as: (*a*) nutrient to secretory (N-S) for the direction from blood to lumen and (*b*) secretory to nutrient (S-N) for the opposite flow from lumen to blood.

In order to study quantitatively ionic movement it is desirable not only to know and control the ionic concentration of the two solutions bathing the membrane but also to measure the electrical potential difference between the solutions. The electrical potential can be measured by placing a saline-agar bridge very close to either side of the mucosa and connecting the bridges to a pair of calomel cell electrodes and a potentiometer. The potential can be varied by means of another pair of bridges placed at a distance from the mucosa. The distant bridges supply an external current from a battery in series with a variable resistance and galvanometer. If sufficient current is supplied, the spontaneous potential of the gastric mucosa can be reduced to zero. The mucosa is then effectively short-circuited and the current required to maintain a zero potential difference is equal to the electrical current that can be continuously drawn from the mucosa. The ratio of external current and mucosal potential is a measure of the membrane conductance.

Using radioisotopes of sodium and potassium, it has been shown that the fluxes of both cations are equal when the two surfaces of the mucosa are bathed by similar salt solutions (frog's Ringer) and the electrical potential difference is brought to zero.[3] When there is an electrical potential difference with the nutrient solution positive to the secretory solution, the nutrient to secretory fluxes of cations are accelerated and the secretory to nutrient fluxes decreased. The response of these cation fluxes to an electrical potential difference is in the direction

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expected for ions moving passively. The movement of sodium would account for about 25% of the mucosal conductance and that of potassium 5%.

By measuring the chloride fluxes with radiochloride it is possible to demonstrate that chloride is being actively transported by the gastric mucosa. When both surfaces were bathed by a bicarbonate-saline solution containing 92 mM of chloride per liter the nutrient to secretory flux was 9.5 microequivalents per cm^2 per hour and the secretory to nutrient flux was 4.0 microequivalents. Using the same solutions and augmenting the spontaneous potential to 60 mv, serosa positive to mucosa, the fluxes were: nutrient to secretory 8.0, and secretory to nutrient 6.2. At zero electrical potential difference, in the absence of an electrochemical potential gradient, the gastric mucosa performs work to bring about a net flow of chloride in spite of the resistance offered by the membrane. Even at 60 mv there is a net transfer of chloride in spite of an electrical gradient adverse to the movement of chloride. If the chloride ion were moving simply by passive diffusion the ratio of the fluxes at 60 mv N-S/S-N, would be 0.1 rather than the observed ratio of 1.3.

The magnitude of the secretory to nutrient flux, 4.0 microequivalents, is unexpectedly large in comparison to the membrane conductance. If this chloride flux were solely due to passive diffusion it would be equivalent to a partial ionic conductance of 0.004 reciprocal ohms per cm^2. In this series of experiments the mean membrane conductance was 0.0025 reciprocal ohms. As the partial ionic conductance cannot exceed the total membrane conductance, the chloride movement from the secretory to nutrient surface cannot be one of simple diffusion. This feature of "isotopic exchange" of chloride by the gastric mucosa suggests that chloride may be moving in chemical combination. The isotopic exchange character of the secretory to nutrient flux prevents direct calculation of the partial ionic conductance of chloride.

The net transport of chloride by the mucosa represents a substantial current of negative ions. Because the isolated mucosa is spontaneously secreting acid there is also an active transport of positive hydrogen ions in the same direction. In the following experiments, using a bicarbonate-free saline containing 114 mM/l of chloride, the chloride fluxes, the rate of hydrogen ion secretion and the electrical short-circuit current were measured simultaneously. The chloride fluxes were: nutrient to secretory 10.6 and secretory to nutrient 6.4, yielding a net chloride flux of 4.3 microequivalents per cm^2 per hour. The rate of hydrogen ion secretion was 1.2 microequivalents. The difference is a net charge transfer of 3.1 microequivalents. The electrical short-circuit current expressed in the same units was 3.1 microequivalents per cm^2 per hour. Therefore the gastric mucosal short-circuit current is quantitatively identified as the active transport of chloride ions in excess of the active transport of hydrogen ion. When the mucosa is not experimentally short-circuited this electrical current is dissipated through the internal passive ionic shunts of the mucosa to become manifest as the distinctive gastric mucosal potential of about 35 mv, the serosal surface positive to mucosal surface.

In the intact mammal, the gastric mucosal potential is about 40 mv, serosa positive to mucosa, during acid secretion.[4] The mucosal potential and conductance are relatively independent of the rate of hydrogen ion secretion. As this potential is unfavorable to the diffusion of chloride, active transport of chloride must be an integral part of the formation of gastric hydrochloric acid. The maintenance of a constant potential independent of the rate of secretion indicates that active transport of chloride and hydrogen ions are closely linked so that there is a constant excess of chloride transported to generate the mucosal potential.

In collaboration with Dr. E. Cotlove, the overall flux of chloride from one side of the mucosa to the other has been examined in terms of movement into and out of the epithelial cell layer across its two surfaces. The gastric mucosa has two extracellular spaces, one accessible from the nutrient surface and one accessible from the secretory surface. The volume of these spaces was determined with radioinsulin. The mean extracellular space accessible from the nutrient surface was 54.1% of the wet weight, and the space accessible from the secretory surface 2.7%. The mean dry weight was 15.3% of the wet weight. These values can then be used to obtain the intracellular concentrations of a given ion and its radioisotope. It is assumed that the isotope content of the extracellular spaces is very close to that of the bulk solutions with which they communicate. The difference between the isotope content of the whole mucosa and that calculated for the extracellular spaces is the estimated intracellular isotope content. When an ion isotope has been placed in one of the bulk solutions, the flux out of the cell surface adjacent to the second solution is the product of the rate of isotope appearance in the second solution and the ratio of intracellular carrier ion and intracellular isotope concentrations. The flux into the cell across this same cell face is the algebraic sum of the flux out of the cell and the net difference between the overall fluxes across the entire mucosa.

Table I presents a series of measurements on the movement of chloride and thiocyanate through the short-circuited isolated gastric mucosa. The determinations for chloride and thiocyanate were obtained from two distinct sets of experiments. For each ion the overall fluxes and the relative cell radioactivities were obtained from paired mucosae. The mean overall flux, from one bathing solution to the other bathing solution, and the relative cell radioactivity for the direction N-S were obtained by placing the isotope in the nutrient solution. The values for S-N were obtained when the isotope was introduced into the secretory solution. The relative cell radioactivity

is the intracellular isotope concentration expressed as a fraction of the isotope concentration of the bathing solution into which the isotope was initially introduced.

Table I

Ion	Concentration mM/l		Direction of flux	Over-all flux microeq., $cm^{-2}hr^{-1}$	Relative cell radioactivity
	Solution	Cell			
Cl	116	67	N–S	8.0	0.1
			S–N	5.2	0.9
SCN	15	9	N–S	0.6	0.4
			S–N	0.3	0.6

It is apparent that the movement of chloride across the secretory surface of the cells is much greater than the movement across the nutrient surface. The findings for thiocyanate are in the same direction but the asymmetry is less marked. While these findings do not establish that active transport of chloride occurs preponderantly at the secretory surface of the cells they suggest that this may be the case. The previously reported observation of an apparent slow exchange of gastric mucosal chloride *in vivo* with radiochloride administered intraperitoneally,[5] may be an artifact due to the rapid exchange between cell chloride and unlabelled chloride in the gastric lumen and the slower exchange between capillary and cell across the nutrient surface.

In order to understand the mechanism of gastric acid secretion it would be desirable to know whether the active transport of chloride is accomplished by either the tubular secretory cells or the surface mucous epithelial cells or by both cell types. Our present evidence does not localize active chloride transport to a particular cell of the mucosa. It has been suggested by one school of investigators[6] that chloride may be secreted by the parietal cell and the hydrogen ions by the surface epithelial cells.

The active transport of chloride demands that energy released by cellular metabolism be transferred to the transported chloride ion. Because of the striking hydrogen ion concentration attained during acid secretion attention has been generally directed to the hydrogen ion secretion at the expense of the concomitant chloride secretion. Recent studies of metabolism during acid secretion[7,8,9] indicate that as much as 20% of the energy of oxidative metabolism is consumed in establishing the hydrogen ion gradient. Many of the metabolic inhibitors which block acid secretion also abolish the spontaneous mucosal potential[10] suggesting that interruption of the source of metabolic energy interrupts active chloride transport.

A hypothesis which has been frequently advanced to explain the transport of hydrogen ions is that they are formed on the secretory border of the cell by an oxidation-reduction reaction.[6,7,11] The experimental evidence which supports this hypothesis is the response of the rate of hydrogen ion secretion to variation of the electrical potential difference across the mucosa.[12] Augmenting the spontaneous potential increases the rate of hydrogen ion secretion and depressing the potential inhibits the rate of secretion. Before assuming that the effect of potential is simply one of driving an oxidation-reduction reaction it will be necessary to know considerably more about the effect of the potential on the ionic metabolism of the gastric mucosa. In addition to altering the rate of hydrogen ion secretion, varying the mucosal potential also changes the intracellular concentration of chloride. In a series of experiments, isolated gastric mucosae were bathed by solutions containing 116 mM/l of chloride. Those mucosae whose potential was maintained at zero had a calculated intracellular chloride concentration of 67 mM/l. The intracellular concentration rose to 86 mM/l when the spontaneous potential was augmented to 60 mv, serosa positive to mucosa.

The fact that the active transport of chloride can be identified with the generation of a continuous electrical current prompts consideration of a mechanism which could generate an electrical current. One possible mechanism is to transport the ion by a carrier which exchanges this ion for an ion of another species which it transfers in the opposite direction. The forced exchange of the ion pair would establish concentration gradients for each of the ions, but it would not lead to a net transfer of charge. If the membrane were in addition selectively permeable to one of the ion species, that ion could diffuse backward along its concentration gradient with the result that there would be no net transfer of this member of the ion pair. The transport of the non-diffusing ion would then represent a net transfer of charge and be identifiable with an electrical current.

If the latter type of forced ion exchange were to be the explanation for the active transport of chloride through the gastric mucosa, the most probable partner ion to exchange with chloride would be bicarbonate. The initial evidence for such a mechanism was favorable.[13] Exposing the secretory surface of the isolated gastric mucosa to a saline solution containing bicarbonate when the nutrient surface is bathed by a saline solution containing no bicarbonate reversibly depresses the mucosal potential and short-circuit current. The flux of C^{14} labelled bicarbonate between two solutions containing bicarbonate and buffered by carbon dioxide is greater in the secretory to nutrient direction through the short-circuited mucosa. The net transfer of bicarbonate-carbon dioxide is in the opposite direction to the transport of chloride.

The interpretation of the flux of a weak electrolyte such as carbonic acid is more complex than that of a strong ion. The weak electrolyte may cross the membrane either as the ionized or un-ionized moiety. In general cellular membranes are considerably more permeable to lipoid soluble uncharged moieties in contrast to the charged water soluble moiety. If there is a pH gradient across the cell membrane, the concentration of the un-ionized moiety on either side of the membrane will be different when the total con-

centration of weak electrolyte is the same. The flux of weak electrolyte from the solution containing the larger concentration of un-ionized electrolyte will be greater permeability to the un-ionized form.

In the course of studying the metabolism of the secreting isolated mucosa, it has been noted that endogenous lactate selectively accumulates in the nutrient solution which has the higher pH.[9] By tracing the movement of lactate from each of the two solutions with radiolactate it is possible to analyze the factors determining the movement of this weak electrolyte in greater detail. In the following experiments the short-circuited mucosa was bathed by solutions containing 10 mM/l of lactate. In one set of experiments, the pH of the secretory solution was titrated to an initial pH of 3.0 before being placed in contact with the mucosa.

In the second set of experiments both the nutrient and secretory solutions were buffered with an additional 20 mM/l of glycylglycine and the initial pH of the nutrient solution adjusted to 6.4 and that of the secretory solution to 7.8. The pH of the solutions given in Table II are the mean pH's at the end of the 6-hour experiments.

Table II. Lactate Flux in Microequivalents-cm^{-2}hr^{-1}

Flux			*Solution pH*	
N-S	*S-N*	*Ratio*	*Nutrient*	*Secretory*
0.018	0.39	22	7.3	2.9
0.020	0.38	18	7.1	7.2

The exogenous lactate fluxes between solutions of pH 7.3 and 2.9 indicate that the selective accumulation of endogenous lactate in the nutrient solution is not the result of a greater permeability of the nutrient surface of the cells to lactate. The persistence of the markedly dissimilar flux rates of lactate when the spontaneous pH gradient is reversed to 7.1/7.2 indicates that the weak electrolyte lactic acid is being subjected to some form of active transport.

It is possible that there exists within the gastric mucosa specific transport mechanisms for lactic and carbonic acid. Nevertheless the secretion of a variety of drugs by the dog stomach is related to the acidic or basic nature of the drug and the dissociation constant.[14] This suggests that the mechanism for transporting weak electrolytes through the gastric mucosa is non-specific and dependent on acid secretion. The gastric mucosa would transport weak electrolytes independent of the pH of the bathing solutions if there existed within the mucosa "transport micelles" with the following characteristics. The hydrogen ion concentration of the micelles should be maintained at a high level. One surface of the micelle should be selectively permeable to the un-ionized moiety of weak electrolytes and the opposite surface be equally permeable to both ionized and un-ionized weak electrolyte. Because a gradient of weak electrolyte will tend to develop across the selectively permeable surface of the micelle, weak electrolytes would be continuously pumped uphill. The energy required to move the weak electrolyte against an overall gradient between the two bulk solutions would derive from the hydrogen ion gradient. Tentatively we can identify the "transport micelle" with the gastric glandular crypts. The secretion of hydrogen ion would maintain a distinctive pH in spite of the diffusion of buffer. The cells lining the crypt would be expected to be preferentially permeable to un-ionized weak electrolyte and the ostia of the tubule not to differentiate between ionized and un-ionized electrolyte.

If this interpretation of the transport of weak electrolytes by the gastric mucosa is correct it serves to localize the site of hydrogen ion formation to the tubular secretory cells. The structural features of the gastric mucosa would appear to account for the transport of carbonic acid. Because of this limitation, the transport of carbonic acid is not sufficient to support a hypothesis of forced anion exchange.

SUMMARY

By use of radiochloride it has been possible to demonstrate that the isolated gastric mucosa actively transports the chloride ion. The active transport of chloride generates an electrical current which is the source of the distinctive gastric mucosal potential. The active secretion of chloride is an integral part of the elaboration of gastric acid and study of this anion pump has become possible since the introduction of isotopes.

Indirect evidence relevant to an understanding of the mechanism of hydrogen ion secretion can be developed from the study of weak electrolyte movement. The gastric transport of weak electrolytes has been clarified by use of radioisotopes.

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Time Relation Between Potassium K^{42} Outflux, Action Potential and Contraction Phase of Heart Muscle as Revealed by the Effluogram

By W. S. Wilde, J. M. O'Brien and I. Bay,* USA

An aspiration of long standing among physiologists has been to relate in time the electrical and contractile processes of muscle to underlying biochemical events. A. V. Hill[1] constructed the chemical changes indirectly from the heat exchanges measured with delicate thermocouples. Fenn and Cobb[2] detected losses of potassium from muscle after prolonged repetitive stimulation. Keynes and Lewis[3] applied isotopic activation analysis to detect ion changes in squid nerve subjected to multiple stimulations. Hodgkin[4] for squid axone and Draper and Weidmann[5] for heart muscle deduced possible ion movements from potential changes recorded with intracellular electrodes. Fleckenstein *et al.*[6] and also Mommaerts[7] analyzed phosphorus fractions in skeletal muscle quick-frozen at stages during the twitch. Each muscle contributed but one point on the graph. Statistical fluctuation is encountered among muscles and the timing is coarse.

In attacking this age-old problem we have devised a new method of rapid-fire sampling of potassium radioactivity in the outflowing perfusate from a tagged heart. K^{42} in a highly pre-enriched turtle ventricle is carried by the coronary perfusate onto a traveling filter paper strip, which is cut into timed segments for radioactivity counting. A constant level of a second radioactivity is pre-added to the perfusate to act as an indicator of volume of outflow caught on each sample segment. The plot of K^{42} concentration in the outflow against time is called an "effluogram". Turtle ventricle has the obvious advantage of a slow electrocardiographic complex which may be further slowed with cold and of a huge mass of potassium to yield measurable outflux. Microvolumetric pipetting is avoided in liquid samples which may become vanishingly small (3 microliters) and we collect up to 20 samples during a single contraction. A distinct pulse of K^{42} release accompanies each single contraction (Figs. 2 and 5).

THE EFFLUOGRAPH TECHNIC

High intensity $K^{42}Cl$ (70 mc·in 8.7 meq K/kg) prepared from K_2CO_3 exposed in the Low Intensity Testiog Reactor at Oak Ridge is injected into the peritoneal cavity of a 2 kg freshwater turtle, *Pseudemys scripta elegans* (Wied). The injection is by way of a rear leg pit. The needle enters parallel to the plastron so as to miss the bladder. After 3–4 hours a 7 cm circular window is drilled through the plastron to expose the heart quickly and with a minimum of spillage of radioactive body fluid. Heparin is injected via an abdominal vein before excision of the heart. This work and the later cannulation of the excised heart under a dissecting microscope are done behind lead and plastic shielding.

The inactive perfusate irrigates the turtle myocardium via the coronary vessels alone and not by way of the ventricular cavities. Usually a single coronary artery arises from the innominate (brachiocephalic) artery about 3 mm from the ventricle (Fig. 3). The coronary cannula is about 2.5 cm of polyethylene tubing drawn under the mild heat of a micro burner to a fine tip (0.5 mm) which enters the natural orifice of the coronary from inside the innominate. The cannula is ligated with nylon thread under sufficient tension to constrict its tip but not to occlude it. The inserted cannula easily slips onto a blunted hypodermic needle for later attachment to the perfusion line. About 10 per cent of hearts must be discarded because two branches of the coronary arise independently from the innominate.

We use a ventricle rendered quiescent by Stannius ligature placed about the auriculo-ventricular (A-V) junction. Fortunately this ligature need not interrupt the coronary venous outflow. Three major and 5 to 7 minor coronary veins empty into the sinus venosus. Usually these collect in a cord surrounded by epicardium before crossing the A-V junction. The ligature about the junction may be placed conveniently under this cord so as not to interfere with coronary venous flow (Fig. 3) yet at the same time block coronary arterial flow to the auricles by other possible routes. Because of the several coronary veins, cannulation of their combined outflow is impossible. Instead, the venous flow follows its natural course to the sinus and auricles from where it is lead off onto a solid glass stylus which writes pen-like on the filter paper strip (Figs. 3 and 4). A bulbous end of the stylus is constricted for anchorage by the Stannius ligature after this end of the stylus has been

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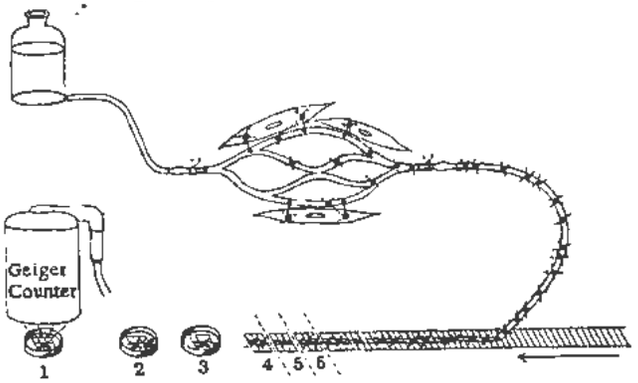

Figure 1. Principle of effluography

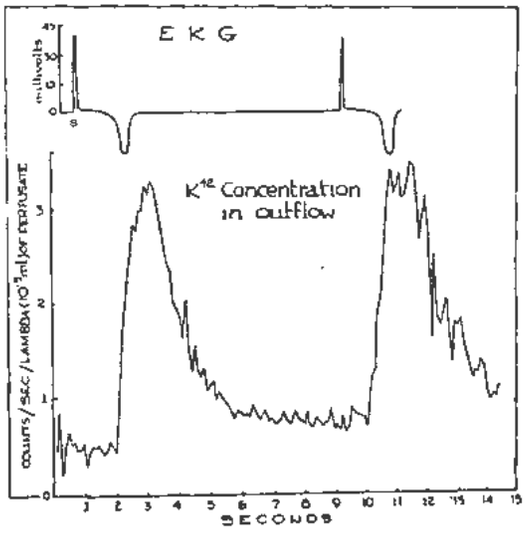

Figure 2. The effluogram

inserted through the tip of the right auricle and into the ventricle. The sinus venosus and auricle drape as an umbrella about the thickened middle of the stylus to direct the venous outflow downward. They are trimmed closely to yield a minimum of dead flow space. The stylus is kept wetted with albumin-Ringer to yield a steady flow. It is held steady against the filter paper strip by a wire loop which encircles the stylus near its tip. Flow is not drop-wise but continuous (Fig. 5, lowest recording). As in mammalian hearts some of the inflowing coronary arterial perfusate enters the ventricular cavity by Thebesian-like channels. Thus in test runs with several hearts the stylus collected anywhere from 13 to 100 per cent of the entire coronary inflow.

The well-known endurance of the excised turtle ventricle, even in the absence of circulation, is not shared by its coronary system. Often flow can not be initiated at all and when present will deteriorate during the first hour. Perfusion must begin as promptly as possible after excision of the heart. After many modifications over a period of 3 years, our best perfusion fluid now contains in meq/l:

Na	124.3	Cl	117.0
K	4.0	HCO_3	14.25
Ca	6.8	H_2PO_4	3.85
Mg	2.0	SO_4	2.0
Cations	137.1	Anions	137.1

Glucose	0.13%
Dextran, clinical	2.0%
Bovine albumin	2.0%
Thiamin HCl[9]	0.048 mg %
Papaverine. HCl[9]	0.001 mg %

The solution is equilibrated against 5% CO_2 and 95% O_2. The force to drive the perfusion comes from this gas under pressure in a steel cylinder with a sensitive valve regulator. With a pressure of 140 mm Hg, the 2.6 gm heart used for Fig. 5 produced a flow of 2.5 ml/min or 4 microliters per sample collected every 0.096 sec.

Our filter paper strips measure 3.12 meters long. A ribbon of paper 9 cm wide is first cut from a special roll of Eaton-Dikeman-No. 624 filter paper. Time lines 5 mm apart are printed across the ribbon with a heavy brass cogwheel that rolls continuously along the ribbon as its cogs are inked with a large ink pad. Waterproof masking tape seals the back of the ribbon against leakage but usually the perfusate does not wet through the paper. Finished strips 15 mm wide are then cut from the ribbon with a knife and straight edge.

The strips are mounted on a canvas belt 3.12 meters in circumference stretched horizontally between two kymograph drums ("Monodrum" with synchronous electric motor by Gorrel and Gorrel). The belt is 32 cm wide and accomodates as many as

Figure 3. Coronary cannula and stylus

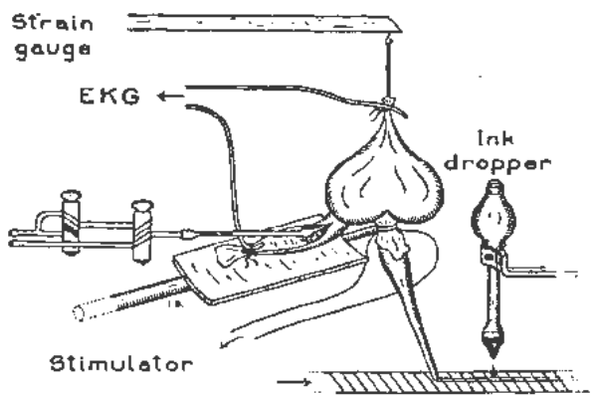

Figure 4. Heart mounted over kymograph

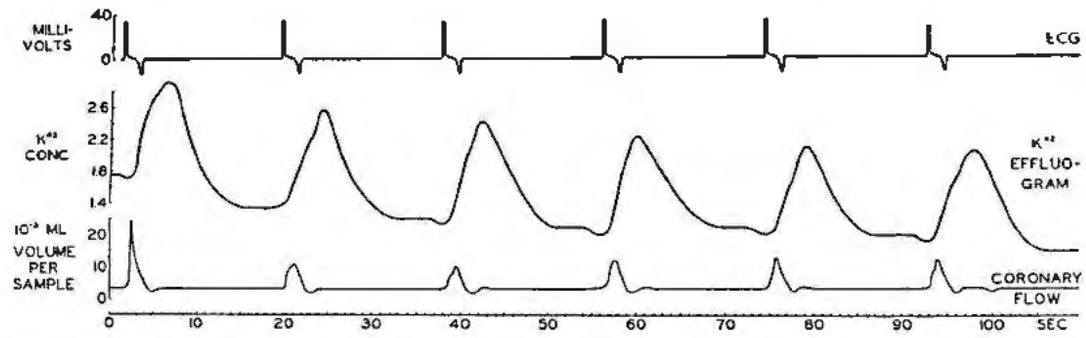

Figure 5. Effluograms: six consecutive systoles electrically stimulated. Lower recording is of volume collected per sample or per 0.096 sec

17 strips either as belts or as a continuous spiral. An optimum drum speed for the K^{42} dosage mentioned above is 15 mm in 0.096 sec. If later the sample segments are cut from the strip with scissors so as to comprise three intervals between ink marks (15 mm), they will represent perfusate collections of 0.096 sec duration. The segments are thus 15 mm square and can be placed in serially numbered planchets for radioactivity counting.

The heart is suspended over one end of the canvas belt as shown in Fig. 4. The assembled parts shown attach to a movable carriage so that the stylus may be maneuvered up and down or side-wise to reach any paper strip. The arterial trunk drains onto a tray to carry away and discard any contaminating Thebesian flow escaping from the coronary system into the ventricular chamber. The heart is mounted in such a way that it may be quickly dismounted and carried to the dissecting microscope for re-inspection of the cannulation, etc. This is accomplished by tying the thread for the Stannius A-V ligature to an eyelet at one end of a small brass mounting pin. The free end of the pin fits snugly into a hole at the end of the tray so as to secure the heart.

Stimulation is bipolar across the A-V junction with an electronic condenser discharge type stimulator. The excitation wave thus travels toward the apex. The ECG lead is base to apex. Positivity at the apex gives an upright deflection which thus exists through most of the QRS complex.

The ECG and contraction myogram (See "Strain gage", Fig. 4) are recorded with a two-channel Sanborn electrocardiograph. The stimulus signals simultaneously on the ECG paper and on the filter paper strip for purpose of synchronization. The signal device for the paper strip has been an "ink dropper" which is thrust downward by an electromagnet, or more recently a felt ink wick attached to the end of a light lever similarly activated. An ink mark is thus made on the sample segment corresponding to the time of stimulation. This is numbered sample 11 of 200 samples usually counted for each systole recorded.

Our interpretations depend critically on knowing K^{42} concentration in the outflow. The volume indicator on the sample segments provides this. Because of possible changes in transudation during systole we prefer a label with extracellular rather than circulatory distribution. For that matter the beta count of I^{131} albumin suffers variable self-adsorption as the outflow wets deeper into the paper. The high energy beta of phosphate P^{32} seems ideal. With the concentration of carrier listed in our perfusate, P^{32} reaches a steady concentration in the outflow within 10 minutes. If Na^{24} and P^{32} are used together as volume indicators during an actual effluographic run including the systole their ratio stays constant over the usual 200 samples collected.

A conveniently calculated form of relative K^{42} concentration results from the equation $K_0/V_0 = D(S-dV)/dV$, in which K_0 is K^{42} counts and V_0 is counts of volume indicator on the sample segment at zero time. K and V are first counted promptly together before the K^{42} decays. Their combined count minus background is S. Five days later when all but 0.1 per cent of the K^{42} has decayed, the residual long-lived V is counted (P^{32} in this case). V is multiplied by the factor d which corrects it for radiodecay since zero time. Thus $(S-dV)$ is the initial K^{42} count. Finally D corrects for the K^{42} decay which occurs while S values for the several samples are being counted. The true concentration of K^{42} is $(K_0/V_0)c$, where c is the concentration of volume indicator in the perfusate at zero time.

The computation even for one sample is far more onerous than our abbreviated formula above implies. We have had runs comprising 15 strips or 3000 samples and often resort to the IBM electronic computer service.

We have used at one time up to three automatic sample changer counting assemblies (Tracerlab, Inc.) using pre-set counts but under continual observation that the count setting is optimal so as not to lose too much K^{42} because of decay. The effluogram peaks are usually counted at 4096 counts and the resting level at 2048 counts per second.

The 15 mm square sample segments fit loosely into the 2.54 cm diameter of a Tracerlab cup planchet. Under the end window Geiger tube used, the position of the segment in the planchet and the position of the wet outflow streak on the segment itself do not by test affect the count of the sample.

DISCUSSION OF RESULTS

A distinct parcel of K^{42} outflux occurs in relation to each heart systole (Fig. 2). The K^{42} concentration wave rises sharply to a peak 4 to 10 times the amplitude of the resting exchange level. Six separate samples contribute to the upswing. Twenty samples are collected during the entire electrical (QT) complex. The entire release comprises 40 samples each of 0.096 sec duration. Figure 5 includes 6 ventricular systoles taken in succession one every 20 seconds. They are all remarkably similar. The stimulus current itself does not directly cause the K^{42} release. No stimulus was applied for the second wave in Fig. 2. It was an idioventricular beat. The release waves are not a mere artifact due to the influence of flow rate changes on the rate of wash out of tag. Such a phenomenon would not yield rising K^{42} concentrations during faster flows as seen during the gush of systole (lower curve, Fig. 5).

The K^{42} charted in the effluogram has suffered a dispersal due to the variable distance traveled by the several K^{42} release elements after they enter the various coronary capillaries. We attempt to construct an ideal curve possessing the same pattern as this dispersal. This is then used as a pattern to extrapolate the K^{42} release backward in time. The construction of the pattern curve is illustrated in a model (Fig. 6, upper curve) in which a bundle of strings is threaded, one thread through each capillary loop. A segmental element of each string is blackened to mimic momentary release of K^{42} into the midlength of each capillary loop. Flow is then mimicked by pulling the strings collectively to the right. The resulting dispersal of elements is added vertically to yield the histogram charted at the right above. This curve is analogous to a graph of concentration of K^{42} in the outflowing perfusate as ordinate plotted against accumulated volume as abscissa. This

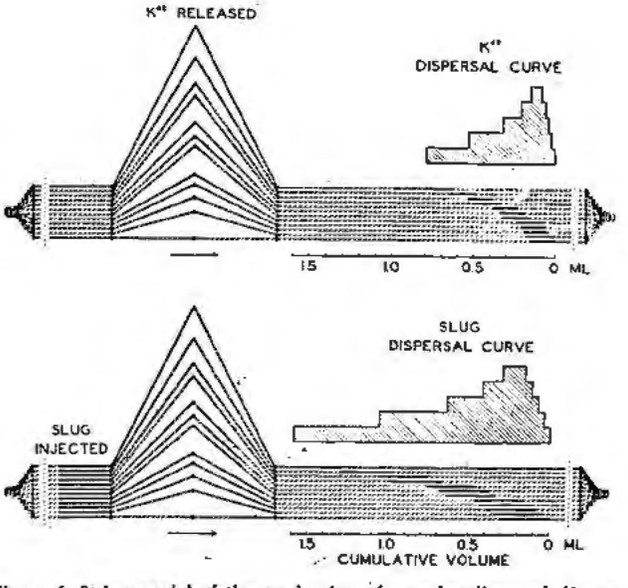

Figure 6. String model of the mechanism of vascular dispersal. Upper curve, dispersal of K^{42} released into capillaries; lower curve, dispersal of injected slug

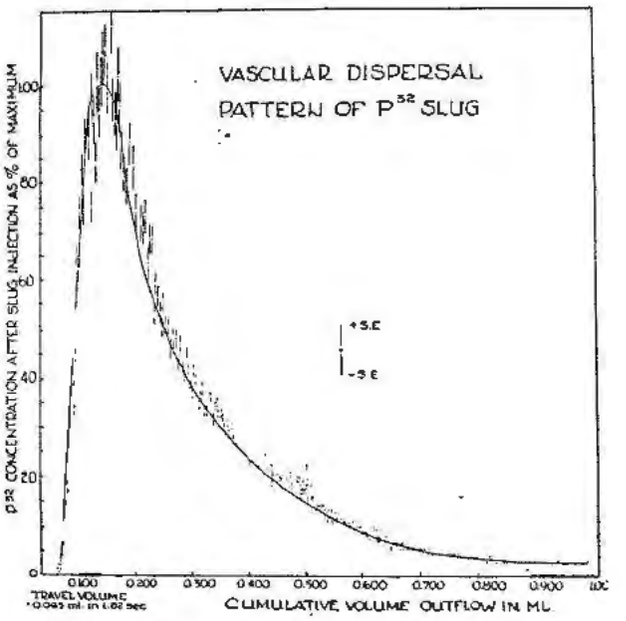

Figure 7. Actual slug dispersal curve

curve cannot be constructed directly by experimental means. Rather we derive its shape from that of a "vascular dispersal" curve delineated as a foreign "slug" of radioactivity traverses the actual coronary bed. A slug of high intensity I^{131}—serum albumin or of $P^{32}O_4$ is caught in the plug of a stopcock situated in the perfusion circuit near the coronary arterial cannula (at left in Fig. 4). A sudden turn of the stopcock sends the slug coursing through the coronary circulation. The resulting plot of P^{32} concentration against accumulated volume of the stylus outflow is shown in Fig. 7. The mechanism of this dispersal is illustrated in the lower model of Fig. 6. Visualize the black slug elements aligned at the left to be traveling through the coronary artery after release from the stopcock. Then as the strings are pulled slowly to the right, i.e., as flow is initiated, the black elements first experience a dispersal in the loops and finally align themselves as shown at the right in the venous outflow of the model. Visualize vertical sections or arrays cut through the strings. Each array is analogous to a volume collected and the sum of the black elements added vertically in the array is analogous to concentration in the unit volume. A plot of concentration against cumulative volume exhibits a pattern similar to the histogram shown to the right of the lower model. In the actual experimental plot (Fig. 7) volume is plotted from left to right thus reversing the curve.

Two significant generalizations emerge: (1) Provided concentration is plotted against cumulative volume, the pattern of the curve is unchanged by fluctuations in flow rate such as is caused by systole. The slug elements hold their respective orientation no matter how variable is the rate of travel of the strings through the flow channels. This is not true for plots of concentration against time. Flow rate fluctuations influence the *time* at which a given

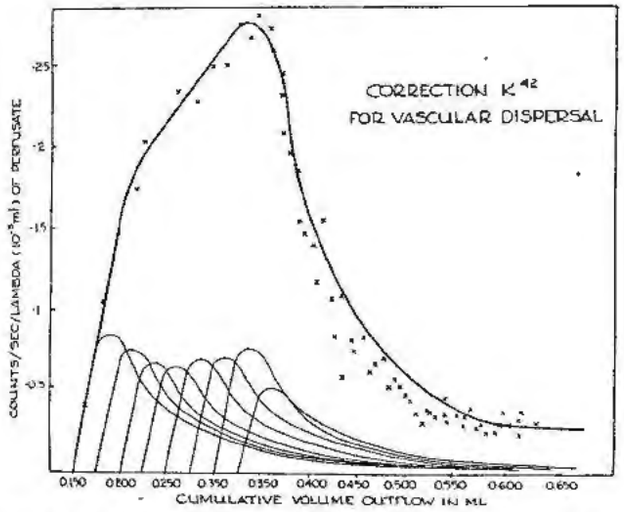

Figure 8. Volume effluogram (above) showing graphical summation of pattern release curves (below)

pattern of slug elements is sampled but not the total volume (accumulated) that has passed through the system when the particular pattern is caught in a sample. (2) For a vascular bed of identical volume capacity on arterial and venous sides, the scatter of the calibrating P^{32} slug, which travels the full distance artery to vein, is double that of the K^{42} released into a capillary which has only half as far to go (compare the width of the two histograms in Fig. 6). The slug dispersal curve may he used as a pattern to judge the dispersal of thc K^{42} releases, provided its width or volume values are reduced by one half.

Suppose the over-all pulse of K^{42} release occurs as a succession of increment sets into the several capillaries. Each increment set will experience a dispersal defined by the pattern curve. A succession of pattern curves must be chosen, of the same uniform width but with amplitudes so adjusted in height that the entire sequence summates to yield the over-all effluogram curve, the latter now also being plotted as K^{42} concentration against cumulated volume (Fig. 8, uppermost curve).

In practice a first pattern curve is fitted at the left under the effluogram curve (Fig. 8). To do this the altitude of the chosen curve is adjusted until its upswing fits the linear part of the upswing of the effluogram. This curve is subtracted graphically from the effluogram. A second curve is fitted under the residue, subtracted, and so on until the entire area is used up.

The area, concentration times volume, under each curve is an amount of K^{42}. This parcel of released K^{42} is thus extrapolated backward as if it all arrived at the stylus tip during the finite interval between the upswings of the first two pattern curves, i.e., K^{42} release elements for all capillaries are extrapolated backward to arrive at the stylus tip at the same time as the element which travels the shortest distance,

A final extrapolation moves this aggregate K^{42} backward a distance equal to the shortest path between the nearest capillary and the stylus tip. This is approximately half the distance between the tip of the arterial cannula and the beginning upswing of the slug dispersal curve. This distaoce is again expressed as cumulative volume.

We now have two points on the volume scale between which this aggregate K^{42} entered the coronary capillaries. We need merely convert these points to time on the filter paper strip and in turn to time on the simultaneous ECG record. If we divide the aggregate K^{42} release by the finite time interval during which it was entering the capillaries we have a rate, i.e., we have a rectangle of which the base is time in seconds and the altitude is rate or K^{42} counts entering the capillaries per second. This rectangle occupies its selected position, the first on the upper right in Fig. 9. The areas or aggregate K^{42} for the succeeding pattern curves are similarly extrapolated backward to take their place as rectangles in Fig. 9. They are derived from the two effluograms in Fig. 2. The areas of each rectangle indicate how much K^{42} entered the capillaries over the time interval indicated.

While the K^{42} pulse in Fig. 9 is adjusted for circulatory travel and dispersal, it is not corrected for time of diffusion from the muscle fiber membrane across the interstitial space and capillary membrane. Unknown surface potentials and permeabilities will influence this. On the other hand, if the potassium ejection is projectile, this travel may be instantaneous. We shall assume this error does not exceed the coarseness of sampling of the paper srtip, namely 0.096 sec. Because of the travel time of the excitation wave from A-V junction to ventricular apex, the time of activation of the several muscle fibers will vary over the time expanse set by QRS (about 5 per cent of the $Q-T$ interval).

The adjusted K^{42} pulse begins during the early T wave (Fig. 9). Its outflux is fastest during the final rapid phase of the T wave. These times probably correspond to the "plateau" and the last rapid repolarization phase recorded with intracellular electrodes. This implies that the fastest outward current of potassium occurs when the membrane potential is rising fastest toward normal resting level. It seems unlikely that a rapidly increasing potential gradient, negative inside the fiber, could be driving K outward. Rather an outward K current must be generating these potential changes. This follows the emphasis of Hodgkin.[4]

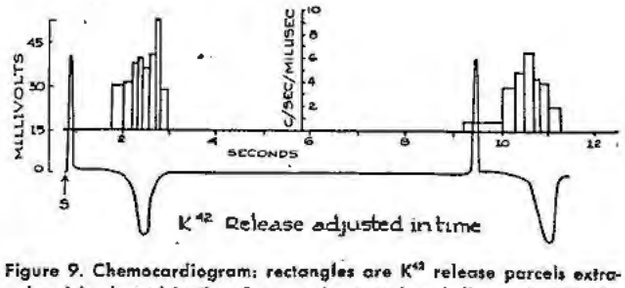

Figure 9. Chemocardiogram: rectangles are K^{42} release parcels extrapolated backward in time for vascular travel and dispersal. Altitudes of each rectangle are rate of release for the interval

We estimate that 0.0012 of the K content of the heart is released per beat or that 1.3×10^{-5} meq K are released per systole from each gram of muscle. Considering the 40 samples into which this release from a 3-gram heart is divided, we are measuring 9.6×10^{-7} meq K per sample. The flame photometer could barely measure 1000 times as much. Rough estimates of the surface area of heart muscle fiber membranes indicate that 44.9 micro-micromoles K/cm^2/beat are released. This is about 10 times the release found by Keynes and Lewis[3] for squid giant axone. Relatively more K release accompanies the slower electrical events seen in turtle heart.

The effluographic method can be applied to any bath or flow system that provides liquid samples. Samples from suspension media for bacteria, yeast, isolated mitochondria or tissue slices can be drained off under mild pressure through polyethylene tubing to the paper strip. Samples may be as frequent and as vanishingly small as the selected intensity of radioactivity allows. No micropipetting is required. The exceedingly small volume of sample required adapts the method to sampling of blood from small animals, or of fluids from ducts of glands or from the ureters of the kidney.

The method as we devised it had no relation to chromatography. However it could be combined with the latter in that various chemicals in the outflow could be chromatographed at right angles to the timed collection to determine not only whether they are released together but whether they are actually linked in chemical bond while undergoing release from the cells of the tissue.

SUMMARY

We have devised a new method for collecting rapid serial samples of radioactive fluids from a bath or flow system. The outflow from a perfused organ, which has been pre-enriched with isotope, is led onto a timed moving strip of filter paper. The paper strip is cut into timed segments which are placed in numbered planchets for counting the unknown radioactivity. A second constant radioactivity, pre-added to the perfusate, is an index of volume for each paper sample. Samples may be as frequent and as vanishingly small as the chosen intensity of radioactivity allows. The record of radioactivity concentration against time is called an "effluogram". A calibration procedure extrapolates each sample back to the time of its release into the capillaries of the organ.

We have shown for the first time, by perfusing a turtle ventricle intensely enriched with K^{42}, that the contraction of heart muscle produces a distinct pulsatile release of potassium. When extrapolated backward to the time of its release into the capillaries, the peak of the K outflux coincides with the fast and late repolarization T wave. The total activity release comprises 0.0012 of the K in the heart. This has been divided into 40 samples each containing about 1×10^{-8} meq K per sample. This sensitivity is 1000 times that of the flame photometer.

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A Study of the Metabolism of Baker's Yeast with the Help of Radioactive Glucose and Ethanol

By J. P. Aubert and G. Milhaud,* France

The use of paper chromatography and autoradiography has made it possible, for the first time, to follow the fate of a metabolite in intact baker's yeast. The method gives a "map" of the various compounds in which radioactivity is incorporated. We set ourselves the task of studying the various compounds formed by yeast with radioactive glucose as a substratum, and of clarifying the influence of aeration conditions. On the other hand, since ethanol is a normal product of glucose metabolism, we determined the substances formed by yeast from alcohol, and investigated whether alcohol was metabolized at the same time as glucose.

TECHNIQUES

The yeast used is a diploid strain of *Saccharomyces cerevisiae* cultured in an aerobic medium on glucose-treated barley-germ water. Before the glucose is exhausted, the cells are centrifuged and twice washed in distilled water. A suspension in a phosphate buffer at 0.025 *M* at pH 6.0 then is incubated in a Warburg flask, in air or under nitrogen at 30°C, in the presence of the substrate. The experiment is stopped before the latter is depleted, the cells are quickly washed with water, and extracted by a process of boiling aqueous alcohol.

The extract is subjected to two-dimensional chromatography on washed Whatman No. 4 paper in the following solvent systems: water-saturated phenol, propionic acid–*n*-butanol–water 1:2:1.4. Autoradiographs then are made.

Uniformly tagged C^{14} glucose was prepared in the laboratory by photosynthesis. C^{14} 1-2 tagged alcohol was obtained by fermenting uniformly tagged C^{14} fructose also prepared by photosynthesis. The purity of these products was suitably checked. Identifications were carried out with compounds coming direct from the chromatogram, or with products derived from these compounds after chemical or enzymatic reactions, by subjecting them to cochromatography or cocrystallization with non-radioactive reference substances. Radioactivity was then measured with a Geiger counter, on the chromatogram or with rate, following elution.

Original language: French.
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EXPERIMENTS ON GLUCOSE

Identification of Compounds Formed

We identified the following compounds:

1. Fructose diphosphate. Coincidence, after cochromatography of the radioactive product with a sample of 1-6 fructose diphosphate, and with fructose, following treatment of the radioactive product with polydase S.

2. Glucose phosphate. Coincidence with glucose following treatment with polydase S.

3. Mannose phosphate. Coincidence with mannose following treatment with polydase S.

4. Uridine diphosphate glucose, galactose, mannose. Following polydase S treatment, six radioactive spots are obtained, the R_f of which are, respectively 0.22/0.17, 0.40/0.23, 0.41/0.35, 0.47/0.37, 0.64/0.42 and 0.78/0.46. Following elution and cochromatography, the 0.41/0.35 spot coincides with glucose, the 0.47/0.37 spot with mannose, the 0.64/0.42 spot with ribose, and the 0.78/0.46 spot with uridine. The 0.22/0.17 and 0.40/0.23 spots, once phosphatase treated, give radioactive products which coincide, following cochromatography, with glucose and galactose.

5. Meso-inositol phosphate. Coincidence, following treatment with polydase S, with meso-inositol, and coincidence of the eluted spot with meso-inositol phosphate.

6. Sedoheptulose phosphate. Coincidence with sedoheptulose, after treatment with polydase S. The radioactive spot, heated for 30 minutes in the presence of Dowex-50 in the acid form splits up: the newly formed spot coincides with sedoheptulosan following cochromatography.

7. Diphosphoglyceric acid. Coincidence with glyceric acid after treatment phosphatase. The R_f of the untreated spot corresponds to a diphosphorylated product. One must thus be dealing with a diphosphoglyceric acid.

8. 3-phosphoglyceric acid. Coincidence of the untreated spot with 3-phosphoglyceric acid, and of the polydase-treated spot with glyceric acid.

9. Phosphoenolpyruvic acid. Coincidence of the untreated spot with phosphoenolpyruvic acid. Following acid hydrolysis in the presence of pyruvic acid as a carrier, the dinitrophenylhydrazone formed and recrystallized several times, is counted: its specific

radioactivity is constant and corresponds to the overall integration of the radioactivity in the carrier.

The following compounds have been identified by cochromatography with the reference product used as a carrier: (10) citric acid, (11) malic acid, (12) fumaric acid, (13) aspartic acid, (14) glutamic acid, (16) glutamine, (17) valine, (18) leucine, (19) norleucine, and (20) isoleucine.

21. Trehalose. Following acid hydrolysis, the product obtained is glucose. The primary product coincides with trehalose following cochromatography. Identification was confirmed by cocrystallization of the radioactive product with trehalose as a carrier.

Influence of Aeration Conditions

There are practically no qualitative differences, whether the yeast is in aerobiosis or anaerobiosis, but the quantitative differences are important. Thus, close to 80% of the radioactivity extracted is found in the following compounds:

Table I. Radioactivity Extracted, Per Cent

	Trehalose	*Glutamic acid*	*Alanine*	*Valine*	*Lactic acid*	*Citric acid*
Aerobiosis	35	23	7	2	1	6
Anaerobiosis	14	4	32	20	13	2

These results show heavy tagging of the trehalose, particularly in aerobiosis. They further suggest, for the other compounds, a predominance of the Krebs cycle in aerobiosis, and of glycolysis, according to Embden Meyerhof, in anaerobiosis. Under aerobiosis, the citric and glutamic acids are those most heavily labeled while, in anaerobiosis, radioactivity accumulates in alanine, valine and lactic acid, which are directly derived from pyruvic acid.

EXPERIMENTS WITH ALCOHOL

Identification of Substances Formed

The experiment is stopped after approximately 90 min of incubation at 30°C in air, when approximately half of the alcohol present has been metabolized. Among the products formed, we identified, by using the same techniques as in the case of glucose, glucose phosphate, mannose phosphate, diphosphoglyceric acid, 3-phosphoglyceric acid, phosphoenolpyruvic acid, uridine diphosphate-glucose-galactose-mannose, sedoheptulose phosphate, citric acid, malic acid, fumaric acid, lactic acid, glutamic acid, aspartic acid, glutamine, asparagine, alanine, valine and trehalose.

The similarity between the chromatograms which correspond to the metabolism of glucose and alcohol is remarkable. The differences are essentially of a quantitative nature. Thus, over 75% of the radioactivity present in the extract is incorporated in the substances of the tricarboxylic cycle and those directly derived from it: glutamic acid 40%, citric acid 18%, aspartic acid 14%, glutamine 4%, asparagine 1%, malic acid 1%. On the other hand, an important phenomenon is observed, that of the reversibility of the glycolysis which goes back to the synthesis of the hexoses and of trehalose: trehalose, 10%, 3-phosphoglyceric acid 1%, phosphoenolpyruvic acid 1%, alanine 1%, valine 1%.

The Metabolism of Ethanol in the Presence of Glucose

In a Warburg flask, 3 mg of yeast was incubated for 25 minutes in the presence of 5.2 μM of glucose and 5 μM of radioactive ethanol (5 μc). The analyses show a consumption of 3.6 μM of oxygen, 3.7 μM of glucose and the formation of 0.1 μc of radioactive carbon. If it be assumed that the formation of alcohol from glucose is a linear phenomenon, even as is the consumption of alcohol by yeast, allowing for the radioactive CO_2 formed and the incorporated radioactivity, it will be found that at least 0.4 μM of alcohol have been metabolized. This quantity is approximately equivalent to that metabolized where the alcohol is the only nutrient, and represents approximately 10% of the glucose consumed.

The autoradiograph shows a concentration of radioactivity in the substances of the citric acid and derivatives cycle: glutamic acid 60%, glutamine 10%, aspartic acid 2%, malic acid 2%. In this case, the absence of all substances related to glycolysis is noted.

CONCLUSIONS

This paper contributes some knowledge of the nature and relative importance of the tagging of the principal compounds formed during the degradation of glucose by live yeast. We have characterized many intermediate compounds in the citric and glycolysis cycle according to Embden Meyerhof. We have found no products corresponding to the oxidative degradation of glucose, except for sedoheptulose phosphate, which thus is identified for the first time in yeast. On the other hand, there is a predominance of the tagging in the compounds derived from the citric cycle in aerobiosis, and of those derived from classical glycolysis in anaerobiosis.

In the case of alcohol, we identified the compounds formed in baker's yeast from the substratum. The radioactivity concentrates mostly in the compounds of the cycle of citric acid and its immediate derivatives. On the other hand, one observes, for the first time in yeast, the formation from ethanol of phosphoenolpyruvic acid, of 3-phosphoglyceric acid, of hexose phosphates, and of trehalose, which prove that glycolysis is reversible. Finally, we have demonstrated that ethanol, a normal product of the metabolism of yeast from glucose, is used up simultaneously with the glucose and creates, under such conditions, some compounds which are related exclusively to the tricarboxylic cycle.

Studies on Pearl Formation Mechanism by Radioautography

By Yaichiro Okada, Tadashi Tsujii, Norimitsu Watabe and Giichi Yoshii,* Japan

Many studies[1,2,3] have been recently carried out on shell- and pearl-formation. The mechanism of the process should be considered as the result of metabolism carried by the cells of the mantle or the pearl sac.

Recent studies[4,5,6,7,8,18,19] deal with the enzymes or other substances relating to the metabolism; in them, Ca^{45}, glycogen and polysaccharide,[10,11] nucleic acid,[12] and phosphatase have been observed.

Bevelander[9] observed the localization and deposition of Ca^{45} and P^{32} in the mantle and shell by the radioautographic method. He found that Ca^{45} was concentrated on the periphery of the mantle and also incorporated into the crystals of calcium carbonate in the newly formed shell, while P^{32} was localized on the inner margin of the mantle in the region of the mucous glands. It was also incorporated in the periostracum surrounding the crystals.

Moreover, biochemical studies were carried out on calcium metabolism in *Crassostrea virginica* Gmelin by Wilbur and Jodey[13] and Jodrey,[14] in *Pteria* (*Pinctada*) *martensii* (Dunker) and *Hyriopsis schlegeli* (Martens) by Matsui *et al.*,[15] and *Hyriopsis schlegeli* (Martens) by Horiguchi *et al.*[16] According to the results with *Crassostrea virginica*, most of the uptake of Ca^{45} by the mantle edge was completed in one hour. After the lapse of 2 or 4 hours the mantle took up as much Ca^{45} as it could accumulate. The rate of disappearance was very high; most of the Ca^{45} diappeared in half an hour.

The present authors have kept *Pteria* (*Pinctada*) *martensii* in aquaria to which Ca^{45} and P^{32} were added, and observed the localization of the radioactive isotopes in the mantle and in the pearl. Above all, the difference of their distribution in different parts of the mantle has been examined. The object of these experiments lies in the application of their results to the practical pearl culture.

MATERIAL AND METHODS

Pteria (*Pinctada*) *martensii* (Dunker), 3 years old, cultured in the bed of the Fisheries Experimental Station, Mie Prefecture, was used for the work.

Preparation of Breeding Sea-Water Containing Radioactive Isotopes

Imported P^{32} solution was diluted 10 times with distilled water, to which was added a 1/1000 sea-water solution. This sea-water showed the radioactivity of 60 cpm/ml. In the same way, sea-water containing Ca^{45} was prepared. It showed 1216 cpm/ml. Radioactivity was measured by the method of Horiguchi *et al.*[16]

Nucleus Insertion and Breeding of *Pteria* (*Pinctada*) *martensii*

On August 1, two spherical nuclei made of paraffin were inserted into each of 40 pearl oysters, *Pteria* (*Pinctada*) *martensii*, in the ordinary way. Experimental animals were cultured for 23 days in the sea, and then brought into the laboratory. They were divided into 2 groups, each of which was bred in a separate aquarium containing sea-water with added P^{32} or Ca^{45}. The volume of the sea-water was 1 liter/1 oyster/2 hours. The temperature of the water varied from 27°C to 29°C during the experiment. The pH of the radioactive sea-water was 8.3. After intervals of 2 and 10 hours, the pearl oysters were placed in normal sea-water for 10 minutes, and then washed well with sea-water.

Procedure for Radioautography

Animals were fixed in 80% alcohol for 34 hours, with alcohol renewed every 8 hours. After the fixation, the shell valves were removed and the mantles were taken out of the body and pasted on glass plates. The mantles, when dried up, were immersed in 8% alcohol-ether solution of celloidin for a moment, to be covered with thin membranes of celloidin. Each mantle was then placed in contact with a Fuji ET-2E 15 contact plate with a sheet of medical paper between them. The plates with mantles were wrapped tightly in black paper.

Some of the fixed pearl sacs or mantles were cut into thin sections (15 μ in thickness) in the ordinary way, and placed on the slide-glasses, deparaffinized by xylol, and covered with thin membranes of celloidin. In this case, stripping plates were utilized for the purpose.

Time of Exposure

The time of exposure followed the preceding model experiment. In the P^{32} experiment, the time of exposure was 8 days in the contact method and 30 days in the stripping method. It was 180 days in the Ca^{45} experiment.

Development and Fixation

Plates were developed by Eastman D-19, or Fuji Rendol, and fixed by the first method of Yagoda[17] or Fuji FH-H.

* Faculty of Fisheries, Prefectural University of Mie.

Control Experiment

As the control, pearl oysters fixed in alcohol were washed with sea-water, and placed in sea-water containing radioactive isotopes for 2 and 10 hours. Then, they were treated in the same way as above.

OBSERVATIONS

Localization of P^{32}

Mantle

Radioautographs of the mantle of the pearl oyster bred for 2 or 10 hours in P^{32} water showed that more silver grains were blackened on the periphery. The periphery of a mantle is divided into the inner, middle, and shell folds, where basophilic and acidophilic glands are localized. P^{32} was found abundant in the epithelial cells and sub-epithelial zone of the inner and outer folds, especially in the inner one.

It was also localized in the glycogen glands of the outer part of the mantle. Two parts can be discerned in the glycogen glands; one is filled up with cytoplasmic contents, while the other is occupied by many vacuoles. P^{32} was mostly found in the former. The concentration of P^{32} in the muscle of the mantle seemed lower than in other parts, but a large amount of P^{32} was localized in the blood vessels and planocytes in the muscles.

No difference was observed in the localization of P^{32} in mantles exposed for 2 hours and for 10 hours.

Pearl and Peorl Sac

During the 23-day cultivation period, pearl sacs had been formed completely in the gonads of pearl oysters, but there were no calcareous layers on the paraffin nuclei. The nuclei were covered with the conchiolin layers only.

In this case, too, P^{32} was localized in the epithelial cells of the pearl sac. Moreover, a large amount of P^{32} deposited in the conchiolin layers of the pearls.

The distribution of P^{32} in the conchiolin layer was not homogenous, and in some part of the layer, it was found at the basal part, and in the other, localized at the upper or middle.

It was also localized in the cytoplasm of eggs in the gonad and in the mid-gut glands.

Localization of Ca^{45}

Thin sections were not prepared in the experiment with Ca^{45}, and localization of this isotope was observed only by the contact method, i.e., no detailed observatinns were made. It was found, however, that Ca^{45} was localized in all parts of the mantle, in larger concentration in the periphery. Ca^{45} was taken up by the nacre of the shell in 2 hours.

CONSIDERATIONS

If P^{32} is incorporated by contamination, it should be localized on the margins of the mounted sections on a slide. In the present radioautographs, however, blackened silver grains were not found along the margin. This suggests that the present radioautographs were registered by the radioactivity of P^{32} taken up in the body of the pearl oyster.

Contraction of the mantle was greater in the P^{32} experiment than in the Ca^{45} one. So it is unadvisable to discuss the distribution of P^{32} by Fig. 1. But, the part where the heavy deposition of P^{32} appears (the left-central part in Fig. 1) seems to correspond with that from which byssus is secreted.

The number of silver grains per $100\mu^2$ blackened by the radioactivity of P^{32} was calculated under the microscope in randomly selected 5 parts in each of the following positions: Outer epithelium, outer sub-epithelial zone, inner epithelium, inner sub-epithelial zone, muscle, blood vessel, basophilic gland and background. These calculations were made in each of 3 specimens of radioautographs which were selected by random sampling. Thus the average number of grains in each position was calculated. The number in the background was 29.2. Table I shows the number subtracted 29.2 from that of each position.

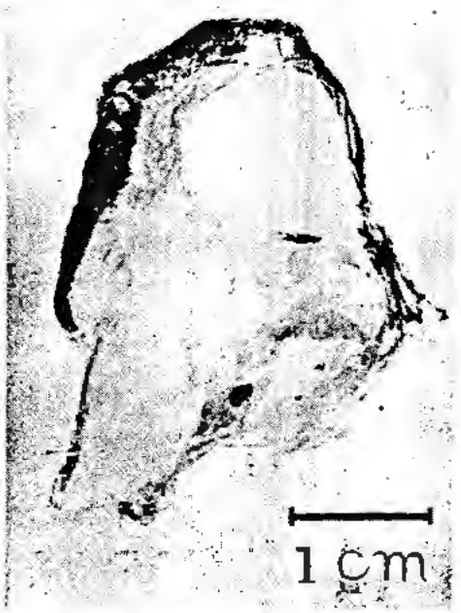
Table 1. Calculation of the Grains per 100 μ^2

Position	*Plate 1*	*Plate 2*	*Plate 3*	*Mean*
Outer epithelium	57.2	70.2	50.8	59.3
Outer sub-epithelial zone	46.8	70.2	64.6	61.6
Inner epithelium	54.6	82.2	43.6	60.2
Inner sub-epithelial zone	49.6	76.8	60.8	68.3
Muscle	41.6	40.6	39.0	39.9
Blood vessel	59.2	68.8	65.2	64.7
Basophilic gland	55.4	84.8	54.2	65.2

Significance of the difference occurring in number by the position was examined by Table I. It was found that a significant difference at the 1% level occurred between the muscle and other position. In other positions there was found no significant difference (5% level). Mean values of outer zone (including outer epithelium and outer sub-epithelial zone) and inner zone (including inner epithelium and inner sub-epithelial zone) are 64.3 and 60.4, respectively. This difference was not significant. However, it would become significant when more examples are examined.

Bevelander[9] reported that P^{32} was especially concentrated in the sub-epithelial zone of inner surface, or mucous gland of the mantle. The present authors also found it abundant in the epithelium as well as in the sub-epithelial zone and basophilic gland. Heavy deposition of P^{32} was found in the epithelium of the pearl sac as well as in the epithelium of the mantle. It was also localized with high concentration in the conchiolin layer of the pearl.

It is reported from the results of chemical analysis that content of phosphorus in the cultured pearl is only a trace, but the results of the present experiment show that P^{23} can easily be taken up by *Pteria* (*Pinctada*) *martensii* to form the conchiolin layer of the pearl. Moreover, the fact that localization of P^{32} in the conchiolin layer is not homogenous shows that its deposition did not take place at the same time over the surface of the pearl, but only partially. This fact will prove the author's idea of pearl formation[19] that the layer of a pearl is not formed evenly



Figures 1a and 1b. 1a (left) radioautograph of pearl-oyster mantle of Fig. 1b showing distribution of P^{32}. Heavy deposits of P^{32} appear in periphery of mantle. Emulsion exposed 8 days. 1b (right) photograph of the pearl-oyster mantle, contact method

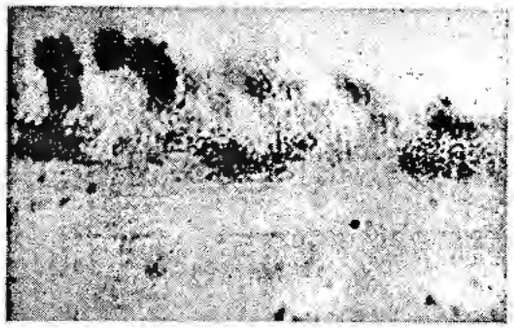


Figure 2. Radioautograph of section of mantle of pearl oyster. Localization of P^{32} is shown by the blackened sub-epithelial areas of inner surface of pearl-oyster mantle. Emulsion exposed 30 days. × 1000, stripping method

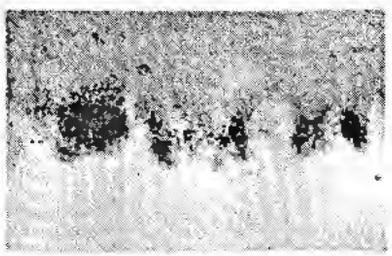


Figure 3. Radioautograph of section of mantle of pearl oyster. Localization of P^{32} is shown by blackened epithelial areas of inner surface of mantle. Emulsion exposed 30 days. × 1000, stripping method

Figures 4a and 4b. 4a (left) radioautograph of pearl-oyster mantle of 4b showing distribution of Ca^{45}. Heavy deposits of Ca^{45} appear all over mantle, especially in periphery of mantle. Emulsion exposed 180 days. 4b (right) photogram of pearl-oyster mantle, × 1, contact method

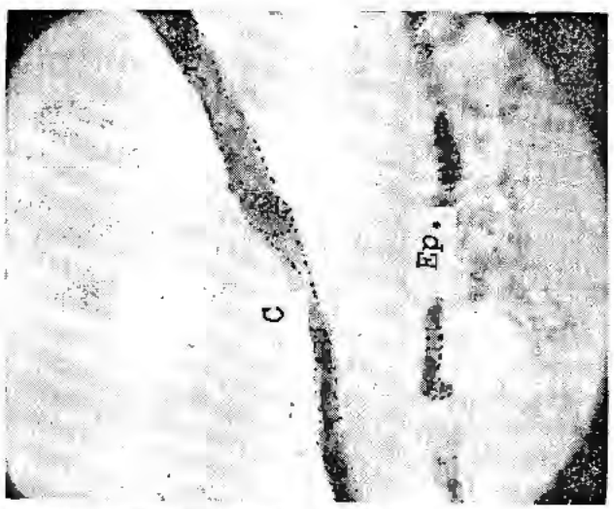


Figure 5. Radioautograph of pearl sac in pearl oyster. Localization of P^{32} is shown by blackened epithelial areas of pearl sac and conchiolin layer of pearl. C, conchiolin layer; Ep, epithelium

over the whole surface at one time, but locally by the local deposition of its constituents.

As for Ca^{45}, Bevelander[9] observed that it was localized generally in the periphery of the mantle of *Anadonta*, and it was incorporated in the calcium carbonate crystals in the regenerated shell of *Pinna*.

The radioautographic studies on the thin sections of the mantle have not yet been finished, so a definite conclusion is yet to be drawn. However, it was found

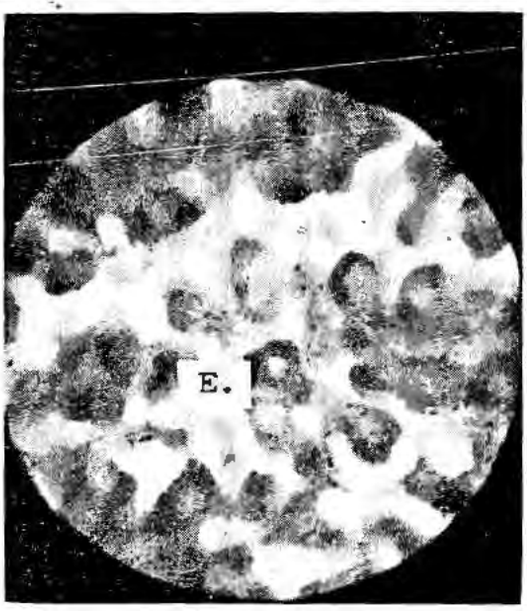


Figure 6. Radioautograph of gonad in pearl oyster. Heavy deposits of P^{32} appear in cytoplasm of egg. E, egg

that uptake of Ca^{45} can be detected by the radioautographic method after culturing for 2 to 10 hours. Ca^{45} was localized generally in the mantle, especially abundant in the periphery.

SUMMARY

1. P^{32} was found taken up by *Pteria* (*Pinctada*) *martensii* (Dunker) after 2 or 10 hours' cultivation in an aquarium.

2. The P^{32} was localized in the outer epithelium, outer sub-epithelial zone, inner epithelium, inner sub-epithelial zone, muscle, blood vessel, basophilic gland and glycogen gland, with the highest concentration in the inner sub-epithelial zone and the least in the muscle.

3. P^{32} was deposited in the epithelium of the pearl sac, and conchiolin layer of the pearl.

4. Ca^{45} was localized generally in the mantle, especially in its periphery.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Mr. Katsuo Takayama, Director of the National Pearl Research Laboratory, and Mr. Ichiro Nomoto, Director of the Fisheries Experimental Station, Mie Prefecture, for their encouragement through the experiments. Thanks are also due to Mr. Yasuo Koseki, Laboratory of Fuji Film Co., for useful discussion.

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Some Observations on the Biological Influences of Radioactive Isotopes Upon Physiological Functions

By R. Sasaki* Japan

The following study[1] was carried out for the purpose of elucidating the absorption of orally administered radioactive Ca^{45} into several tissues or organs of the silkworm larva, i.e., blood, nervous system, sexual organs, muscle, fatty tissue and silk gland, and the injurious effects upon the health which might be indicated by measuring the amount of the oxygen consumption per unit weight of the silkworm larva, pupa and imago.

The experiments were carried out on normal fifth-stage silkworm larvae, to which 0.1 cm^3 of 0.01% $Ca^{45}Cl_2$ water solution was administered by the alimentary canal to the worms in each of the following stages: (1) during feeding on mulberry leaves (10 minutes after feeding), (2) in the surfeit state (2 hours after feeding), (3) in the hungry state (5 hours after feeding) by pouring the solution through the mouth.

In each of these three classes, we arranged six classes respectively: 5, 10, 15, 20, 30 and 60 minutes after administration of the labeled calcium. The worms were anesthetized with ether, and tissues to be analyzed were dissected out on a filter paper and dry substances of the tissues were weighed and the radioactivity of samples was measured by using the Geiger-Mueller counter

At the same time, we measured the amounts of oxygen consumption of these materials by using a Warburg manometer, during the ten days of the larval stage and ten more days after pupation till the imaginal stage, and the amount of oxygen consumption per 100 gm of fresh material for 30 minutes was compared with the normal worm.

ABSORPTION OF RADIOCALCIUM INTO THE BLOOD VIA ALIMENTARY CANAL

About 5, 10, 15, 20, 30 and 60 minutes after administration of radiocalcium to the silkworm larva, 0.1 cm^3 of the blood was taken on a filter paper and dried, and then the radioactivity was measured by using the G-M counter, calculating the percentage of the absorbed and remaining dose to the total dose administered, where the amount of dose is measured in number of counts per minute (see Table I).

According to Table I, the maximum quantity of absorbed Ca^{45} was found in the worms in the surfeit period. In the hungry state, however, radiocalcium rather seemed to be more quickly absorbed into blood than in the other two cases, in the light of the fact that the highest value of count was reached in 5 min after administration, and the calcium absorbed in blood seems to be transferred quickly to several organs in consideration of the gradual decrease of the radiocalcium absorbed in blood. This fact will be clarified by the following experiment.

THE UPTAKE OF RADIOCALCIUM BY VARIOUS VISCERAL ORGANS OR TISSUES

Uptakes of Ca^{45} by nervous system, sexual organ, fatty tissue, muscle and silk gland were measured every 20, 30 and 60 minutes after oral administration of $Ca^{45}Cl_2$ (0.01%). The results thus obtained are shown in Table II.

On the basis of Table II we can assume the following:

The nervous system generally took up the largest amount of Ca^{45}, and the sexual organs, muscle and fatty tissue followed in order. The longest amount of uptake of Ca^{45} by these visceral organs was attained in the case of administration in the hungry or surfeit state.

INJURIOUS EFFECTS OF CA^{45} UPON THE RESPIRATORY FUNCTION

The close relation between the amount of oxygen consumption per unit weight of silkworm and the degree of the health of the worm was found by the authors (Gamo and Nishiyama, 1954): that is, the amount of oxygen consumption of the normal silkworm is larger than the injured worm in the growth period of the silkworm, and vice versa in the diapose period of pupal stage. The fact mentioned above is also ascertained by the following experiment on the silkworm whose injury was caused by radiocalcium (Table III).

By studying Table III, we may conclude that the silkworm in the hungry state will be injured more severely by the administration of radiocalcium, where the degree of health might be indicated by the amount of oxygen consumption measured by using the Warburg manometer. However, our investigation about the physiological cause of injurious effects of radiocalcium on the health of silkworm is now under way concerning the relation between radiocalcium and respiratory enzyme.

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Using radioactive phosphorus as a tracer, a physiological experiment,[2] metabolism study of the larva and pupa of the silkworm, *Bombyx mori*, was carried out. Two diverse methods were principally adopted in the experiments. One is the radiophosphorus injection into the body fluid of the 5th instar of the last larval studium and the pupa, and the other is the feeding of the mulberry leaves containing radiophosphorus for 2 hours. The injection was made through the abdominal legs of larva or the intersegmental portion of pupa by 0.02 cm³ of 3% aqueous solution of $Na_2HP^{32}O_4$. This injected amount of P^{32} corresponds to 25% of the total amount of phosphorus in blood, and under this no injurious effect upon the silkworm physiological condition was brought about.

In the case of oral administration, radioactive mulberry leaves previously cultivated with $Na_2HP^{32}O_4$ solution as fertilizer were given after the lapse of 2 or 3 weeks.

At various times after administration the organs and tissues to be assayed, entire body or fractions thereof, were wet ashed, and then radioactive counts were calculated by a Geiger-Mueller counter at various intervals to obtain precise results.

DISTRIBUTION OF RADIOPHOSPHORUS IN LARVAL ORGANS OF THE 5TH INSTAR

At various intervals, namely 3, 6, 12, 24, 48 and 96 hours after administration either intrahumorally or orally, the organs and tissues were picked out by dissection. The results obtained are briefly summarized as follows: During the first several hours following the injection, a great deal of administered radiophosphorus was found in the alimentary canal and Malpighian tubules as well as in blood. With the lapse of time the amount decreased gradually. In the silk glands, fatty bodies and sexual organs (testes), the aspect was quite different; that is, from 3 or 6 hours after the injection the activities of radiophosphorus injected were increasing until 24 hours. Thereafter the activities of the silk glands and sexual glands increased slightly, but that of the fatty bodies showed a distinct decrease. When the radiophosphorus was administered orally by means of feeding the mulberry leaves which had grown under relatively natural conditions, the absorption of labeled phosphorus resembled that in the case of injection, except that the radioactivity in the Malpighian tubules was at first rather low and at 6 hours came up to a maximum.

The specific activities of radiophosphorus in the various organs and tissues were calculated. In the two different methods used, the figures were very similar, but in the oral administration, the values of the alimentary canal were very low in comparison with that of injection. The values of the Malpighian tubules in the oral administration were also remarkably high at 6 hours, showing considerable decrease before or after that time, while in the case of injection the values indicated the maximum immediately after administration.

From the results with oral administration, changes in specific activity of blood, alimentary canals and Malpighian tubules after feeding on mulberry leaves containing labeled phosphorus resembled each other closely. Three of the silk glands, fatty bodies and sexual organs show the maximum at 24 hours, but these curves almost coincided at 96 hours after administration.

TURNOVER OF PHOSPHORUS COMPOUNDS DURING METAMORPHOSIS

In order to investigate the turnover rate of phosphorus compounds during metamorphosis, radioactive $Na_2HP^{32}O_4$ aqueous solution was injected into the silkworm not only at the time of the 1st, 4th, and 8th and 9th day (full-grown larva) of the 5th instar, but also at the time of the 7th and 14th day (the day preceding emergence) of the pupa. Dissection was performed at 6 hours and 24 hours after injection. As shown in the experimental results, a great deal of inorganic radiophosphorus was converted into organic phosphatides in the larval period, but very little in the pupa. On the 1st day of the 5th instar, the organic radiophosphorus was incorporated in large amounts into organic phosphatides, such as nucleic acid or phosphoprotein. On the middle day of the 5th instar, the greater part was found in phospholipid, and on the last day of the larva, the turnover of inorganic phosphorus diminished on the whole. At the middle of the pupal stage, radiophosphorus was plentifully incorporated into nucleic acids.

The relative specific activities, that is, the percentage of the specific activity of phosphorus compounds in various organs or tissues as opposed to the specific activity of acid-soluble phosphorus in blood at the beginning of the experiment, were tested.

As to acid-soluble phosphorus (including inorganic P), the relative specific activity of blood was the highest and it was almost equal to that of the alimentary canal. This fact shows the possibility that acid-soluble phosphorus of blood alternates quite easily to that of the alimentary canal. Generally speaking, the relative specific activity of nucleic acid phosphorus was the highest on the 1st day of the larva, and then decreased gradually until the last day of the pupa. As to the relative specific activity of the ovaries or testes, the trend was quite different; the values increasing gradually at the middle of the larva period and showing the maximum at the middle of the pupa. The incorporation of phosphoprotein phosphorus, in general, resembled that of nucleic acid, but was slightly different in some respect. For example, the relative specific activity of the silk glands reached the maximum on the last day of the larva. In phospholipid phosphorus the relative specific activities of various organs showed the higest values on the middle day of the larva.

These facts have close connection with physiological phases of the larva and pupa of the silkworm.

Table I. Radioactivity of 0.1 cm^3 of Blood on Each Time After Feeding Mulberry Leaves

Time after administr. of Ca^{45}, min.	10 min		2 hr		5 hr	
	No. of counts	Percentage of absorbed dose	No. of counts	Percentage of absorbed dose	No. of counts	Percentage of absorbed dose
5	4.65	3.18	54.30	37.14	64.50	44.11
10	21.90	14.97	42.50	29.07	36.30	24.38
15	23.75	16.24	52.70	36.05	40.85	27.94
20	29.80	20.38	45.40	31.05	17.75	12.14
30	16.85	11.53	48.40	33.11	45.20	30.92
60	28.85	19.73	58.95	40.32	27.90	19.08

Table II

Time of administr. after feed	Time of measurement after administr. of Ca^{45}, min.	Radioactivity of visceral organs, cpm/gm dry matter					
		Nervous system	Fatty tissue	Muscle	Silk gland	Sexual organ	Total
10 min.	20	5444	1000	294	304	2556	9598
	30	6278	2378	207	312	3357	12,531
	60	5933	5061	2110	463	3926	17,493
2 hr	20	5684	4580	634	357	5091	16,346
	30	7950	4383	2786	532	3098	18,750
	60	8471	4057	6313	563	6933	26,336
5 hr	20	7191	1833	3984	241	3747	16,995
	30	6467	2182	5727	404	5160	19,940
	60	8438	2584	6214	420	7000	24,926

Table III

	Amount of oxygen consumpt. per 100 gm of body wt. during 30 min, cm^3		Index number	
	Normal	Administ. Ca^{45}	Normal	Ca^{45}
Growth period (average value of 2–4th day larva of the 5th stage)	3420.0	3139.1	100	93.2
Diapose period (average value of the beginning of pupal stage)	228.5	380.3	100	166.4
Imaginal stage	5816.9	5188.2	100	89.2

Table IV. Phosphorus Excreted per Day in the 5th Instar

Days of 5th studium	1	2	3	4	5	6	7	8
Excreted P, mg	3.2	5.2	5.3	6.6	6.9	12.1	27.0	32.7

ROLE OF THE ALIMENTARY CANAL IN PHOSPHORUS METABOLISM

As has been pointed out already, the alimentary canal of the silkworm larva has an important role in phosphorus metabolism, as known in the liver of mammals. In this sense, some experiments were devised so that at a certain part of the alimentary canal the absorption and excretion of the radiophosphorus and its metabolite took place.

On the 4th day of the 5th instar, labeled mulberry leaves were administered orally for one hour and thereafter at every hour until 10 hours, 10 individuals were dissected and the parts of the alimentary canal were picked out separately. The greater part of phosphatides in the mulberry leaves was generally absorbed through the anterior and posterior portions of mid-gut.

Further experiments were performed to verify the above-mentioned conclusion. After feeding the labeled leaves with radiophosphorus for one hour, the larvae were ligated at points of appropriate boundaries containing each portion of the alimentary canal, and later, each section had blood drawn at certain intervals, immediately, at 3 hours, 6 hours or 9 hours after ligature. Immediately after ligature, no difference was detected in blood radioactivities of each portion. But, when blood was examined at 3 hours or later after ligature, the radioactivities of blood in the sections containing the anterior and posterior portions of mid-gut became extremely higher than those in the sections of the middle portion of mid-gut and of the hind-gut (small intestine, colon and rectum). From these facts it seems possible to infer that the anterior and posterior portions of mid-gut are more active for phosphorus absorption than other portions of the alimentary canal are.

In order to determine the portion of excretion in the alimentary canal, several techniques were devised. One of the most decisive experiments was that the ligature was made at the appropriate boundary of each portion of the alimentary canal, and then radiophosphate solution was injected into the fluid of each body section. After the lapse of 3 or 6 hours after treatment the ratios of radioactive counts of the content in the gut against those of blood in each section were examined carefully. The middle portion and, next to it, the posterior portion were exceedingly active for secretion of radioactive phosphorus, in comparison with the anterior portion of mid-gut and the hind-gut.

As known by the entomologists, insects, including the silkworm, excrete a great deal of metabolites both through Malpighian tubules, and partially through mid-gut and hind-gut. These secreted substances can not be divided easily. Moreover, in the silkworm larva these substances are excreted together with the non-digested substances in the form of faeces. Therefore, the amount of absorption and excretion of phosphorus in the 5th larval studium was measured after the complicated procedure described below.

At first, the percentages of phosphorus absorption were obtained in the following order: (1) the total phosphorus amount administered orally, (2) the total amount of phosphorus in faeces excreted and (3) the increasing phosphorus amount in the body on the whole, were measured every day. Then, from the ratio of the specific activity of phosphorus in faeces against that in blood (especially the ratio of those of inorganic phosphorus in both materials), the arbitrary absorption amount of phosphorus was calculated. From the procedure mentioned above the percentage of phosphorus absorption was easily obtained, that is, it was about 85% at the 1st day of the 5th instar and then decreased gradually day by day, falling to about 40% at the last day of the larva.

From the above-mentioned results, the excretion amount of phosphorus of one silkworm larva was obtained according to the formula:

(Excretion amount of P per day)
= (Absorption ratios of P)
× (Deglutition amount of P per day)
— (Increasing amount of P in the whole body per day)

The results obtained from the formula are shown in Table IV.

Thus, by using the radiophosphorus, the excreted amounts of phosphorus were found for the first time experimentally. The reason why a great deal of phosphorus is excreted in the later days of 5th instar, is due, on the one hand, to degradation of the silk glands and alimentary canal themselves, and on the other hand to diminution of absorption by the silkworm larva itself.

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The Application of Carbon-14 to Studies on Bacterial Photosynthesis

By Jack M. Siegel,* USA

The role of organic substrates in bacterial photosynthesis is obscured because they function both as sources of carbon and as external hydrogen donors. In particular, the influence of organic substrates on the energy requirements in bacterial photosynthesis has remained largely unexplored. Progress in this field has been deterred by the lack of information about metabolic pathways. A major obstacle to the study of metabolic pathways in the photosynthetic bacteria is created by their singular ability to convert organic substrates to bacterial protoplasm and CO_2 as the sole products. Consequently such studies had to await the availability of carbon-14-labeled compounds.

The present paper is divided into three sections. The first section describes some investigations leading to the elucidation of the metabolic pathway for acetone. The second section deals with studies on energy transfer reactions that take place during the metabolism of acetone and its metabolic intermediates. The third section is devoted to preliminary studies on energy requirements for the photometabolism of organic substrates with special reference to acetone and its intermediates.

A special strain of the photosynthetic bacterium, *Rhodopseudomonas gelatinosa*[1] was used throughout these investigations.

PATHWAY FOR THE METABOLISM OF ACETONE BY *RHODOPSEUDOMONAS GELATINOSA*†

Three alternative pathways were considered for the metabolism of acetone by *R. gelatinosa*:

$$CH_3COCH_3 + C^{14}O_2 \xrightarrow{(a)} CH_3COCH_2C^{14}OOH \xrightarrow{(b)} 2CH_3C^{14}OOH \quad (1)$$

$$CH_3COCH_3 \longrightarrow CH_3COOH + \text{“HCOOH”} \quad (2)$$

$$CH_3COCH_3 \longrightarrow CH_3COOH + C_1 \xrightarrow{+\ C^{14}O_2} CH_3C^{14}OOH \quad (3)$$

These could be distinguished with the aid of carbon-14-labeled CO_2. Of the three pathways only the first and third would result in the formation of carboxyl-labeled acetate, and only the first pathway would result in the formation of carboxyl-labeled acetoacetate.

The studies on the photosynthetic pathway were aided by the finding that acetate accumulated during the photometabolism of acetone at low light intensities. As the light intensity approached zero, the relative accumulation of acetate approached a value of 2 moles of acetate per mole of acetone metabolized. This phenomenon made it unnecessary to add acetate as a trapping agent, and thus permitted a direct comparison of the specific activities of the CO_2 and the acetate.

Experimentally it was found that significant amounts of carbon-14 were incorporated into acetate only when the $C^{14}O_2$ was metabolized in the presence of acetone. In such cases all of the activity resided in the carboxyl group of the acetate. The observed values for the ratio of the molar specific activities of acetate to CO_2 ranged from 0.43 to 0.52, which were in good agreement with the value of 0.5 predicted by Equations 1 and 3.

To study the incorporation of $C^{14}O_2$ into acetoacetate it was necessary to add acetoacetate as a trapping agent. Under these conditions acetate again was found to accumulate. As in the previous experiments significant amounts of carbon-14 were incorporated into acetoacetate and acetate only when the $C^{14}O_2$ was metabolized in the presence of acetone. Degradation of the acetoacetate established that 90% of the activity resided in the carboxyl group and the remainder was located in the carbonyl position. The specific activity of the acetoacetate carboxyl carbon was found to be 1.4 to 1.7 times the specific activity of the residual acetate. The theoretical ratio for the formation of acetate from acetoacetate according to the first pathway is 2.

These results indicate that the photometabolism of acetone by *Rhodopseudomonas gelatinosa* can best be represented by Equation 1. Similar studies on the dark aerobic metabolism of acetone by this bacterium indicated that the same metabolic pathway was followed.[3]

INFLUENCE OF ORGANIC SUBSTRATES ON ENERGY REQUIREMENTS IN BACTERIAL PHOTOSYNTHESIS

Free energy changes have been calculated for the individual steps in the metabolism of acetone.[2] The carboxylation of acetone (Equation 3a) has a positive

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† The results of these studies were published previously[2,3] and are reviewed here only for the sake of completeness.

ΔF of about 10 kcal and requires radiant energy or oxygen. The splitting of acetoacetate to acetate (Equation 3b) has a large negative ΔF and proceeds in the absence of light or oxygen. The subsequent metabolism of acetate also must have a positive ΔF because either light or oxygen is required. The experiments that follow demonstrate that energy which is derived from the decomposition of acetoacetate is conserved and can be utilized for the carboxylation of acetone or for the assimilation of acetate.

Table I. Metabolism of Acetone-1,3-C^{14} Induced by Acetoacetate ($AcAcO^-$) Under Anaerobic Conditions in Darkness

$AcAcO^-$ added μmoles*	Carbon-14 activity in Acetate, cpm	Cells, cpm	Total, cpm	Acetone metabolized, μmoles*	$AcAcO^-$ acetone
50	2070	30	2100	1.51	33
37	1510	75	1585	1.14	33
25	654	53	707	0.51	49
12	466	32	498	0.36	35
6	228	10	238	0.17	35
None	100	0	100	0.07	—

* Each reaction vessel initially contained 20 μmoles of acetone-1,3-C^{14} having a specific activity of 1390 cpm/μmole.

In the first experiment, the bacteria were incubated with acetone-1,3-C^{14} and various amounts of acetoacetate. The incubation was carried out anaerobically in the dark until all of the acetoacetate was metabolized. The residual acetone then was quantitatively removed and the bacterial suspension was assayed for carbon-14. The amount of acetone metabolized was calculated from the total activity fixed and the molar specific activity of the acetone-1, 3-C^{14}. The results, summarized in Table I, show that acetone was metabolized in proportion to the amount of acetoacetate decomposed. The last column indicates the number of moles of acetoacetate required for the metabolism of 1 mole of acetone. With the exception of a single value of 49, the ratio fell consistently in the range of 33 to 35.

The second experiment was completely analagous to the first except that acetate-2-C^{14} was used instead of labeled acetone. In this experiment, the calculations were complicated because the decomposition of acetoacetate resulted in a dilution of the labeled acetate. Consequently, the average value of the specific activity of the acetate-2-C^{14} was used to calculate the amount metabolized. The results, presented in Table II, show that appreciable quantities of acetate were assimilated in the presence of acetoacetate. The degree of coupling between acetate assimilation and acetoacetate decomposition was much greater than that observed for acetone. The deviation from strict proportionality between acetate assimilated and acetoacetate decomposed is believed to result from incomplete equilibration of the acetoacetate and the acetate-2-C^{14}.

The reactions responsible for the observed energy transfers were investigated by means of cell-free enzyme solutions. The enzyme solutions were prepared by grinding the bacteria with alumina followed by extraction with phosphate buffer and centrifugation of cell debris.

The dependence of acetoacetate decomposition on coenzyme A (CoA) was shown by treating the enzyme solution with Dowex-1 resin. The treatment caused a nearly complete inactivation of acetoacetate metabolism. Addition of CoA restored the activity and a maximum value was attained at about 60 units per milliliter of enzyme solution.

The formation of acetyl coenzyme A from acetoacetate and stoichiometric amounts of CoA was determined by adding hydroxylamine at the end of the incubation period. The complete system contained 22 μmoles of acetoacetate, 5 μmoles of CoA and 10 μmoles of glutathione. The results presented in Table III indicate that about 15 μmoles of acethydroxamic acid were produced instead of the 5μmoles that were anticipated. Subsequent studies with graded amounts of glutathione indicated that the additional 10 μmoles originated from acetyl glutathione. In the second experiment the amount of acetoacetate metabolized was determined in duplicate vessels. The results indicate that one mole of acetylthioester was produced for each mole of acetoacetate metabolized.

Active acetate could also be produced by incuhating acetate and adenosine triphosphate (ATP) in the presence of catalytic amounts of CoA and with hydroxylamine present as an interceptor. Similarly succinate plus ATP resulted in the formation of succinhydroxamic acid. The results, summarized in Table IV, show that the conversion was essentially quantitative.

Table II. Metabolism of Acetote-2-C^{14} in the Presence of Acetoacetate ($AcAcO^-$) Under Anaerobic Conditions in Darkness

$AcAcO^-$ added μmoles*	Carbon-14 activity in Cells, cpm	Non-vol. residue, cpm	Total, cpm	Average spec act acetate, cpm per μmole	Acetate metabolized, μmoles	$AcAcO^-$ acetate
50	1900	1070	2970	552	5.4	9.3
37	1370	525	1895	578	3.3	11.5
25	760	340	1100	597	1.85	13.5
12	324	140	464	645	0.72	16.7
None	28	115	143	700	0.20	—

* Each reaction vessel initially contained 80 μmoles of acetate-2-C^{14} having a specific activity of 734 cpm/μmole.

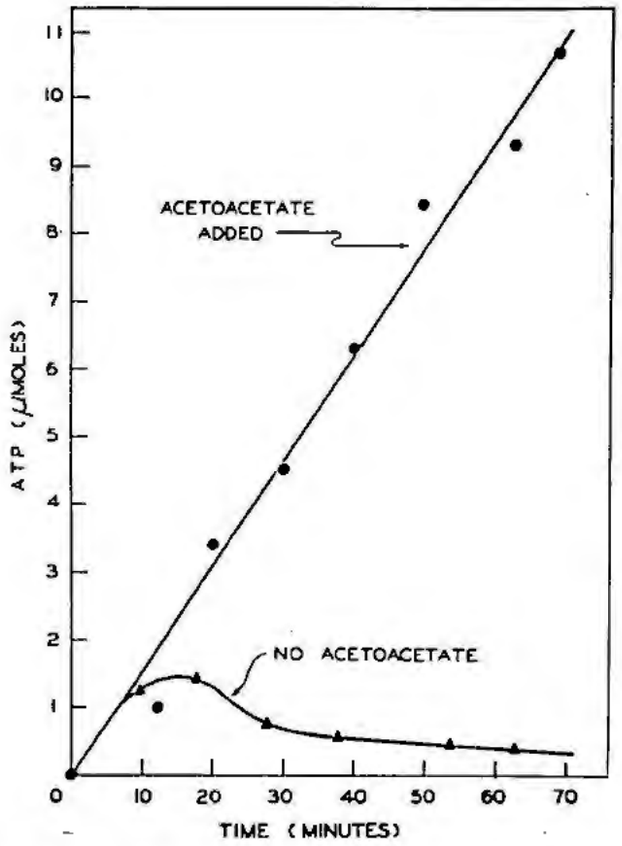

Figure 1. ATP synthesis resulting from the decomposition of acetoacetate. The complete system contained 1 ml of enzyme solution, 20 μmoles of $AcAcO^-$, 10 μmoles of AMP, 10 μmoles of pyrophosphate, and 60 units of CoA. The solution was buffered at pH 7.0 with phosphate and the total volume was 1.8 ml. Incubated at 30°C in evacuated Thunberg tube

The foregoing experiments indicate that the bacterial extracts contain all of the enzymes necessary for the production of ATP from the breakdown of acetoacetate. The extent of this reaction was tested by incubating the enzyme solution with acetoacetate, adenylic acid (AMP), pyrophosphate and catalytic quantities of CoA. ATP was determined specifically by means of the firefly assay system described by Strehler and Totter.[4] The results are presented in Fig. 1. Extensive synthesis of ATP occurred in the presence of acetoacetate but not in its absence. The amount of acetoacetate metabolized after a 1 hour incubation period was determined in a separate vessel and the results indicated that about 1 mole of ATP was generated for each mole of acetoacetate metabolized.

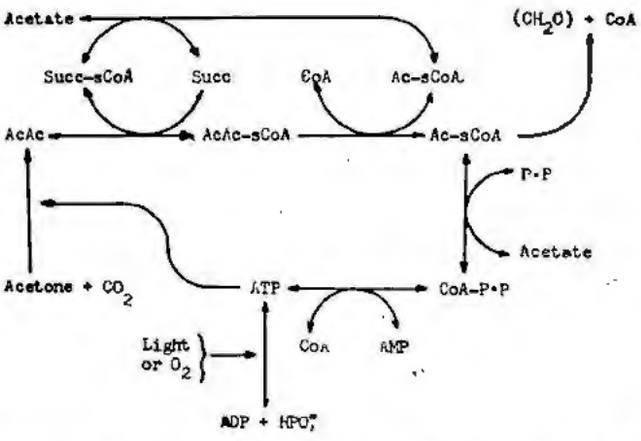

Figure 2. Energy transfer reactions in the photosynthetic bacterium *Rhodopseudomonas gelatinosa*

The various metabolic relationships are summarized schematically in Fig. 2. Starting with acetoacetate it will be noted that there is a net production of 1 mole of acetyl CoA for each mole of acetoacetate decomposed. The acetyl CoA can either be directly assimilated or utilized for the production of ATP. In the latter case the ATP may then be utilized for the carboxylation of acetone.

Recently, several investigators have demonstrated a light-dependent synthesis of ATP both in extracts of photosynthetic bacteria[5] and in chloroplast suspensions.[6] In addition, numerous studies could be cited that demonstrate the production of ATP through oxidative processes. In the present investigation it was found that the anaerobic decomposition

Table III. Formation of Acetyl CoA from Acetoacetate ($AcAcO^-$) by Dialyzed Enzyme Solutions of *R. gelatinosa*

Exp. no.	*Additions**	*Hydroxamic acid, μmoles*	*$AcAcO^-$ metabolized, μmoles*
I	Complete system	15.5	
	Minus $AcAcO^-$(acetate added)	1.0	
	Minus CoA	1.3	
II	Complete system	13.4	9.6
	Minus $AcAcO^-$(acetate added)	0.9	—
	Minus CoA	1.5	1.4

* Experiment I. The complete system contained 0.7 ml enzyme solution (28 mg/ml dry wt), 10 μmoles of glutathione, *ca* 5 μmoles CoA and 21.7 μmoles of acetoacetate in a total volume of 1.4 ml buffered at pH 7.0 with phosphate. Incubated 1 hour in evacuated Thunberg tubes at 30°C. Where indicated 20 μmoles of acetate were added. Experiment II. Same as above with the following exceptions: 0.5 ml enzyme and 10.85 μmoles acetoacetate were added and the total volume was 1.1 ml. Where indicated 10 μmoles of acetate were added.

Table IV. Formation of Acetyl CoA and Succinyl CoA from ATP by Enzyme Solutions of *R. gelatinosa* in the Presence of Hydroxylamine

*Additions**	*Hydroxamic acid*	
	Exp. 1, μmoles	*Exp. 2, μmoles*
Acetate, CoA, ATP	9.7	7.4
Acetate, CoA	1.2	0.3
Succinate, CoA, ATP	8.7	6.5
Succinate, CoA	4.0	2.0

* Each vessel contained 1 ml of enzyme solution, 120 units of CoA and 400 μmoles of hydroxylamine in a total volume of 1.6 ml. Where indicated 10 μmoles of acetate, 10 μmoles of succinate, and 10 μmoles of ATP were present. Incubated 2 hours at 30°C under an atmosphere of N_2.

of acetoacetate also results in the synthesis of ATP. The formation of a common energy carrier in all three processes provides an explanation for the competition between light and oxygen that has been observed by van Niel.[7] It also provides a basis for understanding the observed energy coupling between individual reactions occurring in the metabolism of organic substrates.

ENERGY REQUIREMENTS IN THE PHOTOSYNTHETIC METABOLISM OF ORGANIC SUBSTRATES

An investigation of the photosynthetic rate versus light intensity curves was undertaken as a preliminary step in the measurement of quantum requirements for the photometabolism of acetone and acetate. The photosynthetic rates were determined either by measuring Co_2 uptake or by measuring the incorporation of carbon-14 from labeled substrates. Manometric measurements of acetate metabolism were made with the bacteria suspended in a Co_2: $NaHCO_3$ buffer, and were based on the conversion of Co_2 to $NaHCO_3$:

$$CH_3COONa + CO_2 + H_2O \longrightarrow 2(CH_2O) + NaHCO_3 \qquad (4)$$

In a preliminary experiment it was found that the manometric measurement of acetate metabolism was obscured by a fermentative production of acetic acid. The liberation of acetic acid resulted in the displacement of CO_2 from the buffer. Thus at low light intensified CO_2 evolution was observed rather than CO_2 uptake.

To circumvent this difficulty acetate-2-C^{14} was employed, and the amount metabolized was computed from the amount of carbon-14, incorporated in the acid insoluble cell material. Manometric measurements also were made, and the light intensity curves derived from the two types of measurements were normalized at high light intensities. The results are presented in Fig.3. It was anticipated that at very low light intensities the uptake of carbon-14, i.e., the actual utilization of acetate, would fall on the extrapolation of the curve at high light intensities. Contrary to this expectation, the uptake of carbon-14 corresponded very closely to the manometric data at all light intensities.

A similar study was made with succinate-2-C^{14}. The results are presented in Fig. 4. In this case it was found that the uptake of carbon-14, i.e., the actual utilization of succinate, deviated only slightly from linearity at very low light intensities. The manometric data, however, followed the same pattern previously observed with acetate.

Light intensity curves also were determined for acetone-1,3-C^{14}. The acetic acid, produced metabolically from the acetone, and the insoluble cellular material were assayed separately for carbon-14. The radioactivity in the cellular material represents assimilation of acetate while the sum of the radioactivities of both fractions represents the amount of acetone metabolized. The pair of curves obtained by this treatment of the data are presented in Fig. 5. A slight deviation from linearity at low light intensities is observed for the metabolism of acetone, but the assimilation of acetate derived from acetone no longer displays this phenomenon.

The presence of two components in the rate versus light intensity curve for acetate indicates that two separate light dependent processes are involved. From the ratio of the slopes it may be concluded that the process predominating at low light intensities has a quantum yield that is 7 to 10 times greater than the one at high light intensities.

A possible interpretation is that the photometabolism of acetate can take place by two pathways, and one pathway has an intermediate in common with the fermentative process. This assumption leads one to the unpleasant conclusion that the metabolism of acetate by one pathway is 7 to 10 times more efficient than the major pathway.

An alternative interpretation is that the two components represent separate light dependent processes occurring in a single pathway. The metabolism of acetone to cellular material provides a good example of such a phenomenon. According to this point of view the high quantum yield process may consist of a one-step reaction whereas the low quantum yield process may consist of a complex of energy-requiring reactions.

The manifestation of the high quantum yield process under these circumstances may be explained in the following manner. The intercept obtained by extrapolation of the curve at high light intensities to zero light intensity may represent the dark incorporation of acetate that normally occurs as a concomitant of its photometabolism. The deviation

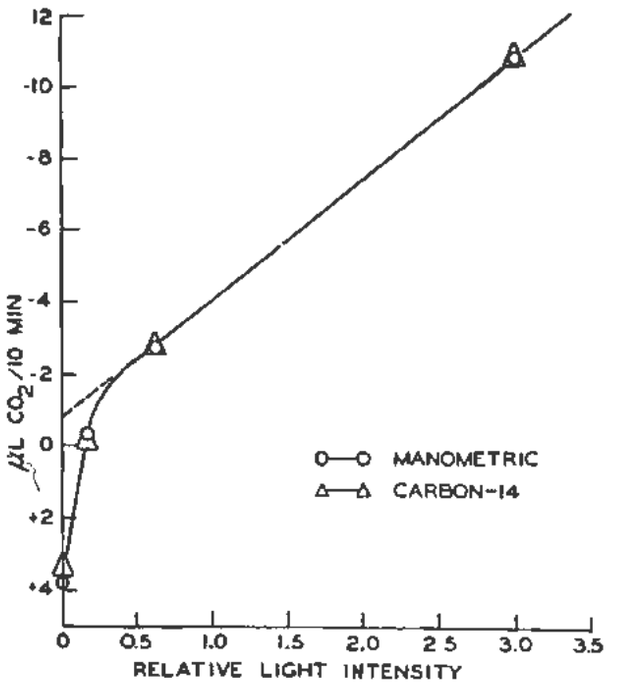

Figure 3. Rate of photosynthesis with acetate-2-C^{14} as a function of light intensity

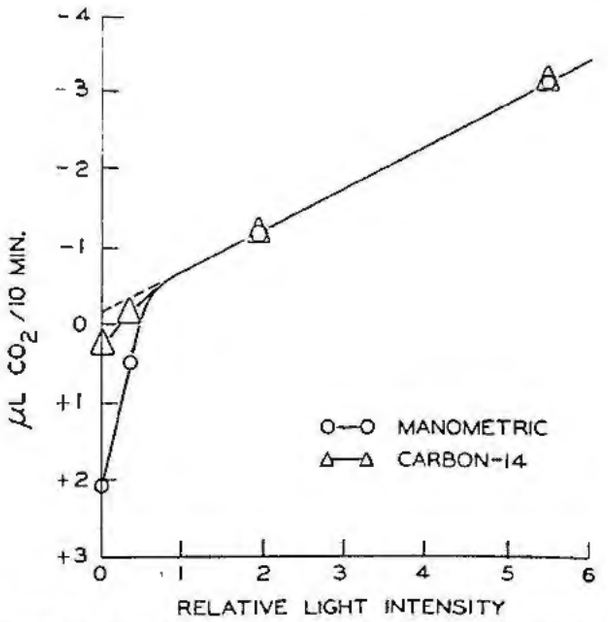

Figure 4. Rate of photosynthesis with succinate-2-C^{14} as a function of light intensity

of the actual uptake from the extrapolated value then would appear to be a suppression of the dark incorporation of acetate. Such a phenomenon could occur if the rate of dark fixation were dependent upon the concentration of an intermediate common to both the photosynthetic and non-photosynthetic pathways. In that event it could be assumed that the concentratinn of the intermediate was nearly zero in the absence of light and increased to some steady state value as the light intensity was increased. In the diagram that follows (Fig. 6) acetyl CoA is proposed as the hypothetical intermediate.

In the absence of light the concentration of acetyl CoA would drop to a low level and reaction 4a would become the rate limiting step in the fermentative production of acetate. When the bacteria are illuminated the concentration of acetyl CoA increases due to the participation of reaction 7. Further increases in the light intensity will cause the concentration of acetyl CoA to increase until a saturation value is reached. The increased steady state concentration of acetyl CoA has several consequences: (1) it increases the rate of reaction 4b thereby diminishing the net fermentative production of acetate; (2) it increases the rate of the acetyl CoA transfer reaction (reaction 3) thereby stimulating the incorporation of labeled acetate into endogenous reserves and (3) it increases the rate of hydrolysis of acetyl CoA. The latter two phenomena should result in a light stimulated dilution of radioactive acetate in the medium. This effect of light has been observed.[8] An increased concentration of acetyl CoA also permits an acceleration of reaction 5. In this case, the rate is governed by the light intensity as well.

This scheme also accounts for the assimilation of acetate during the dark anaerobic decomposition of acetoacetate. The decomposition of acetoacetate would maintain a high steady state concentration of acetyl CoA. This would enable acetate to be incorporated into endogenous reserves by the same mechanism outlined above.

These considerations lead one to the prediction that the light intensity curve for acetoacetate-2,4-C^{14} would consist of a single component whose intercept would coincide with the extrapolated intercept of the acetate curve at high light intensities (see Fig. 3). This prediction will be tested as soon as the labeled acetoacetate becomes available. A partial answer was provided, however, by the experiments with acetone-1,3-C^{14} (See Fig. 5). It was observed that the light intensity curve for acetate-2-C^{14}, produced metabolically from acetone, consisted of a single component with an intercept at zero assimilation. The apparent

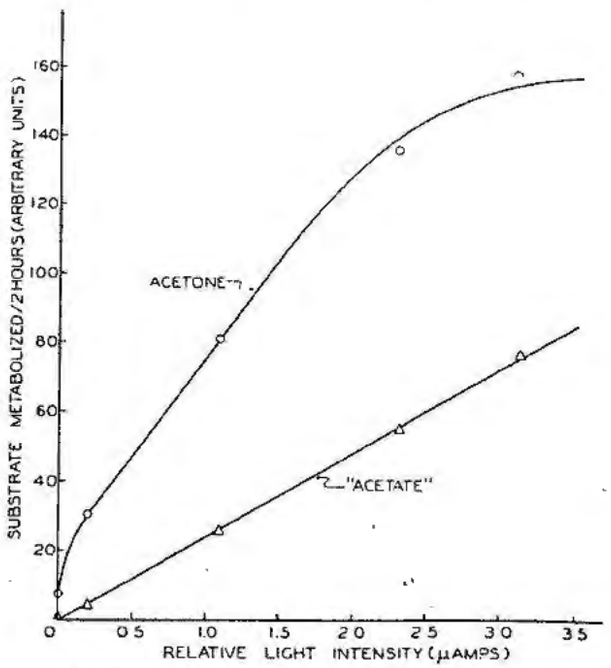

Figure 5. Rate of photosynthesis with acetone-1,3-C^{14} as a function of light intensity

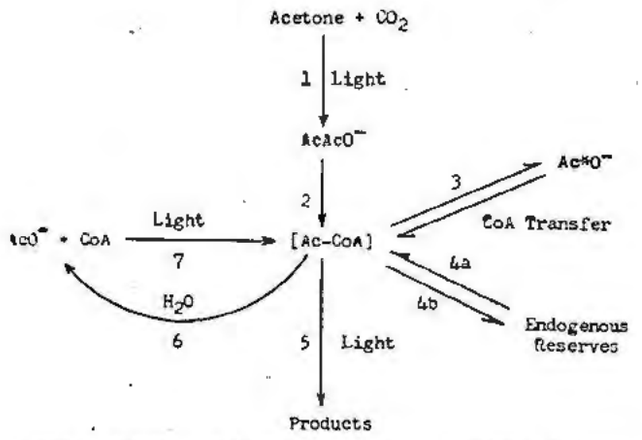

Figure 6. Metabolic relationships with photometabolism of acetate and related substrates

absence of a dark assimilation of acetate in this instance, can be ascribed to the light requirement for the formation of acetate from acetone.

SUMMARY

The metabolism of acetone by *Rhodopseudomonas gelatinosa* was investigated by means of carbon-14 labeling techniques, and its metabolic pathway was elucidated. Acetoacetate and acetate were shown to be intermediates.

Studies with carbon-14 labeled acetone and acetate revealed that energy derived from the decomposition of acetoacetate could be utilized for the carboxylation of acetone or for the assimilation of acetate. Further studies with cell-free enzyme solutions suggested the mechanisms by which this could take place.

Finally, the utilization of labeled substrates in the study of light intensity curves provided the means for relating the energy transfer reactions, noted above, to energy requirements in the photometabolism of organic substrates.

From these studies it may be concluded that during the photometabolism of organic substrates energy is required for discrete reactions that occur in their metabolic pathway. The required energy may be supplied by the absorption of light, by aerobic oxidations or by energy derived from intermediate exergonic reactions of the substrate itself.

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Tracer Atoms Used to Study the Products of Photosynthesis Depending on the Conditions under which the Process Takes Place

By A. A. Nichiporovich, USSR

The plants of various systematic groups are distinguished by the enormous diversity of their biochemical characteristics. There is marked regularity and distinctness in the changes effected in plant metabolism during ontogenesis, or when the plants are grown in different geographic zones, under different conditions of nutrition, light, watering, temperature, etc.

It was and still is supposed that all the mentioned biochemical variety of plants is an outcome of differences in the secondary transformations of the direct products of photosynthesis. On the other hand, the latter are supposed to be alike in all plants or at least to consist of various carbohydrates.[1] On the other hand, there have long been surmises to the effect that the variety of the biochemical peculiarities of plants finds its origin in the variety of the direct products of photosynthesis. Thus, Schimper,[2] Chrapowicki[3] and others believed that besides carbohydrates, the direct products of photosynthesis include proteins and organic acids.

One of the first attempts at experimental demonstration of the variety of the direct products of photosynthesis belongs to V. V. Sapozhnikov.[4,5] He exposed the leaves of plants in an atmosphere of CO_2, illuminated them for 12 to 24 hours, and by chemical analysis established an increase not only of carbohydrates, but of proteins as well. The quantitative ratio between the produced carbohydrates and proteins altered depending on the age of the leaves, the supply of nitrogen-bearing salts, the light intensity and the type of plant. Findings testifying in favour of the possible photosynthetic formation of amino acids and proteins, were likewise obtained by a number of other authors: Lyovshin, [6,7] Kabos,[8] Burström,[9,10] Myers,[11] Pechenitsina,[14] Andreyeva,[12,13] Voskresenskaya,[15,16] Osipova[17,18] and others.

Thus there have long been appearing facts which raised the question of where, at what stages of the transformation of carbon assimilated in the course of photosynthesis, begins the biochemical specificity connected with the characteristics of different plants existing in various physiological conditions and environmental circumstances. One may inquire if this specificity is due to the variety of transformations of some first direct products of photosynthesis common to all plants and universal in their physiological significance—or to diversity in the very earliest stages of the process of photosynthesis and the variety of its direct products?[19,20]

The large theoretical and practical importance of this question cannot be denied. Its solution must determine our concepts of the very content and volume of the process of photosynthesis, of its mechanism and the systems partaking in its operation, the part it plays in the vital activity of the plant, the possible methods and outcomes of influencing plants by regulating the course of their carbon nutrition, etc. But while such problems were being resolved by means of drawing up balances of substances with the aid of conventional chemical analysis, any reliable solution of the question of what are the direct products of photosynthesis was practically impossible. Exposures of the plant leaves were necessarily long and therefore it was not possible to provide solid proof that the observed changes in the chemical composition of the leaves were an outcome of primary syntheses and not of secondary transformations.

Obviously, a decisive role in the solution of the above stated problem could be played by the method of labelled atoms and the use of labelled carbon in particular. Investigations in this direction were begun in the USSR in the Plant Physiology Institute of the USSR Academy of Sciences already in 1950. The first experiments were directed at elucidating the possible variety of ways of inclusion of C^{14} into different groups of substances.

In one of the experimental series,[21] leaves were exposed to light in an atmosphere of $C^{14}O_2$ for 20 minutes. After exposure, the leaves were fixed with 85% ethyl alcohol and successively submitted to extraction by 85% ethyl alcohol, petroleum ether, and boiling water.

The petroleum ether fractions contained only traces of activity and were not considered further. The alcoholic extract, which contained up to 90% of the total activity, was precipitated first by lead acetate and afterwards by copper sulphate. Each of the mentioned fractions contained a mixture of substances, but it may be considered that the alcohol-

Original language: Russian.

Table I. Distribution of C^{14} in Various Fractions Extracted from Different Plants. Leaves Exposed for 20 Minutes to the Light in an Atmosphere Containing $C^{14}O_2$

	Activity in fractions (pc from total)				
			Alcoholic		
Plants	Insol.	Water	Precip. with lead acet.	Precip. with $CuSO_4$	Others
Phaseolus vulgaris	3	36	51	5	5
Asclepias cornuti	4	20	73	2	1
Brassica napus	5	19	70	4	2
Nicotiana rustica	5	10	82	2	1

soluble proteins were in the precipitate with lead acetate; the alcohol-soluble carbohydrates were mainly concentrated in the precipitate obtained from the alcohol fraction with copper sulphate; the water fraction mainly held starch, dextrines, and water-soluble proteins; the insoluble residue contained cellulose, proteins insoluble in water and alcohol and others.

The distribution of the radioactivity for various plants is given in Table I. The data in the table show that the transformations of the carbon assimilated by plants in the process of photosynthesis are very diverse. This is obvious, for instance, from the sharp differences in C^{14} distribution in the water fraction and the precipitate obtained by using lead acetate. In 1955, Norris *et al.*[22] published a paper in which chromatographic analysis of photosynthesic products obtained after 5 minutes' exposure of the photosynthesic organs of many plants of various systematic groups likewise established considerable differeoces in the nomenclature of their products.

Our laboratory has found differences in the distribution of C^{14} calculated for substances of the alcohol fraction when the leaves exposed were young and intensively developing, as well as in mature leaves[21] (Table II). The principal concentration of activity in the young leaves was in the precipitate caused by lead acetate and, in adult leaves, in the precipitate from $CuSO_4$. These findings show a more intensive incorporation of C^{14} into proteins in the young leaves, and into carbohydrates in the adult leaves.

Other experiments with sunflower leaves,[15] furnished data pointing at differences in the course of transformations involving the carbon assimilated by photosynthesis under the influence of light with various spectral composition, in particular in the filtered light of an electric lamp (580–720 mμ; "red light") and mercury-quartz lamps (400–575 mμ; "blue light").

In this experiment, the substances taken into account were those extracted with water. One of the fractions included substances precipitated from the water extract with lead acetate, the latter being removed with hydrogen sulphide, while the other was comprised of neutral compounds (carbohydrates) the substances with alkaline and acid properties here being removed by passing them through ion-exchange resins.

The results are presented in Table III, which shows that, as early as three minutes after exposure, considerable differences begin to appear in the distribution of C^{14} in the substances of various fractions. The data of the table indicate that the spectral composition of the light has significant influence on the course of the transformations of carbon assimilated in photosynthesis. Short-wave rays, in particular, even on the 3rd minute of exposure considerably diminish inclusion of C^{14} into the fraction in which carbohydrates are the main component and increase it in the water-soluble prntein fraction (precipitate with lead acetate).

Further investigation[15,16] of the problem has shown that with the exposure (five min) of leaves to red or blue light there is a difference in the distribution of C^{14} among the amino and organic acids. In blue light the fraction incorporated in amino acids is considerably higher than in red light, while the fraction incorporated in organic acids is lower. This may be explained by the more intensive synthesis, in blue light, of amino acids (alanine in particular) at the expense of the organic acids.

A number of experiments clearly show that the course of the transformations of carbon assimilated in the process of photosynthesis are significantly influenced by the conditions of the plant's mineral and particularly nitrogen nutrition.

Figure 1 presents curves characterizing the results of measurement of the counting rate due to radioactive carbon on one-dimeusional paper chromatograms containing substances from an alcohol extract obtained from the leaves of beans cultivated with insufficient and normal nitrogen nutrition and exposed to light and in an atmosphere with $C^{14}O_2$

Table II. Distribution of C^{14} in Substance of Various Fractions from Young and Adult Leaves of Plants Exposed to the Light for 20 Min

	Per cent of activity in various fractions of alcohol extract					
	Precipitation with lead acet.		Precipitation from $CuSO_4$		Residue after precipitation	
Plants	Young leaf	Adult leaf	Young leaf	Adult leaf	Young leaf	Adult leaf
Nicotiana rustica	87	56	10	39	3	5
Zea mays	83	53	3	40	6	7
Asclepias cornuti	43	34	51	59	14	7
Sorghum vulgare	73	72	22	24	5	4

for 5 or 20 minutes. The data presented show that intensified nitrogen nutrition causes a number of characteristic alterations in the transformations of photosynthetically assimilated carbon. Thus, we see that the trend of the aforementioned transformations is rapidly and significantly altered, depending on a multitude of conditions, i.e., both on the type, nature and condition of the plant itself and on environmental conditions.

But even the experiments described above, where the plants were exposed from 30 seconds to 20 minutes, do not completely solve the question as to which of the observed phenomena must be referred to photosynthetic carbon transformations and which to secondary nonphotosynthetic transformations.

Certain clarity may be brought into the question by the data of N. G. Doman *et al.*[23] who found marked differences in the distribution of C^{14} in substances of various fractions from the leaves of different plants exposed to the light for one second. Even in this short period, the distribution of C^{14} in the substances of various fractions from various plants is different. Likewise varying was the redistribution of the assimilated C^{14} in fractions during subsequent exposure to light in an atmosphere with $C^{12}O_2$ for 4, 14, 29, 59, 299 seconds.

The diversity of products formed in the leaves of various plants exposed for one second is also revealed by ionophoretic separation of substances on paper with the subsequent production of radioautographs. Thus we have reason to believe that the quantitative relations of products formed as a result of photosynthetic carbon assimilation may vary beginning at the earliest stages of its transformation, and this testifies to the possible multitude of the direct products of photosynthesis. But in order to make a more definite statement on this subject, it is necessary to give a precise definition of what may and must be considered to be the direct products of photosynthesis, all the more that there may be different interpretations of this problem.

From the purely photochemical or energetic viewpoint the direct products of photosynthesis could

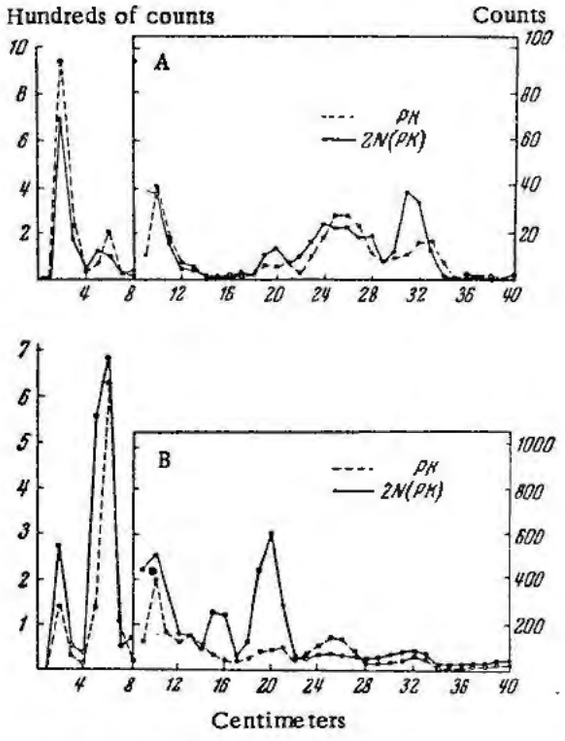

Figure 1. Distribution of C^{14} activity on one-dimensional chromatogram of an extract from the leaves of beans grown at different levels of nitrogen nutrition: A, exposure 5 min; B, exposure 20 min. Approximate distribution of substances on chromatogram: 1–3 cm, spot of application; 5–7 cm, saccharose, arginine, asparagine; 9–11 cm, glucose, asparagic acid, glycine; 14–17 cm, glutaminic acid, treonine; 18–22 cm, alanine, proline; 24–28 cm, trypthophane; 30–34 cm, malic acid

be considered the first products of the reduction of CO_2 or the carhoxyl group which, as a result of photochemical electron or hydrogen transfer, accumulate the energy of the light absorbed by the chlorophyll. Phosphoglyceric aldehyde, for example, might be considered as one of these direct products of photosynthesis and its formation be assumed as the end of the process of photosynthetic transformations, the reduction of carbon and binding of energy

Table III. Distribution of C^{14} in Fractions of Substances from Sunflower Leaves Exposed to Red and Blue Light

Time of exposure sec	Per cent of activity in fractions					
	Precipitated with lead acetate		Neutral		Absorbed in ion-exchange resins	
	Red light	Blue light	Red light	Blue light	Red light	Blue light
	Experiment 1					
5	46	47	7	6	47	46
30	43	41	7	9	50	50
90	12	18	11	11	77	71
180	15	23	50	16	35	61
	Experiment 2					
5	69	40	2	0	28	60
30	72	52	7	4	21	44
90	33	24	14	11	53	65
180	15	22	50	20	40	58

Table IV. C^{14} and N^{15} Content of Chloroplast Proteins: Leaves Exposed to Light and Fed with C^{14}-labelled Carbohydrates or $C^{14}O_2$

	Isotope content of chloroplast proteins					
	Exposure in $C^{14}O_2$		*Exp. in $C^{12}O_2$, leaves enriched with labelled invert*			
			Exp. 1		*Exp. 2*	
Experimental variants	C^{14}	N^{15}	C^{14}	N^{15}	C^{14}	N^{15}
Original condition of chloroplast proteins	0	0.1292	60	0.1140	16	0.0874
Exposure in darkness	0	0.1292	63	0.1026	14	0.0950
Exposure in light	414	0.4940	60	0.1862	14	0.2546

having been accomplished. The subsequent transformations of carbon must proceed at the cost of the plant's usual enzyme systems, so that these transformations seem to give no cause for relating them to photosynthesis proper. The above definition of the boundaries of photosynthesis seems to be logical. Yet it is purely formal and based only on physicochemical criteria. But the process of photosynthesis is first of all physiological and it is this criterion that should prevail in determining its content and boundaries.[20] From the physiological viewpoint photosynthesis is the process of carbon nutrition of plants including the primary formation of organic substances from CO_2 and H_2O and the accumulation in them of the energy of sunlight.

The direct photosynthetic apparatus of the plant, i.e., the place where the main reactions of photosynthesis go on, is the chloroplast. It is in the chloroplasts or on their surfaces that during photosynthesis are primarily formed such substances as sugar and starch which according to classical concept are the direct products of photosynthesis. The chloroplast subsequently supplies and nourishes the cell with these substances which are then used for different purposes and in different ways. Between the main photochemical reaction of photosynthesis and the formation of the first product of the reduction of CO_2 (for instance, glyceric aldehyde) and particularly of carbohydrates, there is a whole more or less complex chain of enzymatic reactions which may likewise take place in the plant in the course of secondary transformations of carbon without the participation of the photosynthetic apparatus.

However, this does not mean that sugar and starch are not direct products when they are primarily formed in the chloroplasts during the photosynthetic assimilation of carbon. In this case they are recognized as direct products of the complicated work of the photosynthetic apparatus of plants, the beginning of which is primary fixation, photochemical reduction of carbon and whose completion is the formation of stable products with definite physiological characteristics, with which the chloroplast supplies the cell. Therefore, if we could demonstrate that during photosynthesis in the chloroplast there other substances are also directly formed (for instance, amino acids, proteins etc.), which further pass into the protoplasm as nutritive or physiologically active substances, we could speak of the diversity of the direct products of the work of the photosynthetic apparatus, as the apparatus of the plant's carbon-nutrition.

It was this problem that our laboratory sought to solve in its works[12,13] with the combined use of the radioactive isotope of carbon (C^{14}) and the heavy isotope of nitrogen (N^{15}). In these experiments the leaves were supplied with ammonium sulphate labelled with N^{15} (through the stalks, by means of infiltration or floating on a solution). After that the leaves were exposed to light for 1–2 hours in an atmosphere with usual ($C^{12}O_2$) or radioactive carbon dioxide ($C^{14}O_2$). After exposure the chloroplasts were separated from the leaves by means of a centrifuge. The proteins of the chloroplasts were carefully purified by successive extraction with alcohol, ether and then treated with diastase to remove starch.[17] Besides this from the centrifugate produced after the extraction of chloroplasts from the leaves cyto plasmic proteins were extracted by heating. The final stage of each experiment consisted in the determination of the presence of N^{15} (excess atom per cent of N^{15} and C^{14} (cpm/mg) in the chloroplast and cytoplasmic proteins.

The experiments showed that during photosynthesis the proteins of chloroplasts are considerably enriched with both C^{14} and N^{15} which testifies to the intensive new formation of amino acids and proteins in the course of photosynthesis. It is important to note that the inclusion of C^{14} into proteins is intensified only if it is supplied to the plant in the form of $C^{14}O_2$ in the course of photosynthesis. But if radioactive carbon is supplied to the plant as part of a carbohydrate, in spite of the intensive formation of proteins in the light (which is testified by the increase in the proteins of a surplus atom per cent of N^{15}) there is no intensified uptake of C^{14} into protein and this observation indicates that the proteins are formed at the expense of the intermediate products of photosynthesis, and not of the available sugars (Table IV).

In other experiments gladiolus leaves, previously kept for 22 hours in darkness, were placed in the light for 2 hours in an atmosphere with $C^{14}O_2$. In this manner the leaves were by natural means enriched with radioactive carbohydrates and other still earlier products of photosynthesis.

Table V. Supply of C^{14} to Chloroplast Proteins from Products of Preceding Photosynthesis

	Activity of osazones (c/mg) at exposure of leaves		Chloroplast protein activity (c/mg) at exposure		N^{15} surplus of atom % in chloroplast proteins at exposure	
	In dark	In light	In dark	In light	In dark	In light
Initial test	2008		25		—	
Exposure 1 h 30 min	1929	656	71	91	0.0000	0.0180
Exposure 3 hr	1984	493	129	120	0.1292	0.5890

After that half of the leaves were deposited on a solution with N^{15} in an atmosphere with $C^{12}O_2$ under light and in darkness. After one and a half or two hours the specific activity of osazones, sugars and proteins obtained from the leaves, and likewise the amount of N^{15} in the proteins of the chloroplasts were determined. The results of the experiments are given in Table IV.

The table indicates intensive photosynthesis in the leaves. This is demonstrated by intensive protein synthesis (increase of N^{15} in proteins under light) and the sharp decrease in the specific activity of osazones (evidently due to the "dilution" of labelled carbohydrates with common ones, forming in the course of photosynthesis) (Table V). However, the chloroplast protein activity did not increase under light as compared with the protein radioactivity in darkness. Therefore, the synthesis of chloroplast proteins proceeds mainly at the expense of the direct intermediary products of photosynthesis. This supplies new proof that chloroplast amino acids and proteins are synthesized in the course of photosynthesis. Another confirmation of this is the sharp suppression of protein synthesis in chloroplasts when photosynthesis is inhibited by phenylurethane which is seen from the data of Table VI.

The summary of such data, which are not exhausted by those cited above, leads us to think that apart from carbohydrates, amino acids and proteins are likewise formed in plant chloroplasts. This formation goes on directly, independent of carbohydrates, possibly owing to divergence of the course of the transformations of intermediary products of photosynthesis. One path in this case leads to the formation of carbohydrates, while the other or others lead to the formation of other compounds and proteins, in particular.

Alteration in conditions of environment, nutrition and the state of the plant itself may change the intensity of the course of photosynthetic transformations of carbon in this or that direction and lead to differences in the qualitative relationships between the direct products of photosynthesis. Thus, for instance, variations in the plant's nitrogen supply have considerable influence on the rate of the photosynthetic formation of proteins in the chloroplasts, which is seen from the rate of the incorporation of C^{14} in Table VII.

In the experiment described, plants of *Nicotiana rustica* were cultivated with both normal and lowered nitrogen supply. The leaves of the control specimens were exposed to light in an atmosphere with $C^{14}O_2$. The leaves of the experimental variant prior to a similar exposure were kept with their stems submerged in $(NH_4)_2SO_4$ for 14 hours.

If at earlier stages of the photosynthetic transformations of carbon — at stages preceding the formation of carbohydrates — the transformation of intermediate products of photosynthesis diverges and leads to the production of a number of stable compounds having independent physiological significance, then we may suppose that apart from carbohydrates and proteins there may be other substances among them as well. In confirmation of the latter supposition are the results of an experiment, where an attempt was made to ascertain the possibility of direct formation of ascorbic acid in the course of photosynthesis (I. V. Ogolevetz). The interest in ascorbic acid is caused by the following:

1. Its close genetic affinity to carbohydrates (Devyatin,[24] Isherwood, Chen, Mapson[25] and others).
2. The availability of numerous data testifying to its formation chiefly in illuminated leaves and localization in the chloroplasts.[26]

Table VI. Incorporation of C^{14} and N^{15} into Chloroplast Proteins under Inhibition of Photosynthesis by Phenylurethane

Experimental variants	Content in chloroplast proteins	
	N^{15} surplus atom %	C^{14} cpm/mg
Exposure of leaves to light in atmosphere with $C^{14}O_2$	0.0407	471
The same with addition of phenylurethane	0.0074	275

Table VII. Incorporation of C^{14} in Leaf Chloroplast Proteins of *Nicotiana rustica* Cultivated with Varying Nitrogen Nutrition

Rate of nitrogen applied in cultivation	Specific activity in chloroplast proteins			
	Controls		With leaves saturated in $(NH_4)_2SO_4$	
	cpm/mg	%	cpm/mg	%
Full	173	100	182	105
½	212	100	466	220
½	284	100	408	145

3. Its possible active role in the process of photosynthesis as one of the components of oxidation–reduction systems participating in the transfer of hydrogen (Krasnovsky and Brin,[27] Arnon *et al.*[28], Luger[29] and others).

The procedure adopted consisted in the following: the leaves were exposed for different lengths of time to light in an atmosphere with $C^{14}O_2$. Later, when the ascorbic acid was identified on paper chromatograms C^{14} incorporation was studied. Curves were drawn based on three points each and were further extrapolated to the zero ordinate on the assumption that the passage of the extrapolated part of the curve through the zero point should testify to the direct photosynthetic formation of the ascorbic acid, while its passage through a point not corresponding to the beginning of exposure, as in particular, through the point where the formation of carbohydrates may be stated to have begun, should indicate its secondary origin, for instance, from carbohydrates. The data in Fig. 2 show that some of the experiments gave kinetic curves testifying to the direct photosynthetic formation of ascorbic acid, as the incorporation of C^{14} in it began simultaneously with initiation of photosynthesis.

At the same time when leaves exposed in the described manner were kept in the dark for four hours a marked shift to the right in the dynamic curves (Fig. 3) was observed revealing intensive subsequent transformations of the ascorbic acid.

Thus ascorbic acid may be considered one of the direct products of photosynthesis, its formation possibly being an important prerequisite for the normal course of that process itself.

At present we do not yet know the details of the origin of the variety of photosynthesis products. Only a number of suppositions may as yet be made to this effect. One of them is that CO_2 is primarily fixed not by one special receptor which it carboxylates, but by several. The further reduction of the carboxyl groups, which may be in several substances,

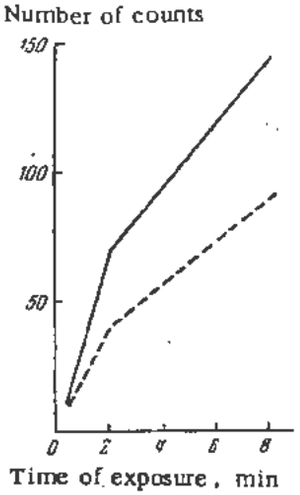

Figure 2. Penetration of C^{14} into asparagic acid in the leaves of gladiolus exposed to light in an atmosphere with $C^{14}O_2$ for various lengths of time

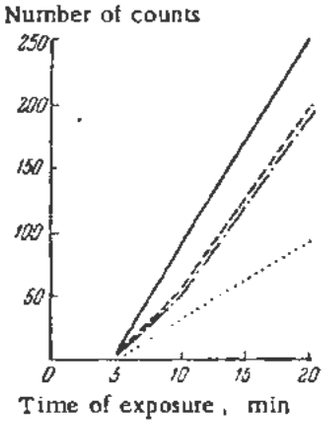

Figure 3. Presence of C^{14} in ascorbic acid from gladiolus leaves exposed to light in an atmosphere of $C^{14}O_2$ for different periods and then held in the dark for four hours

may be one of the starting points of the multiplicity of the direct products of photosynthesis.

A certain confirmation of this viewpoint is furnished by the presence of C^{14} in various substances already after fixation in darkness. It may be supposed that the comparatively simple substances found among the intermediate compounds may be mere fragments of some more complex compounds which are, strictly speaking, the real products of photosynthesis, but which quickly decompose during usual preparatory processing of materials. This supposition, thought it has been formulated by a number of authors[21,30,23] may not be considered as experimentally proved.

The second supposition consists in the following. At a certain stage of the general process of photosynthetic transformation of carbon (which in many details has been successfully elucidated by the American research workers: Calvin, Benson and others), the ways of transformation of intermediate products diverge: for instance, on one hand the transformations proceed along the lines of forming trioses and carbohydrates; and on the other towards the formation of pyruvic, malic, perhaps oxalo-acetic, glycolic acids and corresponding amino acids: alanine, asparagic, serine, glycine.

The second way seems to be the most probable.

Thus, for instance, T. F. Andreyeva,[12] working with hydrolysates of chloroplast proteins after exposure of leaves in an atmosphere of $C^{14}O_2$, observed that C^{14} is mainly incorporated into glycine, serine and alanine.

Interesting data were obtained by D. Racusem and S. Aronoff[31] which show that light promotes the formation of amino acids with branching chains and aromatic rings. Light also had strong influence on the incorporation of amino acids into protein. The relative amounts of various acids entering protein in the light differs from that of amino acids entering in the dark.

From the foregoing one may conclude that the process of photosynthesis is not only accompanied by the formation of several direct products, but that

some of them (for instance proteins) possess specific properties. This problem merits serious attention.

SUMMARY

A large amount of available data shows that the direct products produced by the photosynthetic apparatus may include not only carbohydrates, but also a number of other substances formed in the chloroplasts and translocated from the latter to cytoplasm.

The formation of various products in the photosynthetic organs, the chloroplasts, (omitting the stage of carbohydrates) seems to be biologically reasonable as such synthesis may proceed in extremely favourable energy conditions, because not only enzymes but photochemical reactions may then participate.

Due to the pronounced influence of rays of different wavelength on the assortment of products of photosynthesis, it may be supposed that the complex cycles of the transformation of carbon take place with the participation not only of the main photochemical reaction of photosynthesis leading to the accumulation of energy in products, but also of secondary photochemical reactions not always leading to the accumulation of energy, but directing some of the links of the chain of carbon conversions along specific lines.

Since chloroplasts have different structures and different enzyme systems, and as their condition may alter with age of the plants and the conditions of their cultivation, [32,33,34,35 18] the character and direction of their work as of photosynthetic organs may considerably vary and the cells may thus be supplied with materials varying both in composition and quality.

This view on peculiarities of the plant's photosynthetic apparatus does not alter our concepts on the important and in many cases decisive role of the diverse secondary syntheses, but broadens our information on the nature and procedure of the initial stages of the carbon nutrition of plants and our knowledge about photosynthesis.

Thus, the photosynthetic apparatus not only supplies plants with carbohydrates, the universal and uniform nutritive material. Besides the formation of carbohydrates (which usually play a leading part among the products of photosynthesis), the photosynthetic apparatus forms other important products as well, altering their composition and quality depending on the kind of plants, their state and environmental conditions.

Thereby the photosynthetic apparatus defines the biochemical nature of plant metaholism and determines their complex and diverse relationships with the environmental conditions and especially their reactions to conditions of illumination (intensity, spectral composition, frequency of illumination).

The further development of this important and complicated problem must be conducted with extensive use of the method of labelled atoms.

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The Photosynthetic Cycle

By Melvin Calvin and J. A. Bassham,* USA

Photosynthesis is usually defined as the biochemical reaction

$$CO_2 + H_2O \xrightarrow{h\nu} (CH_2O)_x + O_2$$

This represents the conversion of carbon dioxide and water to carbohydrate and oxygen by green plants in the light. The reaction is separated both chronologically and chemically into two parts:

The photolysis of water

$$H_2O \xrightarrow{h\nu} 2[H] + \tfrac{1}{2}\, O_2$$

and the reduction of carbon dioxide

$$CO_2 + 4[H] \longrightarrow CH_2O + H_2O$$

Each of these two reactions represents a complex series of reactions with many steps. The term [H] is used to denote reducing agents generated in the photochemical decomposition of water. These reducing agents probably undergo several transformations before they are used in the reduction of carbon dioxide.

The reactions involved in the reduction of carbon dioxide have been studied and the results of these studies have been reported in a series of papers on "The Path of Carbon in Photosynthesis."[1,2,3]

The radioactive-carbon isotope, C^{14}, was used throughout this investigation. To a lesser extent, radioactive phosphorus, P^{32}, was also employed.

As a result of this work, it is now possible to write the complete path of carbon reduction in photosynthesis, with all intermediates and enzymatic reactions, from carbon dioxide to sucrose. The study of carbon reduction and its relation to respiratory transformations of carbon compounds has provided evidence regarding the nature of the reactions involved in the decomposition of water and the formation of the primary reducing agents and other energy-rich compounds required for carbon reduction.

FIRST PRODUCTS

The methods used in studying the path of carbon in photosynthesis are here described briefly. In nearly all cases the initial condition is an actively photosynthesizing plant in which photosynthesis has been maintained long enough to establish a "steady state." In this steady-state condition the concentrations of various intermediate compounds in the pathway from carbon dioxide to sucrose are constant. The plants commonly used in these experiments are the unicellular green algae, *Chlorella* or *Scenedesmus*, but leaves of higher plants are sometimes used.

In the first type of experiment to be discussed, $C^{14}O_2$ is added to the unlabeled CO_2 that the plant has been using. After a measured short period of photosynthesis with $C^{14}O_2$, the plant is killed by sudden treatment with boiling ethanol. All enzymatic processes are thereby quickly halted. Extracts of the plant material are made, concentrated, and then analyzed by two-dimensional paper chromatography and radioautography. The techniques of two-dimensional chromatography and radioautography of plant extracts labeled with C^{14} have been described earlier,[1] as well as the identification of the numerous labeled compounds.[1,4,5,6,7] The radioautographs obtained from experiments of 10 seconds and 60 seconds photosynthesis with $C^{14}O_2$ are shown in Figs. 1 and 2. The 60-second experiment illustrates the importance of various sugar phosphates and acid phosphates in carbon reduction. The 10-second experiment shows the predominance of phosphoglyceric acid at short times. If the percentage of C^{14} in phosphoglyceric acid (PGA) of the total C^{14} incorporated during phostosynthesis for various short periods of time is extrapolated to zero time, it is found that at zero time all the C^{14} should be in phosphoglyceric acid. This compound is therefore identified as the first compound into which carbon dioxide is incorporated in photosynthesis.

Figure 3 shows the distribution of the labeled carbon in the three carbon atoms of the glyceric acid obtained from the phosphoglyceric acid in a 15-second experiment. Half of the C^{14} is in the carboxyl group and the other half is divided equally between the other two carbon atoms. From the same experiment some hexose (fructose and glucose) was obtained and degraded. The distribution of carbon in the two 3-carbon halves of the hexose was found to be very much the same as it is in the three carbons of glyceric acid. This result immediately suggests that the six-carbon piece is made from the two three's by joining the two carboxyl-carbon atoms. This is simply a reversal of the well-known aldolase split of fructose diphosphate in the glycolytic sequence, a part of which is shown in Fig. 4. Here the phosphoglyceric acid is reduced with the hydrogen from the photochemical reaction to phosphoglyceraldehyde, which is then isomerized to form dihydroxyacetone phosphate (DHAP). Condensation of phosphogly-

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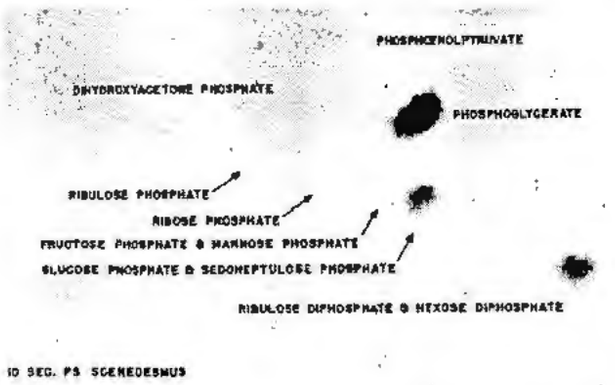

Figure 1. Chromatogram of extract from algae, indicating uptake of radiocarbon during photosynthesis (10 seconds)

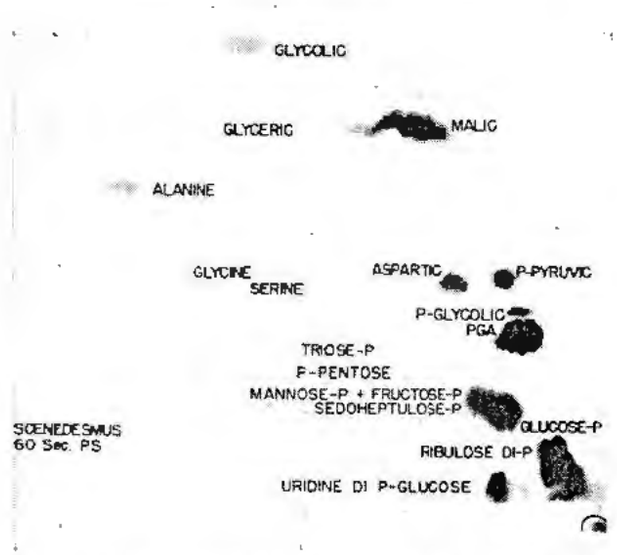

Figure 2. Chromatogram of extract from algae indicating uptake of radiocarbon during photosynthesis (60 seconds)

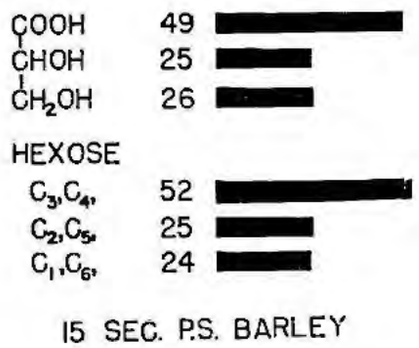

Figure 3. Distribution of labeled carbon in photosynthesis experiments

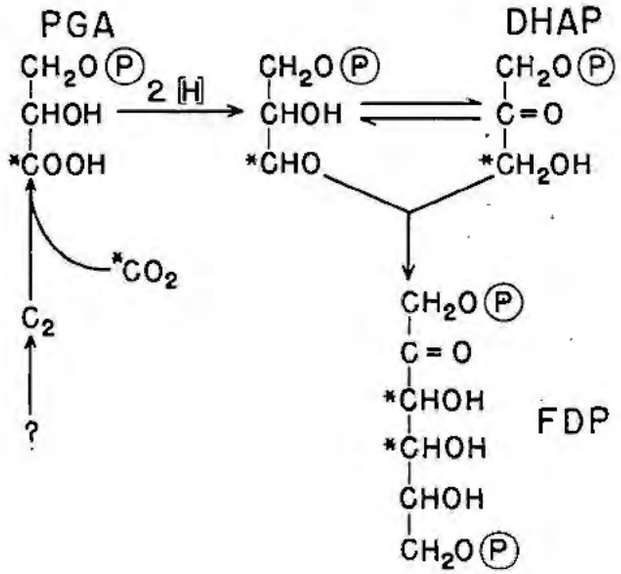

Figure 4. Path of carbon from CO_2 to hexose during photosynthesis

ceraldehyde with DHAP then results in formation of the hexose, fructose-1,6-diphosphate. Thus, the two carbon atoms which were originally carboxyl-carbon atoms finally fall in the middle of the hexose chain. It is quite clear that there must be some compound that accepts the carbon dioxide to form the glyceric acid. Furthermore, that compound must be regenerated from the PGA (phosphoglyceric acid), triose phosphates, and hexose phosphates, or some other compound formed from them. It is thus evident that there is a cyclic process involved in the reduction of carbon dioxide.

Before considering the nature of this cyclic process it is of interest to mention the steps leading from fructose diphosphate to the final product of photosynthesis, sucrose. These steps were identified after the intermediate compounds were isolated by paper chromatography and radioautography. Figure 5 shows the relationship that was found. Here are shown the phosphoglyceric acid, fructose diphosphate, and the various transformations that lead ultimately to glucose-1-phosphate. This compound reacts with uridine triphosphate to make uridine diphosphoglucose. Uridine diphosphoglucose (UDPG) is found on the paper, with the glucose moiety labeled after very short $C^{14}O_2$ exposures. UDPG can then react in one of two ways: either with fructose-1-phosphate to form sucrose phosphate which then is phosphatased to sucrose, or directly with free fructose to form sucrose in one operation. However, since one seldom finds any free labeled fructose, the first of these alternatives appears to be the major pathway for green leaves. An enzyme performing the reaction:

UDPG + fructose phosphate ⟶ sucrose phosphate

has recently been prepared in a partially purified state by Leloir in Argentina. Figure 6 shows the structural formula for the UDPG and its reaction with fructose-1-phosphate. This reaction gives uridine diphosphate and sucrose phosphate with the phosphate on the No. 1 carbon atom of the fructose moiety. The phosphate is then removed to give sucrose. This appears to be the common route to sucrose and is therefore one of the major synthetic

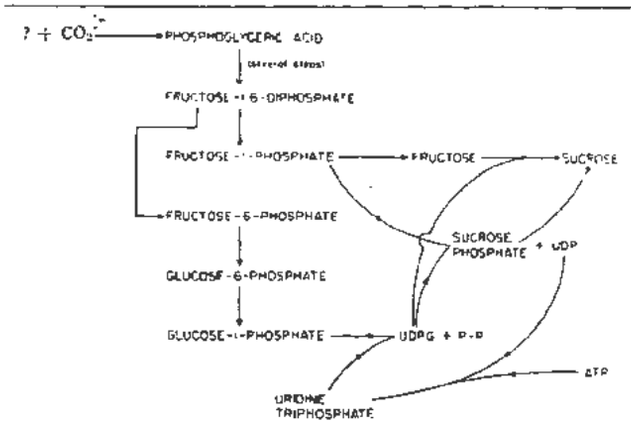

Figure 5. Proposed mechanism for formation of sucrose with uridine diphosphoglucose

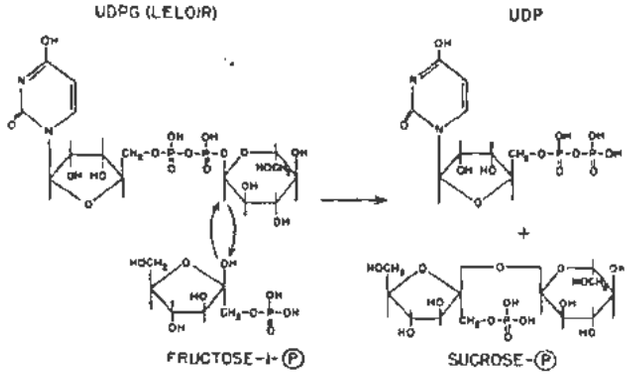

Figure 6. Uridine diphosphoglucose reaction with fructose phosphate

reactions in agriculture, in view of the fact that sucrose provides the substrate for a wide variety of other transformations.

C_5 and C_7 Sugars

We now return to the problem of cyclic regeneration of the carbon dioxide acceptor. The roles of PGA, triose phosphates, hexose phosphates, UDPG, and sucrose have already been identified. Of the compounds labeled by short periods of photosynthesis, there were left only the seven- and five-carbon sugar phosphates. These were sedoheptulose-7-phosphate (SMP), ribose-4-phosphate (RMP), ribulose-5-phosphate (RuMP) and ribulose diphosphate (RuDP).

An attempt was made to determine the order of occurrence of these compounds by the same technique as was used to identify PGA as the first product of CO_2 fixation.

Since the reactions of carbon reduction are so rapid, a flow system was designed to obtain sufficiently short periods of exposure to $C^{14}O_2$ to permit observation of the relative rates of labeling of the various sugar phosphates.[3] The system used is shown in Fig. 7. A suspension of algae was forced by means of a pump from a transparent tank through a length of transparent tubing into boiling methanol. An aqueous solution of $C^{14}O_2$ was injected at a constant rate into the tubing. The time of exposure of the algae to $C^{14}O_2$ was determined by the rate of flow of algae through the tubing and the length of tubing between the point of injection and the killing with methanol. In this way exposure times ranging from 1 to 20 seconds were obtained. When the radioactivity found in each of the sugar phosphates was extrapolated to zero time of exposure, however, no choice could be made between the pentose, hexose, and heptose phosphates. It appeared that all were formed at the same time.

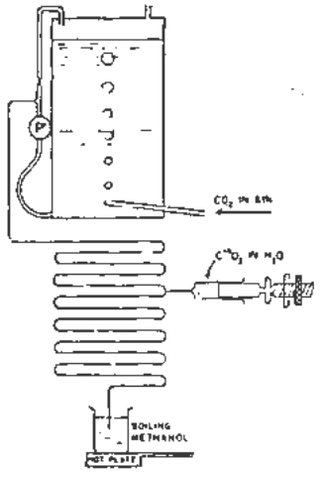

Figure 7. Schematic diagram of flow system for short exposures of algae to $C^{14}O_2$

It was necessary, therefore, to turn to degradation studies of these various sugar phosphates labeled in the very short exposures.

A detailed analysis of the distribution of radioactivity among the carbons of these sugars is shown in Fig. 8. Here, besides PGA, are the five-carbon sugar, ribulose diphosphate (RuDP); the seven-carbon sugar, sedoheptulose phosphate (SMP); and the skeleton of a six-carbon sugar, corresponding either to glucose or fructose (these are the major six-carbon sugars that we find). The stars give some indication of the order of appearance of radioactive carbon in these compounds, and it was from an analysis of these data that it became possible to deduce relationships between the various compounds.

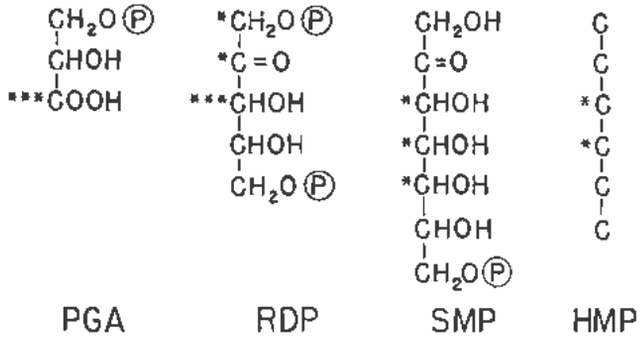

Figure 8. Distribution of radioactive carbon in certain sugars

In much the same way as we deduced the relation between the three-carbon PGA and the six-carbon sugars we were able to deduce the relationships between the five-, seven-, six- and three-carbon compounds that are shown here. It is quite clear at a glance that there is no simple structural relationship between the five- and the seven-carbon compounds and the other sugars. At least, there is nothing as simple as the relationship between the three-carbon PGA and the six-carbon hexose. There is no sequence of carbon atoms in the C_5 or C_7 sugars that could be considered as simply the intact C_3 or the intact C_6, respectively. Until we realized that the C_5 might have more than one origin we were not able to deduce a possible route for its formation. This route is shown in Fig. 9. By taking two carbons off the top of the C_7 and adding them onto a three-carbon piece labeled as is phosphoglyceraldehyde, we would get two five-carbon pieces—one ribulose and one ribose—with their labeling distributed as shown. The average of their labeling would be the actual one found. This evidence, therefore, indicates that the origin of the ribose and ribulose phosphates is in a transketolase reaction of the sedoheptulose phosphate with the triose phosphate to give the two pentose phosphates. These can be interconverted by suitable isomerization. Thus, the pentoses are formed from heptose and triose.

As was shown earlier, the hexose is formed from two trioses. The question then remains: where does the heptose come from? And here, again, a similar detailed analysis was made of the carbon distribution within the heptose molecule as a function of time. This analysis led to the realization that the heptose must have been made by the combination of a four-carbon with a three-carbon piece. The question arose then: where do the properly labeled four-carbon and three-carbon pieces come from? The four-carbon piece could only come by splitting the C_6 (hexose) into a C_4 and a C_2.

This is accomplished by the transketolase enzyme which removes the two "top" atoms from the fructose molecule and adds them to a molecule of glyceraldehyde-3-phosphate to produce a molecule of ribulose-5-phosphate (RuMP). The four-carbon piece that remains (erythrose-4-phosphate) has the distribution of radiocarbon that is required by the observed labeling in the four "bottom" carbon atoms of sedoheptulose.

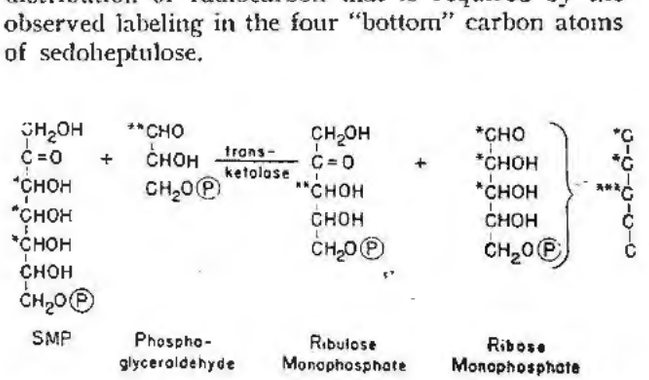

Figure 9. Formation of 5-carbon sugars from sedoheptulose phosphate

The three-carbon piece required for the three "top" carbons of sedoheptulose might be dihydroxyacetone phosphate. In this case the condensing enzyme would be aldolase and the product would be sedoheptulose diphosphate.

Alternately, the three-carbon piece might be obtained by the splitting of hexose by the enzyme transaldolase, which would transfer the three top atoms of fructose-6-phosphate to the four-carbon piece (erythrose phosphate) formed from the four bottom atoms of another fructose molecule. In this case the product would be sedoheptulose-7-phosphate.

It is not possible at present to choose unequivocally between these two possibilities. However, evidence obtained from degradations from various radioactive sugar phosphates isolated from soybean leaves exposed to $C^{14}O_2$ for a very short time indicate that the proposal requiring aldolase may be correct.

These degradation results are tabulated in Table I below.

Table I. Distribution of C^{14} in Sedoheptulose Isolated from Soybean Leaf

	Time of exposure to $C^{14}O_2$	
	0.4 sec	*0.8 sec*
H_2C—OH	0	2
C=O	0	2
HO—C—H	33	39
HC—OH	8	18
HC—OH	49	38
HC—OH	0	2
H_2C—OPO_3H^-	0	2

In either of the alternate sugar rearrangements, carbon atoms No. 4 and No. 5 of sedoheptulose are derived from No. 3 and No. 4 of fructose, respectively. However, in the aldolase version, carbon atom No. 3 of sedoheptulose is derived from carbon No. 1 of dihydroxyacetone phosphate. Alternatively, in the transaldolase version, carbon atom No. 3 of sedoheptulose is derived from carbon No. 3 of fructose and therefore should have the same label at all times. Since the latter condition is not experimentally fulfilled, the aldolase reaction appears to be the correct one. However, it must be noted that this argument rests on the assumption that the concentration of the intermediate erythrose phosphate is small compared with that of fructose-6-phosphate. Also it may be noted that a small amount of labeled sedoheptulose has been obtained from hydrolysis of the sugar diphosphate area, indicating the presence of labeled sedoheptulose diphosphate. The presence of this compound may be accounted for by assuming its formation by aldolase from dihydroxyacetone phosphate and erythrose phosphate. Therefore, this route is tentatively accepted for the formation of sedo-

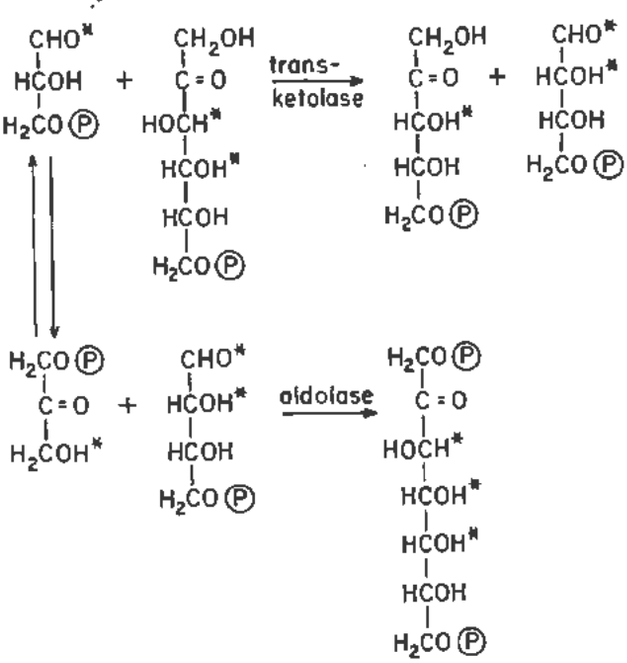

Figure 10. Formation of a heptose from triose and hexose

heptulose. The described transformation is shown in Fig. 10. Here are shown the two trioses that can make one hexose. One hexose then reacts with another triose to give pentose and tetrose, by means of the action of the enzyme transketolase. Tetrose and triose are then condensed by aldolase to give sedoheptulose. The net result of the reactions shown in Figs. 9 and 10 is the formation of three molecules of pentose from five molecules of triose.

Identification of CO_2 Acceptor

All the results thus far were obtained with the first type of experiment, in which $C^{14}O_2$ was added to plants for a very short period (1 to 60 seconds) before the plants were killed. A second type of experiment was used for the identification of the CO_2 acceptor. In this case once again the starting condition was an actively photosynthesizing algae suspension in "steady-state" condition. In addition, the intermediate compounds were "saturated" with C^{14}. This was accomplished by leaving the plants in contact with an atmosphere of C^{14}-labeled CO_2, maintained at constant specific activity and CO_2 pressure, for more than an hour prior to the start of the experiment. Under this condition, the concentration of each labeled intermediate compound can be determined from the radiocarbon found in that compound on subsequent analysis by chromatography and radioautography.

After this initial C^{14}-saturated steady state was obtained, aliquots of the algal suspension were taken at frequent intervals for analysis. Then some environmental condition such as light was suddenly changed. Aliquots of the algae were taken every two or three seconds for about a minute, and then at less frequent intervals. Analysis of these aliquots showed the way in which the concentrations of the various intermediates varied as a result of the environmental change.

In the first such study[2] the light was turned off. It was found that the concentration of PGA increased very rapidly while that of ribulose diphosphate (RuDP) decreased rapidly. The results of a later, somewhat more refined, experiment are shown in Fig. 11.[8] Here it is seen that the concentration of RuDP decreases to below a detectable amount (<1% of its initial value) in about 30 seconds. These changes in concentrations can be accounted for if we assume the following: the reduction of PGA to triose and the formation of RuDP are reactions requiring light; RuDP is converted to PGA via a carboxylation reaction that does not require light.

These relations are shown in Fig. 12. PGA is reduced to triose phosphate (at the sugar level); the triose phosphate then undergoes a series of rearrangements, such as the ones described earlier, through the hexose, pentose, and heptose, back again to the ribulose-5-phosphate. This is all at the sugar level of oxidation and requires very little energy for its operation. There is then some light requirement for the formation of RuDP from RuMP. The reduction of PGA requires both reducing power, reduced triphosphopyridine nucleotide (TPNH), and adenosine triphosphate (ATP), while the formation of RuDP from RuMP requires ATP, as will be seen later. Both these cofactors are produced at the required rate only when the light is on. Thus, when the light is turned off the rate of formation of RuDP and the rate of reduction of PGA decrease. On the other hand, the rate of carboxylation of RuDP to

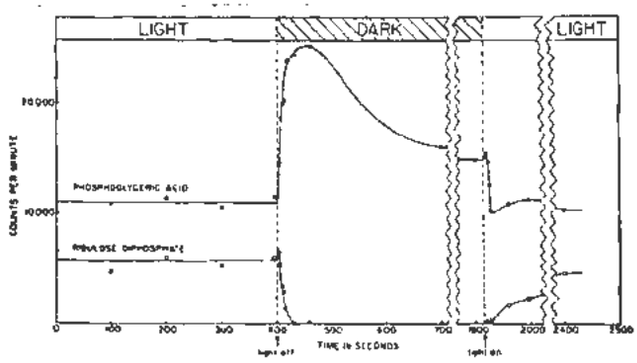

Figure 11. Light-dark transients in PGA and RuDP concentrations

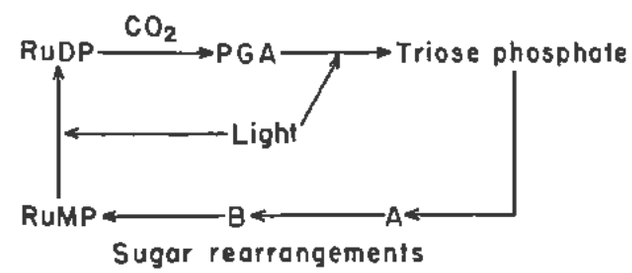

Figure 12. Suggested cyclic scheme for relationships in photosynthesis

form PGA continues unaffected except by the concentration of RuDP.

From the above scheme it was possible to predict the result if the light were left on but the CO_2 pressure were suddenly decreased. In that event, the carboxylation of RuDP to form PGA should decrease but the formation of RuDP and the reduction of PGA should be unaffected. Consequently the concentration of RuDP should rise while that of PGA should fall. This experiment was performed[9] and the expected result, shown in Fig. 13, was obtained. When the CO_2 pressure is decreased, the first compound to increase in concentration is RuDP and the second is its immediate precursor, RuMP. Last to rise in concentration is triose phosphate which is one of the precursors of RuMP. The first compound to decrease in concentration is PGA and the second is its immediate product, triose phosphate. Next to decrease is RuMP, and last to decrease is RuDP. These changes provide excellent confirmation for the proposal of the cyclic system.

There remained some question whether the carboxylation of a molecule of RuDP produced two molecules of PGA or whether some other reaction might occur *in vitro* in which only one molecule of PGA is produced along with a molecule of triose. In order to test this alternative, a rather careful experiment was performed[8] in which the rate of increase of PGA when the light was turned off was compared with the steady-state uptake of CO_2. During the first few seconds after turning off the light, the rate of increase of PGA should approximately equal the rate of its formation during steady-state conditions, provided reduction of PGA could be suddenly halted. The ratio of molecules of PGA increase per second/molecules of CO_2 taken up per second should indicate the number of molecules of PGA actually formed per molecule of CO_2. If this ratio experimentally approached 2 at short times, or even exceeded 1, we would have evidence for the formation of two molecules of PGA for each molecule of RuDP carboxylated. The ratio was calculated from the data showa in Fig. 11 and the measured CO_2 entry rate, and was found to be between 1.5 and 2. Thus, kinetic *in vitro* evidence is provided for the carboxylation reaction

$$\text{RuDP} + CO_2 + H_2O \longrightarrow 2\ \text{PGA}$$

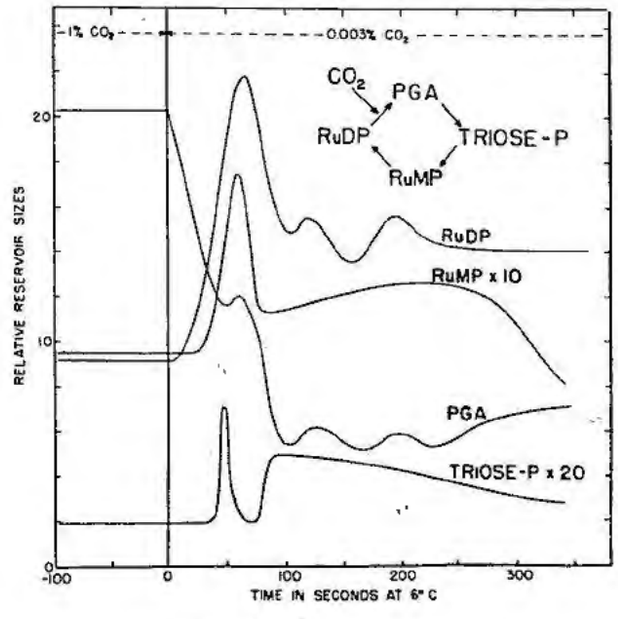

Figure 13. Transients in the regenerative cycle

THE CARBON-REDUCTION CYCLE

The complete carbon-reduction cycle is shown in Fig. 14. Here are shown all the details, including the intermediate compounds and enzymes required for the various transformations. The net result of each turn of the complete cycle is the introduction of 3 molecules of CO_2 and the carboxylation of 3 molecules of ribulose diphosphate, leading to the formation of 6 molecules of phosphoglyceric acid.

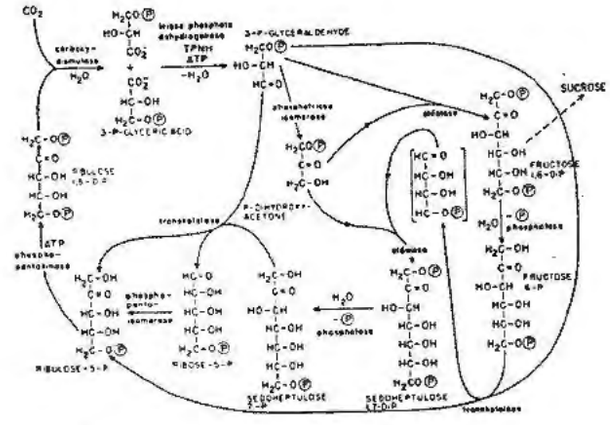

Figure 14. The complete photosynthetic carbon cycle

These 6 molecules of PGA are then reduced to provide 6 molecules of triose phosphate. Of these, 5 are eventually converted to ribulose diphosphate, thus completing the cycle, while the sixth finds its way ultimately into sucrose and represents the net gain in reduced carbon per turn of the cycle. All the enzymes shown had been previously isolated separately except for the carboxylation enzyme which converts CO_2 and ribulose diphosphate to PGA.

Carboxydismutase

About a year ago, using tracer studies, we sought and found a cell-free preparation, both from algae and from other green plants, that was capable of catalyzing the production of PGA specifically from ribulose diphosphate (RuDP) and sodium bicarbonate. The RuDP used in these experiments was isolated by chromatography from green-plant extracts. The technique was to expose the RuDP and the enzyme preparation to $NaHC^{14}O_3$ and show that carboxyl-labeled PGA was formed (Fig. 15). The traces of malic, citric, and aspartic acids and alanine formed indicate the presence in the preparation of

some Krebs-cycle enzymes which could convert some of the PGA initially formed to other compounds. Indeed, upon longer exposure (> three minutes) to these crude preparations, much of the PGA was converted. The formation of a little labeled malic acid in the absence of substrate (RuDP) indicates the presence of pyruvic acid and malic enzyme.

Because in this experiment the tracer was in the CO_2 and not in the RuDP, it did not give direct information about the fate of the five carbon atoms of ribulose. It was therefore necessary to do the experiment with labeled RuDP and unlabeled CO_2. This was not very satisfactory in the first instance when the crude preparation was used. Although labeled PGA was formed, a good many other labeled compounds were formed as well, because of the presence in the preparation of enzymes that could act on ribulose diphosphate and compounds formed from it. In particular there was present a phosphatase which permitted the formation of ribulose-5-phosphate. This compound, in the presence of transketolase and aldolase (and possibly transaldolase), would rapidly find its way into hexose, heptose, and triose. The triose may have given rise to some PGA by oxidation. Although attempts to bypass this difficulty by inhibiting the initial phosphatase reaction on RuDP were partially successful, they were not conclusive, because of the insensitivity of the

$$\text{RuDP} \xrightarrow{HCO_3^-} \text{PGA}$$

system to fluoride ion (F^-). It was therefore necessary to proceed with the attempt to free the preparation from any other enzymes capable of acting upon RuDP except the one(s) required for the PGA-forming reaction (from CO_2). This was accomplished first from neutral extracts of New Zealand spinach (*Tetragonia expansa*), and later from extracts of sonically ruptured algae. The enzyme appears in the protein fraction, salted out of neutral extracts, between approximately 0.3 and 0.4 of saturation with $(NH_4)_2SO_4$. The results of an early experiment with such a preparation acting on labeled RuDP are shown in Fig. 16.[10] Here the fate of the ribulose carbon is clearly its conversion to PGA when both enzyme and $NaHCO_3$ are present. There appears to be some sugar monophosphate present in all the experiments, partly because of its presence in the original RuDP sample and perhaps partly because of the presence of some residual phosphatase in the enzyme preparation. Later experiments have given preparations that convert essentially *all* of the ribulose carbon into PGA and nothing else.

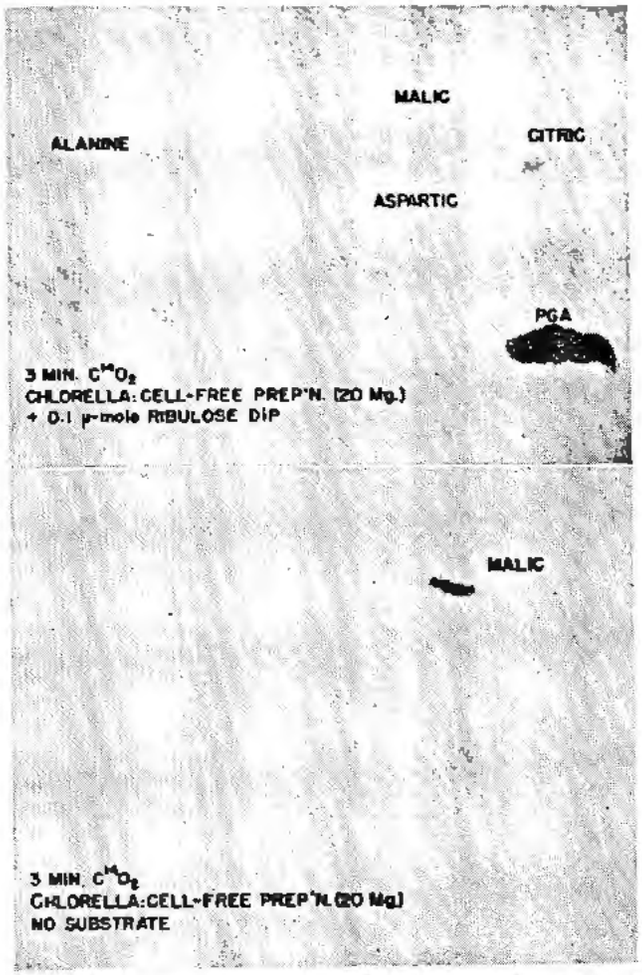

Figure 15. Chromatograms indicating formation of carboxyl-labeled PGA

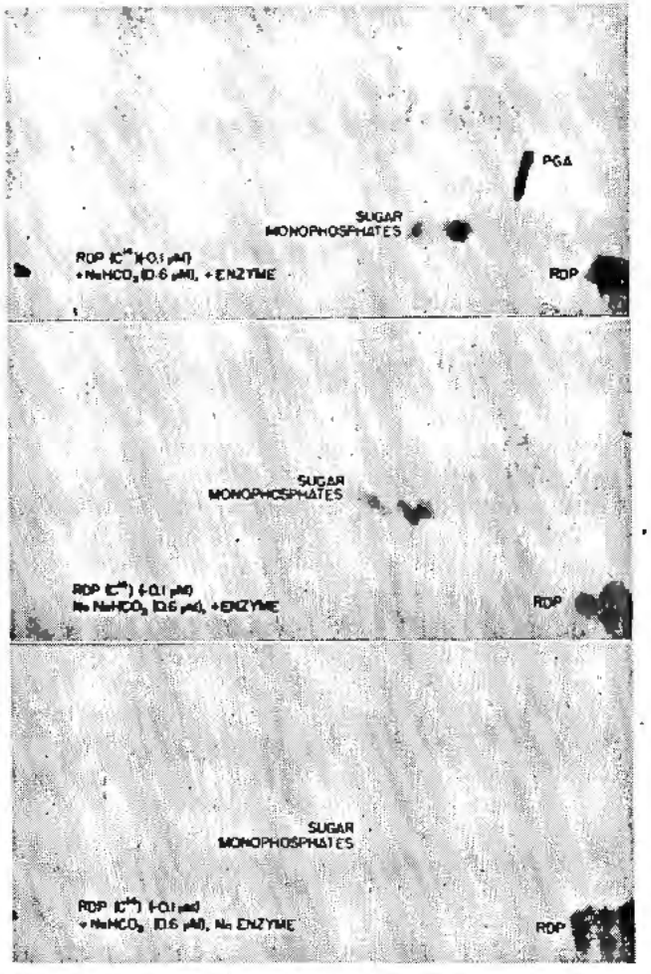

Figure 16. Chromatograms showing effect of enzyme action on ribulose diphosphate

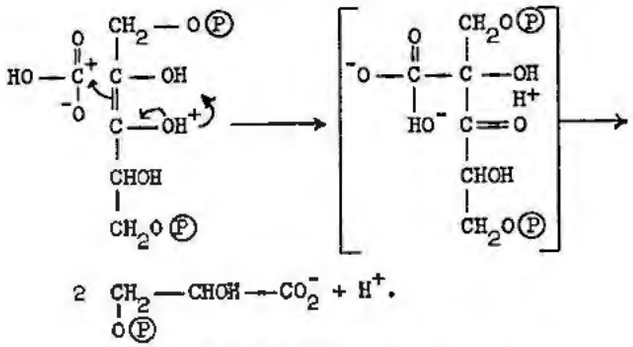

Figure 17. Photoreduction equation

It thus appears that the original formulation of the reaction is at least a likely one (see Fig. 17). Because the carboxylation reaction takes place at the expense of the oxidation of carbon atom No. 3 of the ribulose to the carboxyl level, the name "carboxydismutase" suggests itself as uniquely descriptive. It is interesting to note that the enzyme is not readily demonstrated in animal tissues (rat liver), and that it can be obtained from spinach in association with the highly organized intact chloroplasts,[11] from which it is extremely easily separated. It does not appear to be especially sensitive to versene, *o*-phenanthroline, or cyanide, but it is sensitive to *p*-chloromercuribenzoate, an inhibition that is reversed by cysteine.

Chemical Requirements to Run the Cycle

We now have the cycle in its details (Fig. 14), and we now know precisely what reagents are required to make the cycle turn. It can be seen that the requirement for the reduction of a PGA molecule to a triose is one molecule of triphosphopyridine nucleotide (TPNH) and one molecule of adenosine triphosphate (ATP). The only other energy requirement comes at the point of conversion of RuMP to RuDP, where another molecule of ATP is used. A calculation of energetic compounds needed per CO_2 molecule entering will show that the net requirement for the reduction of one molecule of CO_2 to the carbohydrate level is four equivalents of reducing agent, or four electrons, and three molecules of ATP. The four electrons are supplied by two molecules of TPNH. All these required cofactors must be made ultimately by the light through the conversion of the electromagnetic energy in some way. It must be emphasized that in this requirement for reducing carbon there is no particular requirement for a photochemical reaction other than the production of the two reagents. If we could supply those two things from some other source than the photochemical reaction, we should be able to make this whole sequence of operations function. We have reason to believe that this is indeed being done by the use of the required collection of enzymes. But a suitable situation exists in nature also. The situation is such that we must have simultaneously a high level of this particular reducing agent—which we now know can be triphosphopyridine nucleotide (TPN) and ATP at the same time and the same place.

Running the Cycle Without Light

There is one known system in nature, aside from the green plants, in which that situation occurs. This situation exists in one of the photosynthetic purple bacteria that does not make oxygen, but does reduce carbon dioxide with molecular hydrogen. Figures 18 and 19[12] show that it is possible to have the reduction of CO_2 take place either through the agency of light or through the agency of a chemical oxidation system. The organism is the purple bacterium, *Rhodopseudomonas capsulatus*. The initial slope corresponds to the reduction of carbon dioxide in the light. In this case both hydrogen—as the reducing agent—and light are required. As soon as the light is turned off, the reduction of carbon dioxide stops. Figure 19 shows the same organism. This is a dark fixation. Here it is exposed only to helium and hydrogen, and there is an initial fixation which immediately saturates and stops. When oxygen is then admitted to the system, the fixation again continues in the same way as it does with light. The intermediates in the dark are very much the same as in the light. The hydrogen presumably provides the reducing power that is needed. The oxygen is required to oxidize some of that hydrogen to make ATP, and the two together can make the carbon dioxide cycle function. This suggests that a prime function of the light, in this case where hydrogen is the reducing agent, is to supply the oxidizing agent necessary for the production of the required ATP.

QUANTUM REQUIREMENTS

In order to estimate what a minimum quantum requirement for photosynthesis may be, on the basis of the information we have so far accumulated about the detailed chemistry of the process, at least one assumption is necessary. This is related to the mode of interaction of electromagnetic radiation and matter. It is that a single quantum can excite not more than a single electron. Another assumption

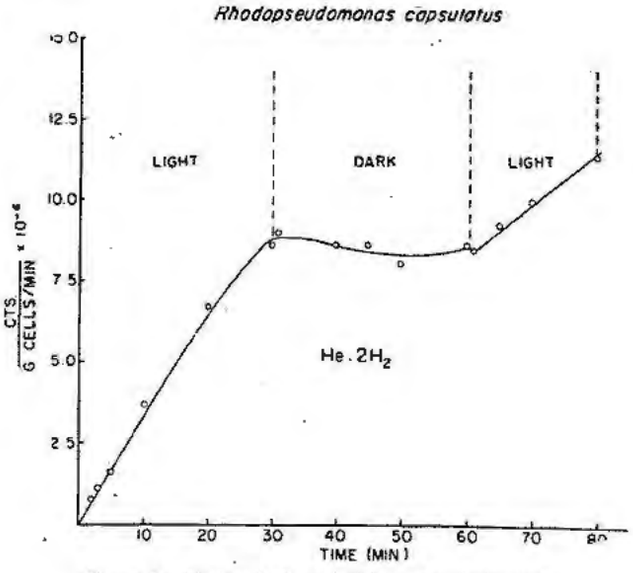

Figure 18. Photoreduction of CO_2 by purple bacteria

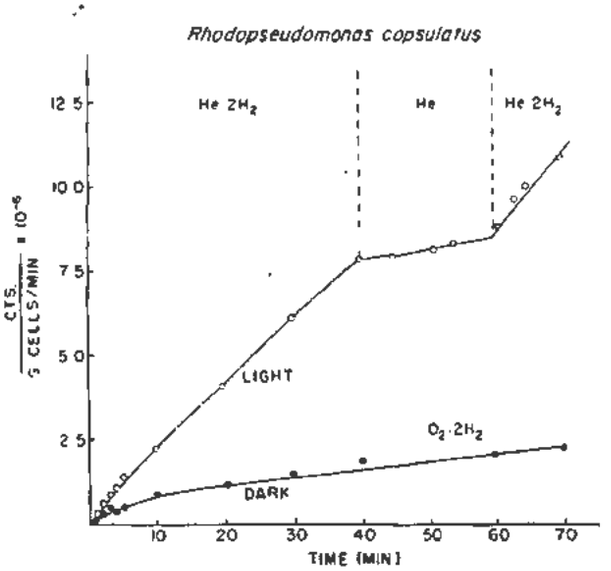

Figure 19. Chemical reduction of CO_2 by purple bacteria

about the behavior of the excited electron is required, namely, that it does not by some chemical (or physical) dismutation process give rise to more than one equivalent of reducing power at the potential of TPNH. And if that is the case, inspection of the requirements mentioned above allows one to predict what the minimum quantum requirement for such an operation would be. Four electrons are needed for the reduction, and three molecules of ATP.

Something about the various ways in which ATP can be produced is already known. For example, during the transfer of two electrons from DPNH to an atom of oxygen, two or three molecules of ATP can be produced. Therefore, one can suppose that when all the energy for the operation of this cycle comes from light, the minimum quantum requirement must be six or seven. That is, four electrons are needed for the reduction and two or three more for the three molecules of ATP that are required. However, it should be possible to find conditions under which the quantum requirement for the reduction of CO_2 and the evolution of oxygen would be as little as four, provided there were some other source besides the light for the three molecules of ATP. These conditions have been realized.[13] The quantum-requirement determination was carried out by use of an apparatus in which one could measure directly, without any ambiguity, the production of oxygen by a direct measurement of a unique quality of the oxygen, paramagnetism, rather than merely by a gas pressure. Also it was possible to measure directly the amount of carbon dioxide absorbed by measuring a property of the CO_2 in the gas phase, in this case its infrared spectrum.

As a result of these measurements, it was found that the quantum requirement ranged experimentally from 7.4 at high light intensities, where photosynthesis exceeded respiration by a factor of 12, to 4.9 at low light intensities, where photosynthesis and respiration were nearly equal. At zero light intensity the value of the quantum requirement extrapolated to four. (This value of four as the quantum requirement at low photosynthetic rates is in no way comparable to the values between three and four reported by Warburg and his associates[14] at very high P/R ratios (>20).) This result indicates that some of the ATP requirement of photosynthesis can be met by reactions of respiration which produce ATP but that the four electrons of reducing agent must be supplied by the light reaction or, with special organisms, by externally-supplied reducing agents.

QUANTUM CONVERSION

So far only the reduction of carbon has been considered. Since this seems to be quite a separate system from the oxygen-evolution reaction, it might appear that one should not expect to learn much about the photoproduction of the electrons and the ATP from studying the carbon reduction. But there must be a connection between the two. By suitable observations it is possible to see at least one point at which the carbon-reduction cycle makes contact directly with the photochemical apparatus. This is shown in Fig. 20. Here the cycle is shown again.

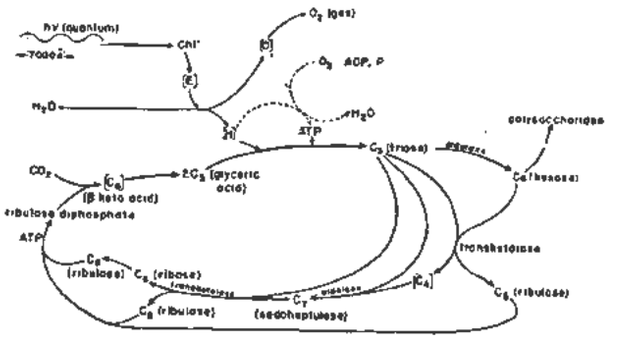

Figure 20. Proposed cycle for carbon reduction in photosynthesis

The quantum is first absorbed by chlorophyll and converts water into something that makes a reducing agent [H] and some oxidizing agent [O]. The reducing agent can reduce the glyceric acid to triose. Some of the reducing agent must be used to make ATP, with oxygen or the intermediates on the way to oxygen, because that is necessary for the cycle to run. What we wish to consider now is this point of contact, [H], between the photochemical apparatus and the carbon cycle and what information about the quantum conversion we can gain from this study.

Light Inhibition of TCA-cycle Incorporation

An experiment was carried out in which a steady state was examined and the changes induced by a sudden change of conditions were observed. Figure 21 shows the result of this experiment. Here is the same type of experiment as before, but with the examination directed toward different substances. Attention is focused on glutamic acid and citric acid, and it will be seen that while the light is on, the rate of formation of radioactive glutamic acid and radioactive citric acid is quite low. But immediately after

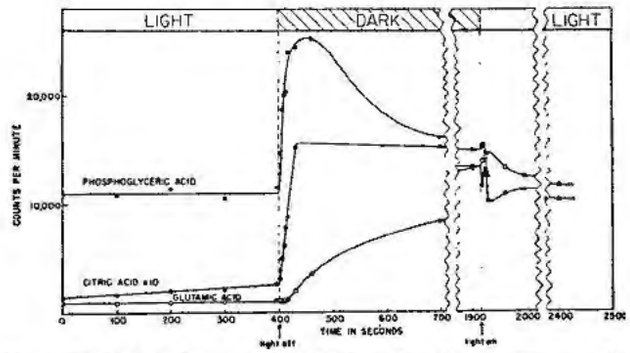

Figure 21. Light-dark transients in PGA, citric acid and glutamic acid concentrations

the light is turned off, the rate of formation of these labeled acids is increased manyfold. Glutamic and citric acids are two compounds very closely related to the respiratory cycle known as the Krebs cycle, and Fig. 22 describes in schematic terms the metabolic relationships leading to the experimental facts we have just seen. Here is shown the photosynthetic cycle and the Krebs (tricarboxylic acid) cycle. The glutamic acid and citric acid are in or related to the Krebs cycle. The photosynthetic cycle does not contain either glutamic or citric acid but does form PGA and sugars. Eventually these direct products of the photosynthetic cycle have to become carbohydrates, proteins, and fats, and ultimately they will get back into the tricarboxylic acid cycle. That is the major route in the light. But immediately after the light is turned off a direct connection between the two cycles is apparently made which allows the PGA to be transformed directly into the compounds of the tricarboxylic acid cycle. Figure 23 shows the details of that mechanism. Carbon can enter the tricarboxylic acid cycle via acetyl Coenzyme A, condensing with oxalacetic acid to give citric acid, thence continuing around this cycle and via a side reaction to glutamic acid. The question is: how is glyceric acid converted to acetyl Coenzyme A? This must happen rapidly in the dark, but not very rapidly in the light. Fortunately we have some idea how acetyl-CoA may be formed from glyceric acid, and Fig. 24 shows this. The glyceric acid is dephosphorylated to form pyruvic acid; the pyruvic acid then reacts with an enzyme system, of which thioctic acid is a coenzyme, to form

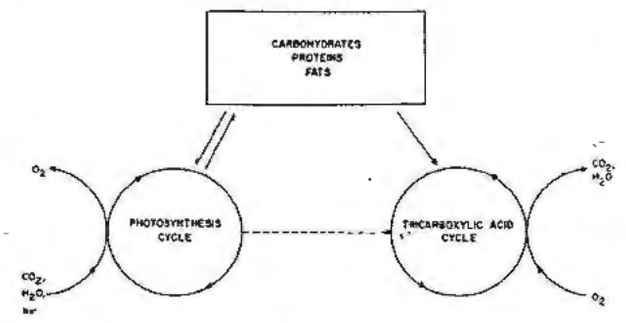

Figure 22. Schematic relationships between the photosynthetic cycle, the tricarboxylic acid cycle, and storage products in the plant

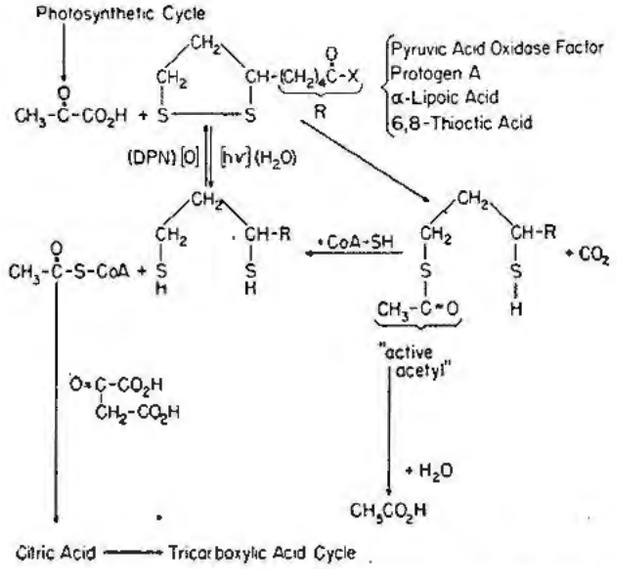

Figure 23. Mechanism of photochemical control of the relationships between the photosynthesis cycle and the tricarboxylic acid cycle

acetyl-thioctic acid and carbon dioxide. The acetyl-thioctic acid then undergoes a thiol ester interchange with CoA to form reduced thioctic acid and acetyl-CoA, which then goes on into the citric acid cycle.

How does light affect these reactions? The conversion of PGA to citric acid provides for the entrance of carbon into the tricarboxylic acid cycle, and if somehow this pathway is closed by reduction of the level of the disulfide, the rate of transfer of radioactive carbon from the photosynthetic cycle to the citric acid cycle will be reduced. This suggests that the light shifts the equilibrium from the disulfide to the dithiol form of thioctic acid by inducing reaction with something other than pyruvic acid, perhaps ultimately water. In the dark, oxidation converts the dithiol form to the disulfide, which can again catalyze the oxidation of pyruvic acid to CO_2 and acetyl-CoA. This system is like a valve that is closed by light, and that controls the flow of carbon from the photosynthetic cycle directly into the tri-

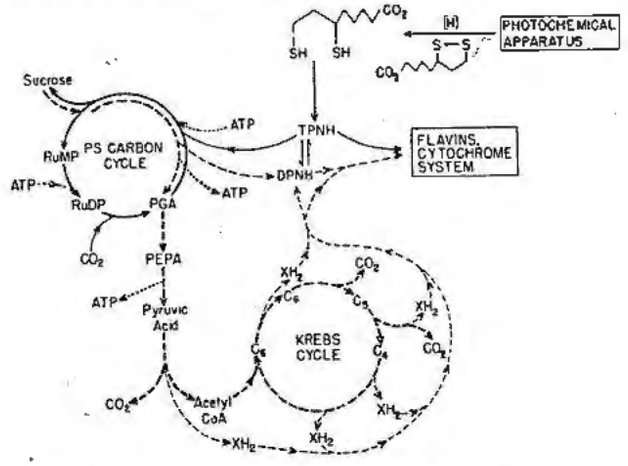

Figure 24. Diagram of the suggested nature of the photochemical apparatus and its relationship to other functions.

carboxylic acid cycle. It suggests further that the disulfide may be closely allied to, if not identical with, the electron acceptor from the photochemical act. Actually a number of experiments have been performed that indicate that this may be so.[16,17,18]

The proposed relations between the photosynthetic carbon-reduction cycle, the photochemical reactions, and the Krebs cycle are shown in Fig. 23. It is suggested that the required ATP is generated by reactions coupled with the oxidation of TPNH or DPNH through the cytochrome system.

It can be seen that the use of radioactive elements, employed as tracers, have made possible the elucidation of the path of carbon reduction in photosynthesis. In addition, information gained from the study of the path of carbon in photosynthesis and its relation to reactions of respiration has provided the basis for proposals regarding the energy transport from the primary photochemical act.

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C^{14} in the Study of the Biosynthesis of Chlorophyll

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Until the development of the labelled atom technique our concepts of the formation of chlorophyll molecules in plants lacked conclusive corroboration. Experimental data had been obtained only by following up the accumulation of plant pigment in the course of ontogenesis and under various environmental influences. After Nensky,[1] Willstätter[2] and Fisher[3] had produced more definite evidence concerning chlorophyll structure it became possible to develop hypotheses regarding the chemical aspects of the process based on analogies with reactions outside the organism. But conclusive evidence could only be obtained by following up the principal stages of the process. This requirement was met by the method of labelled atoms.

Preisser[4] and Pringsheim[5] were the first to observe the appearance of a specific substance with a spectrum similar to chlorophyll in plant leaves raised in the dark. Timiryaziev made a more detailed study of this compound which is rapidly converted into chlorophyll when exposed to light. The conclusion was thus reached that the formation of the pigment molecule proceeds mainly in the dark, undergoing but slight alterations upon exposure to light. Still more detailed studies on this precursor of chlorophyll were carried out by Monteverde,[6] who suggested that it be named protochlorophyll. Monteverde and Lyubimenko found that protochlorophyll accumulates in considerable amounts in the seed envelopes of the Cucurbitae which later on enabled Noack[8,9] and Fischer[10] to elucidate its chemical nature. The identity of this substance wtih the protochlorophyll of etiolated leaves was irrefutably established by Koski and Smith.[11]

Attempts at a chemical interpretation of the first stages of chlorophyll formation were made in the late twenties by Euler[12] and the writers. The constant parallelism in the accumulation of green and yellow pigments in plants as well as the fact that carotenoids precede chlorophyll in etiolated sprouts suggested the possibility (Euler) that the porphine carbon atom skeleton may be formed by closing the isoprene chain of the carotenoids into which nitrogen atoms of nitrous or hydroxamic acid may be incorporated. The idea of the genetic affinity of chlorophyll and carotenoids very interesting in itself was later on confirmed. However, it is much easier to picture the formation of the porphine skeleton, as first pointed out by us in 1926-1928, if we assume the preliminary formation of individual pyrrole nuclei with substitutes appropriate to natural pigments. It may be suggested that these pyrrole nuclei form the ring of a porphine leucocompound which upon further oxidation yields porphyrins. The idea of primary formation of substituted pyrrole nuclei was developed in 1940 by Turner with respect to heme synthesis. These theoretical surmises were finally proved within recent years by the application of labelled atoms to the study of heme synthesis.

Upon administration to animals of N^{15} labelled glycine, Schemin *et al.*[15] found a considerable portion of the isotope in hemin extracted from blood. Wittenberg and Schemin *et al.*[16] showed that, upon degradation of hemin, N^{15} is equally incorporated both into carboxyl-containing and carboxyl-lacking pyrrole nuclei. By applying glycine labelled with C^{14} in methylene as well as in carboxyl, it was shown that eight of the carbon atoms of heme (four in the α-position and four in the methyl bridges) have their source in methylene of glycine. Yet the carboxyl groups of glycine do not take part in the formation of the heme molecule.

Later on it was shown that the remaining 26 carbon atoms of heme originate from acetate, the most important being in this case the methyl group of acetate whereas its carboxyl group gives rise almost exclusively to carboxyl groups of heme. Further elucidation of the process of heme formation in blood was achieved by incorporating into blood of animals succinate labelled in various positions and later, C^{14}-labelled δ-amino-levulic acid.

An important role in this respect was played by the establishment of the chemical structure of porphobilinogen as a monopyrrole with residues of acetic and propionic acid in positions 3 and 4 and with an amino-methylene group in position 2, beside the acetate residue. Still earlier, it has been shown that under certain conditions porphobilinogen outside the body gives rise to uroporphyrin. Dresel and Falk succeeded in observing the formation of porphobilin itself from δ-amino-levulic acid in the hemolysate of chick erythrocytes. Thus all the links of the chain of heme biosynthesis were rather completely established.

The very similarity between the chemical structure of chlorophyll and heme, suggests similar initial stages of their biosynthesis. It was Fischer[10] who first detected the presence of porphyrins in plants,

Original language: Russian.

which in 1940[17] enabled him to suggest that the porphyrins and protoporphyrin in particular, may happen to be the precursors not only of heme, but of chlorophyll as well. Some years later, Granick[18] made the very interesting discovery that large amounts of protoporphyrin together with its magnesium derivative vinyl-theoporphyrin-magnesium a_5 and a number of other porphyrins accumulate in the progeny of various cells of *Chlorella* subjected to roentgen and ultra-violet irradiation. This testifies to the similarity between the initial stages of chlorophyll and heme formation.

Granick[19] carried out interesting experiments in introducing porphobilinogen into *Chlorella* killed by successive freezing and thawing. He demonstrated that a decrease in porphobilinogen content is followed by a proportionate increase in the quantities of protoporphyrin and other porphyrins. However, still very few direct experiments have been carried out with the use of isotopes which would confirm the similarity in the path of synthesis of the chlorophyll and heme molecules prior to the appearance of protoporphyrin.

In 1950 there appeared a short preliminary communication by Salomon *et al.*[20] to the effect that, when acetate and glycine labelled with C^{14} in α-positions were introduced into the nutritive medium of a *Chlorella* culture, the resulting chlorophyll showed noticeable radioactivity. In a more detailed publication the same authors[21] communicated about the addition to *Chlorella* cultures of acetate labelled in methylene and carboxyl, and glycine likewise labelled in various positions. With chlorophyll isolated in the form of crystalline methylchlorophyllid it was shown that the carbon atoms of the above substances are used for the formation of chlorophyll, approximately in the same way as in heme biosynthesis. It was found, however, that carbon of the glycine carboxyl group likewise participates in building up the phorbine skeleton of chlorophyll. In this respect the data obtained by K. Salomon *et al.* are greatly contrasted by events associated with the formation of the blood pigment.

All these findings were obtained with organisms characterised by a peculiar metabolism and belonging to the lowest groups of the plant kingdom. It seemed worth while therefore to experiment with typical higher plants.

We introduced 1-C^{14}-acetate into the cavities of etiolated onion leaves and then illuminated them. After the leaves became green, their pigments were extracted with the aid of acetone. The chlorophyll and carotenoids were further taken up by petroleum ether and separated (after M. S. Zvet) by chromatographing on sucrose. To test the purity of the preparation, the individual pigment zones were eluated and, in addition, chromatographed on paper with a solvent system of methanol saturated with petroleum ether. The paper chromatograms were kept in darkness for a certain time in contact with a roentgen film. The complete agreement of the radioautograph obtained, with the distribution of the colored pigment zones on the chromatogram is indicative of the radiochemically sufficient purity of the preparations.

Table 1. Radioactivity of Chlorophylls A and B from Onion Leaves

No.	*Substance*	*No of exp.*	*Radioactivity of chlorophyll, cpm/mg*
1	Chlorophyll A	2	1×10^6
2	Chlorophyll B	1	2×10^6
	Average		1.5×10^6

As shown by radioactive measurements with the aid of the Geiger-Müller counter a considerable proportion of radioactive carbon of the 1-C^{14}-acetate is contained in chlorophyll. A comparison of the radioactivity of preparations of chlorophyll A and B with their pigment content, as established by means of a Beckman spectrophotometer (model DU), allowed us to calculate the activity of both chlorophyll components. The values of this activity are presented on Table I.

As shown in the table, with the degree of precision obtained, no difference has been noted in the activity of chlorophyll A and B. This indicates the similarity of the biosynthetic chains leading to the formation of chlorophyll A and chlorophyll B molecules in the plant.

Thus, experiments on higher plants likewise confirmed the identity of the initial stages of the biosynthesis of chlorophyll and heme. It seemed necessary further on to carry out a more detailed study of the part played in this process by glycine. The procedure of this experiment differed from the above only in that labelled acetate was substituted by 1-C^{14}-glycine. The results obtained provide a confirmation of the earlier evidence on the participation of glycine caboxyl in the building up of the chlorophyll molecule, although to a considerably lesser degree.

Since in both cases a surplus of the labelled compound was administered to the leaf, which greatly ensured the preservation of its specific activity, the possibility was opened of a direct comparison of the specific activities of chlorophyll preparations obtained in various experiments.

The specific activity of chlorophyll obtained in the glycine experiment amounted to 2.63×10^5 cpm/mg chlorophyll. A comparison of this figure with the average specific activity of chlorophyll obtained with the C^{14}-acetate (3.23×10^6 cpm/mg chlorophyll) shows that the latter is 12.3 times greater than the former. If we consider that the specific activity of the carboxyl carbon of glycine incorporated into the leaves was 2.1 times higher than that of the carboxyl carbon of acetate, the above value increases 25.8 times. This signifies that the carboxyl carbon of glycine is utilized in the biosynthesis of chlorophyll 26 times lesser than the carboxyl carbon of acetate.

The studies on heme biosynthesis revealed that the acetate carboxyl is used only for the formation of two carbon atoms of phorbine. Hence after distribu-

tion among the four pyrrole nuclei (0.025 gram atoms), the glycine carboxyl is used only in the formation of 0.1 gram atoms of phorbine. In other words, according to these data, the glycine carboxyl gives rise only to 0.003 of total carbon of phorbine. This negligible quantity along with the presence of labelled atoms in carotenoids as revealed in the same experiment, is indicative of but an indirect utilization of the glycine carboxyl in the formation of chlorophyll. This provides additional evidence to show the similarity of the paths of chlorophyll and heme biosynthesis.

The entire course of metabolism in the live tissue makes it probable that acetate used in biosynthesis of chlorophyll is derived from carbohydrates. Already in old Russian studies by Palladin[22] and others, there were indications as to the favourable influence of carbohydrate on the accumulation of chlorophyll in the leaves. Although many investigators interpreted this as a mere result of an indirect influence on the part of carbohydrates, it was likewise possible to suggest their direct participation as a source material in the synthesis of pigments.

The method of labelled atoms enabled us to check this assumption in a special series of experiments. Labelled glucose was produced by means of photosynthesis in tobacco leaves (*Nicotiana alata* Link and Otto) in an atmosphere of $C^{14}O_2$ and under conditions ensuring the formation of evenly labelled molecules. This glucose was twice subjected to paper chromatography. The radioautograph of the chromatogram thus obtained indicated the zone of glucose which later on was eluated and used for the experiment.

A typical experiment was conducted as follows. Purified glucose preparation with a total activity of 14,400 cpm was incorporated into etiolated onion leaves. The chlorophyll and carotenoids formed under light, when chromatographed and purified, showed the following radioactivity (Table II).

A comparison between the specific activities of individual pigments obtained in various experiments showed the relative stability of the part which the total activity of the pigment mixture belongs to chlorophyll, xanthophyll or carotene. Whereas, the activity of each individual pigment when related to that of the original substance proved to be greater upon more prolonged exposure to light this is in full agreement with the course of accumulation of pigments during illumination. However, unlike other pigments

Table II. Radioactivity of Pigments after the Incorporation into Etiolated Onion Leaves of Uniformly Labelled C^{14}-glucose, after 48-hr Illumination

Substance	*Radioactivity, cpm*	*%*
Glucose	14400	100.0
Chlorophyll	100.6	0.69
Xanthophyll	35.7	0.25
Carotene	124.0	0.86

Table III. Distribution of C^{14} in the Chlorophyll Molecule

Substance	*Number of C atoms in molecule*	*Radioactivity, cpm*	*%*
Chlorophyll	55	100.6	100.0
Chlorophyllin	35	60.8	60.8
Phytol	20	35.0	34.8

the activity of carotene may have been somewhat exaggerated in these experiments owing to the fact that carotene is not adsorbed by sucrose.

After measuring the total activity of chlorophyll the latter was dissolved in ether and saponified by a 30% solution of KOH methanol. The radioactivity of the chlorophyllin and phytol thus obtained are presented in Table III.

If a calculation be made of the specific activity of the carbon atoms of phytol and chlorophyll on the assumption that all of them equally originate from glucose carbon, very similar figures will be obtained equalling, in the case of phytol, $35.020 = 1.75$, and in the case of chlorophyllin $60.835 = 1.74$. We are not inclined to attach too much importance to this coincidence, yet the order of magnitude of these figures in accordance with the current concepts on the relationship between the paths of biosynthesis of heme, chlorophyll, carotenoids and phytol, should be rather similar.

Our findings[23,24] along with those available in the field of biosynthesis of porphyrins within the animal body, offer a possibility of outlining a hypothetical scheme for the explanation of the mechanism of the participation of glucose in the formation of the phorbine skeleton of the chlorophyll molecule.

In the course of the glycolytic process, or some other process related to it the hexose molecule gives rise to acetate. The latter is involved into the chain of tricarbonic acids, one of the intermediary products of which the succinyl coenzyme A, combines further with glycine, which loses its carboxyl, and this results in the production of δ-amino-levulic acid. The combination of the latter's two last molecules leads to the origination of a primary pyrrole compound, four molecules of which further close into the porphine skeleton of uroporphyrin. Decarboxylation and dehydrogenization of uroporphyrin leads to the formation of protoporphyrin, the introduction into its molecule of an iron atom results in the formation of heme, whereas magnesium stimulates the production of protochlorophyll and then chlorophyll.

The transition from protoporphyrin to protochlorophyll may be regarded as the outcome of the closure of the isocycle between the γ-carbon atom of the porphine skeleton and the α-carbon of the propionic residue in position six of the pyrrole nucleus as well as the oxidation of the β-carbon atom in the same side chain finally hydrogenization of the vinyl residue in position four, possibly due to the fermentative transfer of hydrogen liberated during the above dehydrogenization (Fig. 1).

As regards phytol, the path of biosynthesis of this extremely important alcohol, beyond doubt geneti-

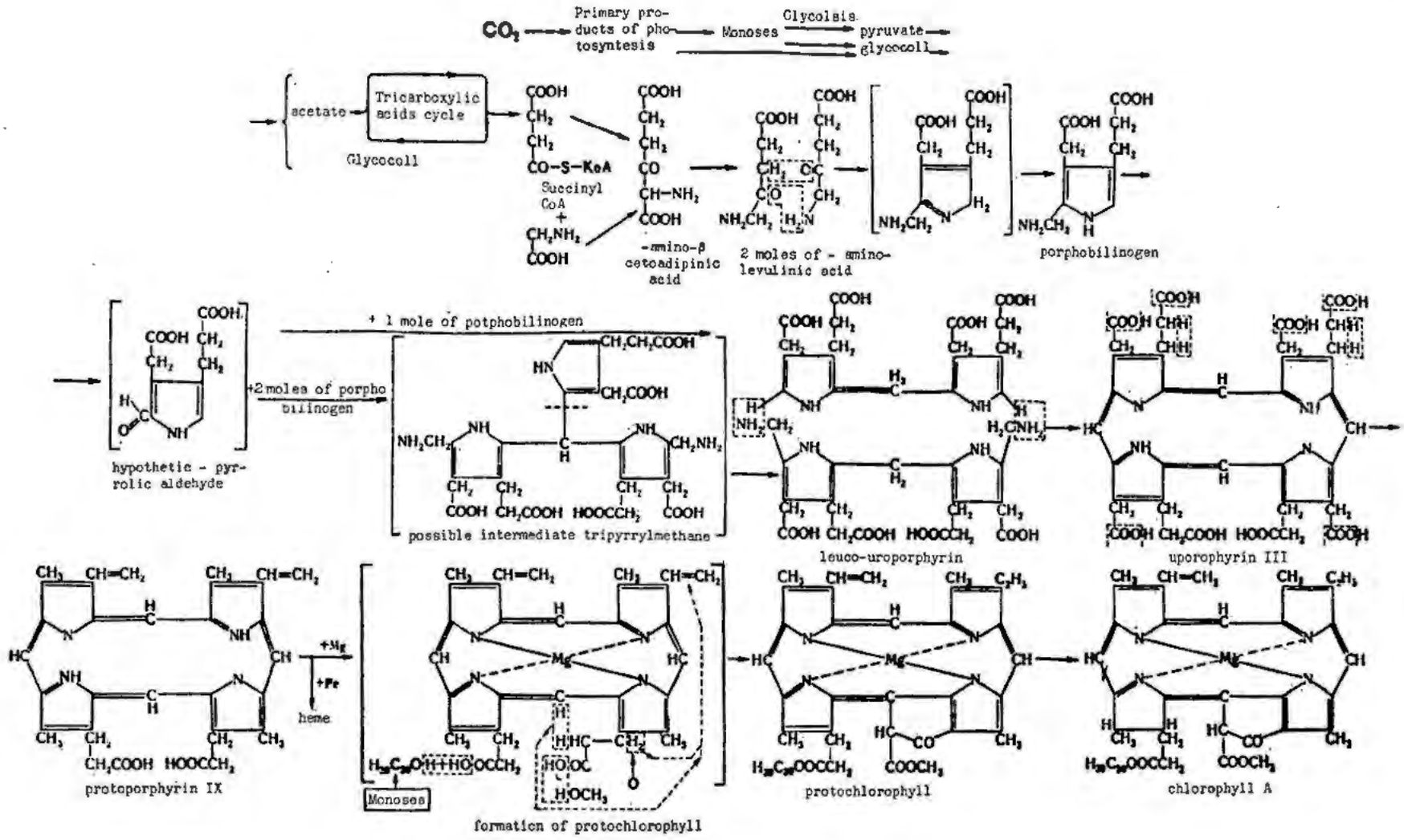

Figure 1. Scheme of chlorophyll biosynthesis

cally associated with the carotenoids as far as we know, had never before been investigated by the above methods of labelled atoms. A number of interesting hypotheses concerning its formation exist.

Thus, Karrer[25] pointed out the possibility of the formation of carotenoids from phytol. On the other hand, Smith[26] and ourselves[27] even in 1930 had put forward the suggestion that phytol molecules may be formed through decomposition and dehydrogenization of carotenoids. With regard to the latter a number of authors have shown the prevailing role of acetate in the formation of their molecules in lower organisms. In our experiments with acetate, we likewise observed the formation labelled carotenoids the assumption is thus justified that the participation of carbohydrates in the synthesis of phytol is ensured by their preliminary transformation into acetate. This confirms our findings as to the nearly equal participation of glucose in the formation of the phorbine nucleus and the phytol chain of the chlorophyll molecule.

All these findings include the biochemical transformations, in the course of which the complex chlorophyll molecule is formed into the general system of reactions of the living cell. The question that arose further was whether the synthesis of chlorophyll proceeds continuously throughout the life of the chloroplast, or whether it is confined to the periods of absolute growth of chlorophyll in the plant. In other words, it seemed necessary to ascertain whether or not the continuous disintegration and formation of new chlorophyll molecules—their renewal—are associated with concomitant variations in the pigment content of the leaves.

If the method of labelled atoms had played an important part in settling the problem as to the sources of carbon utilized for chlorophyll synthesis, in studying the renewal of chlorophyll it presents special advantages. It is only by this method that the newly formed pigment molecule may be distinguished from those formed earlier. It allows also a comparison of their chemical behavior, to find out the regularities underlying the replacement of an old molecule by a new one. It is not surprising, therefore that, of the problems of biochemistry, it was the processes of renewal that long remained unstudied. The idea of chlorophyll renewal had been, for decades, alternately supported or negated by a large number of scientists, but as the discussions did not produce any direct evidence, the problem remained almost as obscure at at the outset. Only the use of labelled atoms offered a new tool to attack this problem.

It was only Turchin[28] and his associates that succeeded in presenting a quantitative estimate of this phenomenon by means of the stable N^{15} isotope. The renewal itself of chlorophyll was discovered qualitatively as early as 1952 by the French scientists Roux and Husson.[30] Thirty-day old winter rye specimens showed a renewal of 45.8% of their chlorophyll in 24 hours. Experiments with sorrel showed a chlorophyll renewal of 95.8% in 72 hours.

Table IV. Radioactivity of Chlorophyll A and B, Extracted from Tobacco Leaves Exposed for 24 Hours to an Atmosphere of $C^{14}O_2$

Substance	*Quantity, mg*	*Activity of chlorophyll, cpm/mg*
Chlorophyll A	3.3×10^{-3}	1.2×10^5
Chlorophyll A	1.7×10^{-2}	1.4×10^5
Average (Chlorophyll A)		1.3×10^5
Chlorophyll B	6.5×10^{-2}	0.5×10^5
Average (Chlorophyll A and B)		1.0×10^5

The object of study in the experiments conducted by ourselves were normal and intensely colored leaves of tobacco (*Nicotiana alata* Link and Otto), in which chlorophyll accumulation had already ceased. For hours they were kept in an atmosphere of $C^{14}O_2$. The chlorophyll and carotenoids were separated by M. S. Tzvet's method on a sucrose column and then subjected to control paper chromography by the above method. The specific activity of these preparations obtained is presented in Table IV.

In order to calculate the actual rate of chlorophyll renewal in the plant, it is necessary to compare the specific activity of carbon in the pigments extracted in our experiments with the specific activity of the carbon of the original compounds.

Assuming 1.0×10^5 cpm/mg as the mean value of chlorophyll activity, we find 1.4×10^5 cpm to be the activity of 1 mg of carbon it contains. With due account for the calculation coefficient of the apparatus and for self-absorption, which in our case amounted to 0.052, the absolute specific activity is found to be 2.7×10^6 disintegrations per minute per mg of carbon. The specific activity of carbon in the CO_2 introduced into the photosynthesic chamber was 5.3×10^7 disintegrations per minute per mg. Thus, the concentration of radioactive carbon in a chlorophyll molecule amounts to 5.1% of the initial concentration. This figure represents the degree of renewal of chlorophyll in the experimental leaves.

The rate of chlorophyll renewal was further calculated according to the equation:

$$\frac{dx}{dt} = k\,(z - x)$$

where x is the specific activity of chlorophyll carbon, z the specific activity of carbon in CO_2, and t is the time of contact between the leaf and $C^{14}O_2$. The equation is easily transformed into the formula

$$k = \frac{-\ln\left(1 - \frac{x}{z}\right)}{t}$$

which may serve for calculating the renewal constant.

The formulas $T\frac{1}{2} = 0.693/k$ and $\tau = 1/k$ enable us to compute the time $T\frac{1}{2}$, i.e., the renewal time of half of chlorophyll available prior to the experiment. In this way the mean length of life of one molecule, τ, can also be computed.

From these calculations, we determined the value of x/z to be 0.051 when $t = 1$ day. Hence we obtain $k = 0.0523$. It follows that the renewal period for half of the available chlorophyll is $T_{1/2} = 13.2$ days, while the mean length of life of one molecule is $t = \pm 19.1$ days.

It is quite possible that the rate of the renewal of chlorophyll may vary in different plants. It may be altered in the course of ontogenesis, as well as by diverse external factors assuming sufficiently high values. This may perhaps explain the experimental findings of Becker and Sheline,[29] who did not succeed in noting any incorporation of the radioactive Mg^{28} isotope into the chlorophyll of the leaves of beans and coleus, although the experiments of these authors lasted more than 11 hours in the first case and 65 in the second.

The results obtained show that the chlorophyll carrying apparatus of the green leaves does not remain stable, the chlorophyll being constantly renewed and decomposed. The synthesis of chlorophyll in the living plants apparently proceeds rather intensively even though the total amount of chlorophyll does not change. The chlorophyll content of a plastid is determined by the relationship between the rates of destruction and formation of its molecules, which in its turn depends on the physiological condition of the cell and the plant as a whole. The carbohydrate metabolism of the leaf provides a continuous supply of material for chlorophyll synthesis, and, vice versa, the decomposing molecules of the latter are, in their turn, used for the formation of other compounds.

The confirmation of the rather high renewal rate of chlorophyll and carotenoids is of great significance. It shows that the plant pigments belong to a group of substances that are not only catalytically very active but also of direct biological importance. This finding substantiates the concept according to which the chloroplast is a mobile system very sensitive to alteration of the internal and external conditions.

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Some Aspects of Sucrose Metabolism in Plants

By H. K. Porter and J. Edelman,* UK

The numerous observations showing that starch formation can be induced in leaves depleted of carbohydrate by floating them on sucrose solutions led to the hypothesis that sucrose was an intermediate in the pathway of starch synthesis. This idea received support from the fact that changes in starch and sucrose in intact plants frequently occurred concurrently, as for example during the diurnal fluctuation of carbohydrate in leaves in the light, and the loss of starch and appearance of sucrose in some ripening fruits. However starch synthesis can also be induced from hexoses and other related substances so that it remained open to doubt if sucrose was an essential step, and it was evident that very active sugar interconversions could take place. By using sugars labelled with C^{14} it is now possible to follow the metabolism of individual hexose, or of the hexose residues combined in sucrose, and this means has been used to study sugar metabolism of tobacco leaf disks.

Uniformly labelled fructose and glucose were prepared from $C^{14}O_2$ by photosynthesis.[1] Sucrose labelled in either the glucose or fructose moieties was prepared by enzyme transfer reactions from inactive sucrose and the labelled hexoses. For synthesis of sucrose labelled in the fructose moiety sucrose phosphorylase was used, and for sucrose labelled in the glucose moiety, the invertase of *A. oryzae*.[2] The labelled sucrose was separated from the enzyme digests by passage through a small charcoal column.

Disks cut from tobacco leaves, previously depleted of carbohydrate, were floated upon 5% solutions of radioactive sucrose, or invert sugar in which one hexose only was radioactive. The experiments were carried out using the Warburg technique and in an atmosphere of oxygen. The high oxygen tension was found to increase the amount of starch formed. Under these conditions sugar was taken up into the leaf disks at a constant rate for at least 24 hours, and the respiration quotient rose from an initial value, in the carbohydrate depleted material, of 0.7 to 1.05. After the first few hours the specific activity of the carbon dioxide evolved reached a constant value, equal or nearly so to that of the sugar supplied. Sugar thus became virtually the sole respiratory substrate and the endogenous respiration was suppressed. Measurements of the specific activity of the sugars and starch accumulating in the disks over a 24-hour period showed that a steady state was reached when the sugar had penetrated across the disks, and that incoming material was then redistributed among the several components in constant proportions. When fully expanded leaves are used the sugars accumulating within the disks, the starch synthesised and the carbon dioxide of respiration account for about 80% of the sugar taken up from the external solution, so that from total change in 24 hours a good estimate can be obtained of the amounts of glucose and fructose free or combined which are diverted to respiration and starch synthesis.

At the end of a 24-hour period the leaf disks were extracted with alcohol and the sugars in the extract separated using paper chromatography. Areas of paper containing each sugar were eluted with water, the eluate being collected on counting plates.[2] After recording the radioactivity the sugars were washed off the plates and estimated. In this way activity and amount were determined on the same sample and the errors in the estimate of specific activity greatly reduced. Starch was isolated from the alcohol insoluble residue and its specific activity determined.

The mean relative specific activities of the several leaf components are presented in Tables 1 and 2.

Table 1. Radioactivity of Starch Isolated from Tobacco Leaf Disks after Floating for 24 Hours on 5% Solutions of Radioactive Sugars, and of the Carbon Dioxide Evolved (Calculated as Hexose)

	Relative specific activities cpm/10^{-5}mg		
Sugar supplied		*Starch*	*Carbon dioxide*
Sucrose (fructose)*	8.0	7.2	7.8
Sucrose (glucose)*	8.3	9.7	9.2
Invert sugar (fructose)*	7.1	7.9	7.7
Invert sugar (glucose)*	11.5	10.2	9.6

Table 2. Radioactivity of Sugars Accumulated in Tobacco Leaf Disks Floated on 5% Solutions of Radioactive Sugars for 24 Hours

	Relative specific activities cpm/10^{-5}mg					
Sugar supplied		*S*†	Fm†	Gm†	F_t†	G_t†
Sucrose (fructose)*	8.0	8.0	22.3	0.9	20.4	1.9
Sucrose (glucose)*	8.3	8.3	2.9	17.8	2.5	18.0
Invert sugar (fructose)*	7.1	6.9	8.7	5.3	11.0	5.0
Invert sugar (glucose)*	11.5	13.3	10.3	11.7	9.8	16.6

* Hexose residue or free hexose uniformly labelled with C^{14}.

† S, sucrose. Fm and Gm, fructose and glucose moieties of sucrose. F_f and G_f, free fructose and glucose.

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From Table 1 it is seen that the specific activity of the starch formed and of the carbon dioxide evolved were equal to one another, and equal or nearly so to that of the sugar supplied in all cases. It can be concluded that starch and carbon dioxide arose solely from the sugar supplied, and were derived from a common source. Moreover glucose and fructose whether initially free or combined in sucrose must have contributed equally to the common source and there could be no question of a preferential use of glucose or fructose for either starch formation or as a substrate for respiration. The sugars entering the cell may therefore be supposed to become incorporated into a metabolic pool, by the formation of derivatives of the hexoses which can be rapidly interconverted. The distribution of labelling in the sugars accumulating in the disks is shown in Table 2. When sucrose was supplied the sucrose retained the asymmetry of labelling and at the same time free hexoses which appeared in considerable quantity had the same activity as the sucrose moieties and are therefore assumed to have been derived from sucrose by inversion. Since there was no exchange of label between the free hexoses, inversion must have occurred at a site removed from that of starch synthesis. When invert sugar was supplied there was synthesis of sucrose as well as starch and this newly formed sucrose was strongly labelled in both moieties whichever hexose was initially labelled. This sucrose must also have derived from the metabolic pool after equilibration of the fructose and glucose structures. Free hexoses were strongly but unequally labelled, a circumstance which is thought to arise from mixing of the incoming invert sugar in which only one hexose was labelled with the products of inversion of the sucrose in which both moieties have become labelled, again on the assumption that inversion takes place out of contact with the interconverting system.

The results of these experiments suggest that sucrose is perhaps not on the direct pathway of starch synthesis, but that the link between carbon dioxide and starch is effected through sugar derivatives, possibly phosphate esters, which in appropriate circumstances give rise to sucrose as well. Such a view is consistent with the observations of Vittorio *et al.*[3] that in tobacco leaves during a short period of phostosynthesis starch became labelled before sucrose and sucrose before hexoses. Under the conditions of the disk experiments, namely where a large and continuous supply of sugar was available the sugar entering the cells was probably at once incorporated into the pool to the maximum possible extent, while the excess passed on unchanged into a reservoir, perhaps the vacuole, where sucrose inversion can occur but not hexose interconversion.

Since the first stage in the utilisation of free sugar as the substrate for starch synthesis must be formation of derivatives which could lead to glucose/fructose interconversion the well known need for oxygen might perhaps be in oxidative phosphorylation to provide adenosine triphosphate for the initial introduction of phosphate into the sugar molecule and so make possible isomerase action. By inhibiting oxidative phosphorylation therefore both starch synthesis and respiration should be inhibited if the common precursor requires phosphorylation for its formation. When uniformly labelled sucrose was supplied to tobacco leaf disks in the presence of dinitrophenol as inhibitor, starch formation was progressively prevented as the concentration of inhibitor was increased, but carbon dioxide output was stimulated.[4] The radioactivity of the carbon dioxide at low dinitrophenol concentrations was equal or nearly so to that of the sucrose, indicating that sucrose was the respiratory substrate, but as the inhibitor concentration was increased the total activity of the carbon dioxide fell (see Fig. 1). Increasing output was therefore accompanied by diminishing use of sucrose and increasing use of an unlabelled material as substrate. Dinitrophenol thus appeared to restrict sugar utilisation both for starch synthesis and in respiration. These preliminary results with an inhibitor of oxidative phosphorylation are thus consistent with the hypothesis that incorporation of free sugar into the metabolic pool involves phosphorylation. They also raise the question of the nature of the substrate whose oxidation is stimulated by dinitrophenol. At present the only information is that the respiration quotient fell as sugar utilisation was restricted, so that it is unlikely to be oxidation of organic acid.

Until 1950 it was thought that invertases (saccharases, sucrases) catalysed only the simple hydrolysis of sucrose (and some related sugars) to glucose and fructose. However, Bacon and Edelman[5] and Blanchard and Albon[6] showed independently that

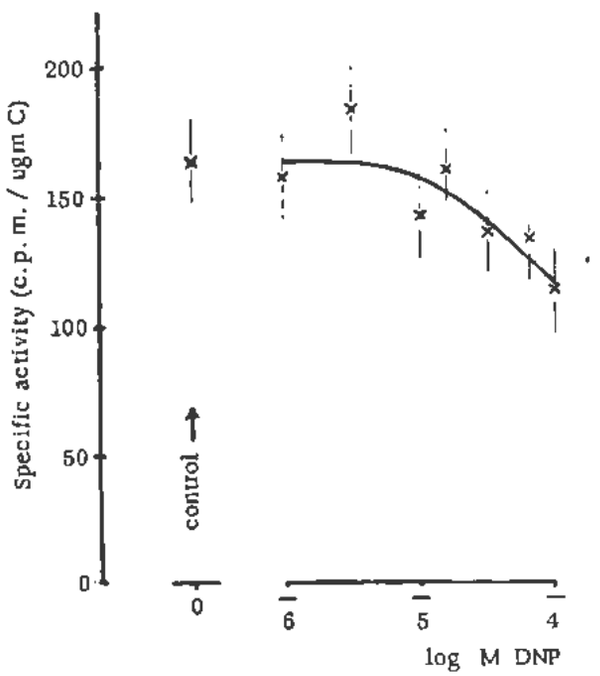

Figure 1. Effect of dinitrophenol on the relative specific activity of carbon dioxide evolved by tobacco leaf disks floating on 5% sucrose uniformly labelled with C^{14} for 24 hours at 25°C

during this hydrolysis, catalysed by invertases from various sources, oligosaccharides of greater molecular weight than sucrose were formed. It was suggested (see review)[7] that these saccharides were formed by transfer of fructose residues from sucrose to suitable acceptors by the following steps:

(*a*) The formation of a fructose-enzyme complex during the cleavage of sucrose

$$\text{sucrose} + \text{enzyme} \longrightarrow \text{fructosyl-enzyme} + \text{free glucose}$$

(*b*) The transfer of the fructose residue from the enzyme to an acceptor molecule

$$\text{fructosyl-enzyme} + A \longrightarrow \text{fructosyl-}A + \text{enzyme}$$

The over-all reaction would thus be

$$\text{sucrose} + A \longrightarrow \text{fructosyl-}A + \text{glucose}$$

It was found that many compounds containing hydroxyl groups would function as acceptors,[7] e.g. sucrose itself, and other sugars such as raffinose, giving rise to higher oligosaccharides; various alcohols, giving rise to esters such as methyl fructoside; or water, resulting in the formation of free fructose. Hydrolysis can thus be considered to be a particular case of fructose-transfer.

Invertases have been classified into two major groups, viz. glucosaccharases (α-glucosidases, attacking sucrose at the glucose moiety) and fructosaccharases (β-fructofuranosidases, attacking at the fructose moiety). An account of the development of this classification has been given;[8] it is sufficient to say that a major factor was the observation that some invertases were inhibited by glucose and some by fructose. It has been generally agreed that the former group contained, among others, invertases derived from moulds, and the latter groups those derived from yeasts.

Research on the nature of the oligo- and heterosaccharides formed during invertase action demonstrated that this classification is not justified, and that the enzymes from both yeasts and moulds act in a similar manner, i.e., by the transfer of fructose residues. Although this conclusion was supported by experiments with radioactive sugars,[9] major differences in the specificity of yeast and mould invertases were demonstrated by the respective actions of these enzymes on mixtures of sucrose and radioactive glucose (radioactivity was rapidly incorporated as combined glucose into oligosaccharides in each case). With mould enzyme, sucrose was the first oligosaccharide to become labelled, indicating that the action responsible was a transfructosylation which may be considered to occur thus:

$$G\rightarrow\leftarrow F + \text{enzyme} \longrightarrow \text{enzyme-}F + G\rightarrow$$

$$\text{enzyme-}F + G^{*}\rightarrow \longrightarrow G^{*}\rightarrow\leftarrow F + \text{enzyme}$$

Combining the equations we have

$$G\rightarrow\leftarrow F + G^{*}\rightarrow \longrightarrow G\rightarrow + G^{*}\rightarrow\leftarrow F$$

where $G\rightarrow\leftarrow F$ denotes sucrose, and $G\rightarrow$ glucose, the arrow indicating the reducing groups and '*' indicating radioactivity. This fructosetransferring capacity of mould sucrase has been used in this laboratory to synthesize glucose-labelled sucrose on a preparative scale from inactive sucrose and radioactive glucose. Later in the reaction saccharides of higher molecular weight (tri- and tetra- saccharides) which are formed become labelled in their glucose components. This supports the theory that these compounds are formed by further fructose transfer, e.g.,

$$\text{enzyme-}F + G\rightarrow\leftarrow F \longrightarrow G\rightarrow\leftarrow F\leftarrow F + \text{enzyme}$$

a hydrogen atom on the fructoside moiety of sucrose being replaced by a fructosyl group. Yeast sucrase did not catalyse the incorporation of free glucose into sucrose, but into a reducing disaccharide. This new sugar was formed by the transfer of fructosyl residues to the 6 position of free glucose molecules, i.e.,

$$\text{enzyme-}F + G^{*}\rightarrow \longrightarrow F\rightarrow G^{*}\rightarrow + \text{enzyme}$$

The over-all reaction was thus:

$$G\rightarrow\leftarrow F + G^{*}\rightarrow \longrightarrow F\rightarrow G^{*}\rightarrow + G\rightarrow$$

The absence of radioactivity in the reducing disaccharide when labelled fructose was added under comparable conditions showed that it was formed by transfer of a fructose and not a glucose radical.

The addition of radioactive fructose to sucrase-sucrose mixtures showed that free fructose itself could act as an acceptor of fructosyl groups to form (labelled) fructosyl-fructose disaccharides of at least two types. As was expected from the theory of fructosyl transfer, none of the glucose-containing oligosaccharides became labelled in the presence of labelled *free* fructose.

The significance of fructose transfer in metabolism is as yet unknown. There appears to be a correlation with the wide occurrence in the plant kingdom of fructose polymers ranging from sucrose, through oligosaccharides, to polyfructoses.[5] Similar glucosidic linkages are present in the naturally occurring plant fructosans (e.g., inulin, levan) and in products of mould and yeast sucrases. Fructose-transferring enzymes which may or may not be identified with invertases are known to occur in plants[7], e.g., Jerusalem artichoke and barley. It is hoped that further investigation of these will throw some light on the metabolism of sucrose and the polysaccharides.

ACKNOWLEDGEMENTS

Our colleagues R. V. Martin, L.H. May, V. C. Runeckles and F. J. Bealing have been associated with one or other aspects of the work.

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Application of Radioactive Isotopes to the Study of Processes of Photosynthesis and Chemosynthesis in Lakes

By S. I. Kuznetsov,* USSR

The process of photosynthesis in phytoplankton of various fresh-water lakes has been most thoroughly investigated by Winberg.[1,2,3] He has shown that in summer the daily prime production of organic matter is very different in different lakes; thus in dystrophic lakes it may be zero and in eutrophic lakes may reach 10–15 mg per litre, as was the case in Lake Chornoye of Kosino (Moscow district).

When phytoplankton is decaying, the organic matter particularly, the instable fraction of it which is easily disintegrated, undergoes aerobic decomposition while still in the lake water. In deep oligotrophic and mesotrophic lakes the organic matter is mineralized almost completely, and bottom deposits in such lakes are highly mineralized.

In eutrophic lakes, up to 20 per cent of organic matter, formed in the process of photosynthesis, is accumulated in deposits where it partly undergoes anaerobic decomposition.

Such reduced substances as hydrogen, methane, hydrogen sulphide, and ammonia, formed in the process of an aerobic decomposition, can serve as a source of energy for chemosynthesis of organic matter by the corresponding groups of bacteria.

However, a number of questions connected with the photosynthesis of phytoplankton, and especially with the bacterial chemosynthesis of organic matter in lakes, remained obscure.

Certain investigators, Rodina,[4,5] for instance, attached much importance to chemosynthetic processes. In the production of organic matter the role of iron bacteria, sulphur bacteria, nitrifiers and other similar organisms was considered to be very important. This supposition, however, was mainly qualitative, or at best, based on the quantitative estimation of the numbers of these groups of autotrophic organisms presented in a lake. Until now, there have been no methods that permitted precise determination of the true dynamics of the production of biomass by autotrophic organisms in a lake.

We were of the opinion that in the majority of basins of the lake type, the principal part in the production of organic matter was played by phytoplankton, and that chemosynthesis was of great importance only in basins with considerable amounts of substances which can serve as a source of energy for autotrophic organisms.

In this connection we investigated, on the one hand, basins of the lake type, such as the Rybinsk Reservoir, Lake Glubokoye, and a number of Siberian lakes, which undergo more or less complete turnover because of the wind, and Lake Belovod, a basin of the bioanisotropic type, where the deep strata have high density, contain much hydrogen sulphide and do not mix with the upper strata even in the period of autumn circulation.

It became possible to solve these problems only after we began to utilize the long-living radioactive isotope of carbon, C^{14}.

METHOD

The method of investigation was based on the assumption that, during short experiments, the processes of photosynthesis and chemosynthesis proceed in isolated water samples with the same intensity as in lakes. This assumption has now been fairly well substantiated by experiments.[6,7,8]

To obtain water for experiments from the required depth, a Ruttner water-sampler was used. Half of the water was then filtered through a "preliminary" membrane filter with 5–7 μ pores in order to remove phyto- and zooplankton. Four white-glass bottles of similar size were taken; two of them were filled with filtered water and the other two with non-filtered so that no air bubbles were left under the stopper. One bottle from each pair was then placed in a black oilcloth bag. Simultaneously, a solution of $Na_2C^{14}O_3$ with specific activity of 1.2 μc was introduced into each bottle in the ratio of 1 ml of solution to 300 ml of water. The same amount of $Na_2C^{14}O_3$ was introduced into the control bottle which was immediately fixed with formalin.

All the bottles were then suspended on a wire attached to a buoy and placed back in the lake at the depth from which water for the analysis was taken (Fig. 1). They were left there for a day or more according to the object of the experiment. By incubating the water samples directly in the lake we tried to bring the experimental conditions as near as possible to the natural conditions as far as temperature and illumination were concerned.

At the end of the experiment the bottles were taken from the lake, the water was at once fixed with

Original language: Russian.

* In cooperation with Y. I. Sorokin, N. N. Lyalikov and M. V. Ivanov.

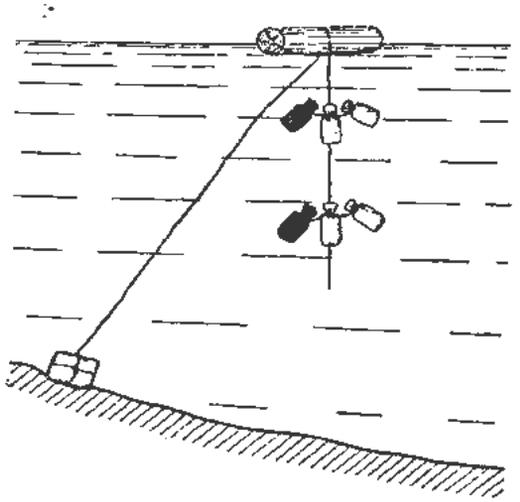

Figure 1. Diagram of arrangement for determination of photosynthesis and chemosynthesis in a water basin

formalin and in the laboratory a certain portion of it was filtered through a membrane filter with pores 0.5 μ in diameter.

The filters were washed with distilled water, dried and treated with the vapours of strong hydrochloric acid (in the manner recommended by Steeman Nielsen[9]) or with $^1/_{10}$ N solution of hydrochloric acid. This was done to remove the carbonates. After repeated drying the radioactivity of the filters was determined.

The plan of the experiments carried out in order to determine the processes of photosynthesis and chemosynthesis in the lake waters can be represented in the following manner (Table I).

The activity of the filter, through which water from the control bottle was filtered, did not, as a rule, differ much from the background readings and served as an indicator of the purity of the experimental conditions. We determined the extent of photosynthesis of phytoplankton or bacteria as the difference between the activities of filters through which water from light and dark bottles was filtered, and the extent of chemosynthesis by the activity of filters through which water from dark bottles was filtered. At the same time the total amount of carbon dioxide in the lake water was determined chemically. The amount of carbon dioxide fixed in the process of photosynthesis or chemosynthesis was calculated from the ratio $x = (ac\ 1000)/db$ mg CO_2/l, where a is total amount of carbon dioxide in one litre of water, b is the total activity of 1 litre of water in cpm, c is the activity of the filter and d is the amount of water filtered through the membrane filter.

To determine the extent of chemosynthesis in bottom deposits, the experiments were conducted differently from those described above. The following method was employed: lake deposits required for the analysis were taken by means of a Ekman dredge.

From 0.5–1 gm of mud were taken from the upper layer and placed in each of two test tubes. To each of these was added 3–4 ml of water and 1 ml of $Na_2C^{14}O_3$ with specific activity of about 0.02 μc. One of the test tubes was immediately fixed with formalin and served as a blank. Then both test tubes were closed with rubber stoppers, put in black bags and placed in the lake. The experiment lasted for two to three days. Then the contents of the test tubes were fixed with formalin and transferred into flasks with 20 ml of 4/10,000 N solution of sodium hydroxide. The flasks were shaken to separate bacteria from mud particles. Then 1–2 ml of the slightly settled liquid was filtered through a membrane filter with pores of 0.3 μ. This filter was then treated with 0.1N solution of hydrochloric acid, dried and the activity was measured with the aid of a counter in order to determine the radioactivity of the organic matter formed in the mud in the process of chemosynthesis.

The control analyses showed that after such treatment practically all bacteria are washed away from the mud particles, because the sediment in the test tube remained non-active when checked under an end-window counter tube.

We have taken into consideration all reservations about possible errors of the method, which are mentioned in Steeman Nielsen's article,[9] and we think that in our experiments the errors do not exceed 10 per cent.

The amount of oxygen dissolved in water was determined by the Winkler method. The dissolved hydrogen sulphide and sulphides were determined iodometrically; water transparency, by means of the Secci disc. The penetration of light in quantities sufficient for photosynthesis was determined according to the method suggested by Juday and Schomer.[10] For this purpose *Scenedesmus quadricauda* was introduced into light and dark bottles with ground stoppers. Each pair of bottles was placed in a lake at a definite depth. The water was analysed on the following day. If the light bottle contained more oxygen than the dark, this indicated that photosynthesis was going on at the corresponding depth.

Table I. The Plan of Experiments on the Rate of Photosynthesis and Chemosynthesis

Treatment of water before experiment	*Conditions of experiment*	*The processes at the expense of which carbon dioxide is assimilated*
Natural water, not filtered	In a light bottle	Photosynthesis of plankton and green or purple bacteria. Chemosynthesis of bacteria
	In a dark bottle	Chemosynthesis of bacteria
Water filtered through a membrane-filter with large pores	In a light bottle	Photosynthesis of green bacteria. Chemosynthesis of bacteria
	In a dark bottle	Chemosynthesis of bacteria

THE RELATION BETWEEN THE PROCESS OF PHOTOSYNTHESIS AND CHEMOSYNTHESIS IN LAKES WITH FULL CIRCULATION

One of the first tasks of our experiment was to determine the intensity of organic substance formation at the expense of chemosynthesis in lakes of various types. Experiments were carried out in the upper strata of lakes where photosynthesis proceeded most intensively. The results obtained are summarized in Table II.

The data in Table II show that, in the surface waters of these lakes, photosynthesis proceeds 30–100 times more intensively than chemosynthesis. The most intensive fixation of carbon dioxide in the process of chemosynthesis occurs in lakes of the eutrophic type where it reaches 0.1 mg CO_2/l/day. In this case, on the one hand, intensive process of organic substance mineralization proceeds under aerobic conditions. The ammonia formed in this process furthers the development of nitrifying bacteria. On the other hand, anaerobic decomposition of plankton occurs in the bottom deposits of the lakes of this type. Later on, the methane and hydrogen formed in this process enter the water and are oxidized in aerobic conditions, supplying energy to those chemosynthetic bacteria which oxidize hyrogen and methane.

In the surface waters of mesotrophic lakes the intensity of chemosynthesis is not more than 0.008 mg CO_2/l/day and in the case of oligotrophic waters the daily value of chemosyntbesis is so small that it cannot be determined at all in spite of the extreme sensitivity of this method.

A somewhat different picture is presented by the deep layers of water in lakes. In the mesotrophic Lake Glubokoye, the organic substance of plankton is mineralized while in water. Io this lake bottom deposits are highly mineralized, and this results in extremely low chemosynthesis in the hypolimnion; the intensity is only 0.001 mg CO_2/l/day (Table X) or is so small that it cannot be determined at all.

Tbe Rybinsk Reservoir is a huge water basin. It is of moderate depth and its bottom is formed by very diverse materials. Here chemosynthesis proceeds more actively at the bottom levels.

Chemosynthesis was measured not only in summer but in winter too, when the reservoir was covered with ice which prevented the aeration of water. The results of the analyses are given in Table III.

The data assembled in Table III show that in the water above mud ehemosynthesis proceeded several times more intensively than in the water directly above soil. Even during winter stratification chemosynthesis proceeded more intensively in deep layers than in surface waters. It is clear that there is a correlation between chemosynthesis and the products of decomposition of the organic matter of mud deposits.

In order to substantiate the above assertion, on 15 April, 1955, when the reservoir was still ice covered, the intensity of chemosynthesis was determined on the samples of water taken from various parts of the Volga branch of the Rybinsk Reservoir. The results of the analyses are given in Fig. 2.

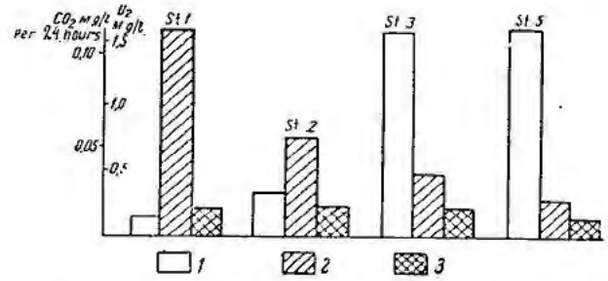

Figure 2. Dependence of amount of chemosynthesis in the water of the Volga Spur of the Rybinsk Reservoir on the quantity of methane and hydrogen dissolved, and on the oxygen content in the bottom water. 1, content of dissolved oxygen in the water; 2, chemosynthesis in natural water, 3, chemosynthesis in degassed water

At shore station No. 1, where the hottom contained the greatest amount of mud particles and the amount of oxygen dissolved in water was about 0.1 mg/l, chemosynthesis proceeded most intensively.

The further from the shore, the more mineralized became the deposits, the amount of oxygen dissolved in water increased, and the dissolved methane and hydrogen noticeably decreased. The intensity of chemosynthesis in the water also decreased. That chemosynthesis was connected with the oxidation of

Table II. Comparative Daily Values of Photosynthesis and Chemosynthesis in Surface Waters of Lakes of Various Types in July-August

Name of lake	*mgCO_2/l/day*		*Type of lake*
	Photosynthesis	*Chemosynthesis*	
Lake Belovod (Moscow district); laboratory experiment	7.203	0.097	Eutrophic
Lake Sekelikan (Buryat-Mongolia)	2.610	0.085	Eutrophic
Lake Kicherskoye (Buryat-Mongolia)	0.266	0.008	Mesotrophic
Lake Sekeli (Buryat-Mongolia)	0.201	0.002	Mesotrophic
Lake Glubokoye (Moscow district)	0.235	0.003	Mesotrophic
Rybinsk Reservoir	4.315	0.024	Mesotrophic
Lake Baikal	0.065	Impossible to give any figure for one day	Oligotrophic

Table III. The Intensity of Chemosynthesis in the Water of the Rybinsk Reservoir, mg CO_2/l

Place of sampling	*Nature of the bottom*	*26.V*	*13.VI*	*3.XI*	*11.XII*	*15.IV*
Channel of Volga	Mud	0.005	0.026	—	—	—
Channel of Logozha	Mud	—	—	0.017	—	—
Channel of Mologa, surface	Mud	—	—	0.009	0.013	—
bottom		—	—	—	0.036	—
Central reservoir	Soil	—	—	0.006	—	—
Shore near Borok	Soil	0.012	—	—	—	0.017

methane and hydrogen originating from mud is clear from the fact that the degassing of water samples led to a sharp decrease in chemosynthesis. And *vice versa*, if a gas bubble of hydrogen or methane was introduced into the water, chemosynthesis increased sharply. The experiment was conducted in the usual way. The bottles were incubated directly in the lake. The results of the analysis are summarized in Table IV.

The data in Table IV show that the addition of methane leads to a fourfold intensification of the chemosynthesis of the organic substance in water. The addition of hydrogen brought about even more a strong intensification of chemosynthesis. All this once again confirms that these gaseous products of anaerobic decomposition of organic substance in mud determine the process of chemosynthesis in natural waters.

The isotope method allowed a more detailed investigation of the process of photosynthesis in lakes. In order to determine the intensity of photosynthesis of small bacterial forms, water, previously filtered through a membrane filter with pores of about 7 μ, was used for the experiment. These bacterial forms include forms which develop in the light under conditions of prolonged incubation when water is inoculated on the "hungry" agar. The results are given in Table V.

The data in Table V show that bacterial photosynthesis in Lake Glubokoye averages about 15 per cent of the total photosynthesis. We cannot explain this figure by small forms of algae penetrating through the preliminary filter. Microscopic inspection of the filter shows that not more than 2–3 per cent of the total quantity of algae cells present in the water penetrate the preliminary filter. These forms can comprise not more than two per cent of the total photosynthesis. This means that the error of the analysis is not more than two per cent. The amount of photosynthesis in filtered water varies from 11 to 23 per cent of the total photosynthesis, and is considerably higher than the possible error of the analysis.

Table IV. Determination of Potential Intensity of Chemosynthesis in the Water of the Rybinsk Reservoir after the Addition of Methane or Hydrogen

Type of experiment	*Chemosynthesis, mg CO_2/l/day*	*Increase in relation to natural water in mg CO_2/l/day*
Water from the reservoir without additions	0.034	—
	0.038	—
Water + H_2	1.191	1.55
	1.188	1.152
Water + CH_4	0.158	0.124
	0.128	0.094
Water + gas from mud deposits	0.117	0.081
	0.094	0.058
Blank (water + formalin)	0	—
	0	—

The isotope method allows investigatium of the intensity of light and the depth to which it penetrates into the lake where photosynthesis is proceeding; it also permits study of the vertical distribution of phytoplankton and determination of whether the process of photosynthesis at great depths is limited by the insufficiency of light.

Table V. Comporative Determination of Daily Value of Photosynthesis of Bacterial Forms for Lakes Glubokoye and Baikal

Name of Lake	*Date*	*Photosynthesis intensity in mg CO_2/l/day*		*Photosynthesis of bacteria as per cent of total photosynthesis*	*The number of phytoplankton cells passing a "preliminary" filter, as % of total number in the original water*
		Total	*Bacterial*		
Lake Glubokoye	13-14. VII.	0.200	0.029	14.5	2.9
	15-16. VII.	0.133	0.021	16.1	2.1
	31. VII. 1. VIII.	0.235	0.026	11.0	—
	1-2. VIII	0.296	0.068	23.0	—
Lake Baikal	13-14. VIII.	0.050	0.001	2.0	—
	14-15. VIII.	0.075	0.001	1.0	—

For this purpose, closed bottles, with water containing natural phytoplankton from the surface layer, were placed in the lake at different depths, and the same bottles but with water taken from different depths are placed in the surface layer of the lake.

The intensity of the photosynthesis is determined by the above mentioned method. The results of one of the experiments with the water from the Rybinsk Reservoir are given in Fig. 3.

If we plot the values for photosynthesis on the abscissa, and the depth of the lake at which determinations were carried on ordinate then curve A which is based on the data for surface water, will show the depth of light penetration and the actual intensity of photosynthesis. Curve B based on the data for deep waters incubated in the surface layer of the lake, will show the intensity with which photosynthesis might proceed at the given depths if the penetration of light was not prevented by the upper layers of water. The area bound by these two curves represents the "light hunger" of phytoplankton.

Figure 3 shows that the phytoplankton of the Rybinsk Reservoir is under a permanent "light hunger", which is due, mainly, to the fact that water is mixed by strong winds.

Thus, on 27 June, 1954 (Fig. 3 a), when the weather was calm, although the greater part of phytoplankton was in the surface 2 metres of water, yet 62 per cent of phytoplankton suffered "light hunger" On 15 July, when the wind force was 5 (Fig. 3b) the percentage of phytoplankton suffering from "light-hunger" reached 90.

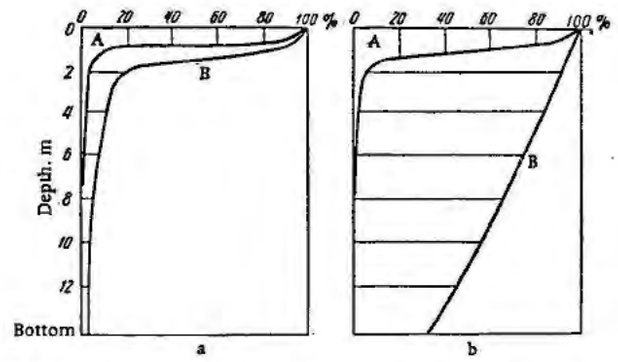

Figure 3. Determination of photosynthesis value, A, and the vertical distribution of phytoplankton, B, in the Rybinsk Reservoir in relation to the circulation of the water due to winds. (a) June 27, 1954, calm; and (b) July 15, 1954, wind force about 5

THE RELATION BETWEEN THE PROCESS OF PHOTOSYNTHESIS AND CHEMOSYNTHESIS IN LAKES WITH A SHARP VERTICAL STRATIFICATION OF HYDROGEN SULPHIDE OR IRON IN WATER

In addition to the gaseous products, methane and hydrogen, chemosynthetic bacteria can also derive energy from hydrogen sulphide or ferrous oxide (which in certain special cases accumulate in hypolimnion of lakes in large quantities.

The most suitable object for a study of this type of chemosynthesis turned out to be Lake Belovod where deep layers of water contain considerable amounts of hydrogen sulphide throughout the year, and Lake Glubokoye where, at the close of summer and winter periods of stagnation, the content of ferrous oxide in hypolimnion reaches several mg /l.

Lake Belovod is one of the few lakes where several authors[11,12,13] have observed abundant development of purple sulphur bacteria at a dept of 12–13 metres. We considered that the use of radioactive carbon dioxide would make it possible, on the one hand, to find out which of the two processes (photosynthesis or chemosynthesis) make possible the development of purple sulphur bacteria and, on the other hand, to determine the daily production of organic matter at the expense of the development of these bacteria. We were right in presenting the problem in this manner because, firstly, the abundant development of purple bacteria usually occurs in lakes at such a depth, where the layers containing dissolved oxygen are in contact with deeper strata containing hydrogen sulphide, which creates conditions favourable for chemosynthesis, and, secondly, it was not clear whether light penetrated to this depth, though according to recent data light is absolutely indispensable for the development of purple sulphur bacteria.

We made analyses of Lake Belovod water in June, July, August and September 1954. Below we give the results of the analyses made on July 10–15, 1954. Their main purpuse was to determine some of the hydrochemical characteristics, the depth of light penetration and the intensity of photo- and chemosynthesis. Results are given in Fig. 4.

Figure 4 shows that the greatest number of *Chromatium okenii* was observed at the depth of 13.0–13.5 m where it reached 430,000 cells/ml. The water at that depth had a pink colour. The oxygen

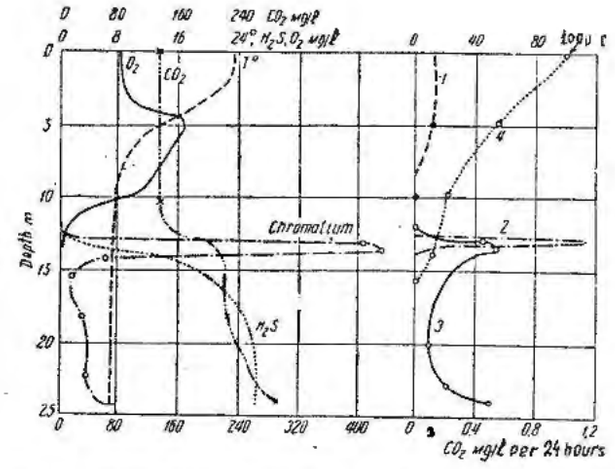

Figure 4. Hydrochemical analysis of the water of Lake Byelovod and character of stratification of photosynthesis and chemosynthesis on July 10-14, 1954. Curve 1, photosynthesis of phytoplankton mg CO_2/l/24 hr; 2, photosynthesis of *chromatium* mg CO_2/l/24 hr; 3, total chemosynthesis mg CO_2/l/24 hr; 4, intensity of light penetration, per cent of total light incident on surface

Table VI. Photo and Chemosynthesis in Lake Belovod, mg CO_2/l/24 hr

Depth in metres	Penetration of light to the depth: Photosynthesis of Scenedesmus in mg O_2/l	Penetration of light to the depth: Penetration in per cent of the light at the surface	Photosynthesis: of phytoplankton	Photosynthesis: Chromatium sp	Total chemosynthesis
0	17.1	100	0.135	—	0.003
5	9.6	55	0.11	—	0.003
10	3.7	21	0.00	—	0.004
13	—	—	—	1.15	0.452
13.5	—	—	—	0.16	0.550
14	2.6	15	—	0.030	0.400
16	0.8	0.4	—	—	—
17	0.0	0.0	—	—	—
20	—	—	—	0.005	0.100
23	—	—	—	0.000	0.220
24	—	—	—	0.000	0.500

maximum was found in the metalimnion. At a depth of 5 metres, the oxygen content was 16.2 mg/l. At a depth of 13.5 metres, the oxygen content dropped to zero, and the presence of hydrogen sulphide was observed (up to 2 mg/l). At still greater depths, concentration of hydrogen sulphide sharply increased and this was accompanied by a corresponding increase in the electric conductivity of water. Water transparency was 10 metres (doubled value). Penetration of light determined by Schormer's method and the intensities of photo- and chemosynthesis are shown in Fig. 4 and in Table VI.

It is clear from Table VI and Fig. 4 that, in the surface layers, where there is no *Chromatium sp* at all, photosynthesis is due to the phytoplankton activity. At the depth of 10 metres there is practically no photosynthesis although light penetrates as deep as 16 metres. The photosynthesis is most intense at the depth of from 13.5 metres, where great numbers of *Chromatium sp* were observed. At the depth of 13 metres in a layer of water which is 0.5-m thick, the photosynthesis of purple sulphur bacteria was almost ten times more intensive than the photosynthesis of phytoplankton in the surface water layer.

Figure 4 also shows that in the hydrogen sulphide zone of the lake the process of chemosynthesis proceeds very intensively. The daily assimilation of carbon dioxide in a dark bottle reached 0.5 mg/l.

Data from the literature made it possible to assume that there are three types of processes whose energy could be utilized here for the synthesis of the organic matter: (1) chemosynthesis of the purple sulphur bacteria themselves; (2) oxidation of hydrogen sulphide by thionic bacteria, which were observed here in great numbers after the water inoculation on Beyerink's nutrient medium; and (3) chemosynthesis under the influence of sulphate-reducing bacteria utilizing the energy of hydrogen sulphate reduction. In this case hydrogen was produced during the anaerobic decomposition of the organic substance of bottom deposits.

In order to find out the capacity of *Chromatium sp* to carry on the process of chemosynthesis in the conditions of Lake Belovod, experiments were conducted in the following manner:

Water taken from the depth of 12.5 m, where at the time of experiment there was the greatest number of *Chromatium* cells, was filtered through a preliminary filter with pores of 5–7 μ before the experiment was started.

Most of the purple sulphur bacteria were left on the filter, and small forms, including thionic bacteria, easily passed through the filter pores.

The experiment with filtered and non-filtered water was conducted in the ordinary manner. The bottles were placed in the lake at the depth of 12.5 m and remained there for 24 hours. The results of the analysis are given in Table VII.

Low intensity of photosynthesis in filtered water, which was only 10 cpm/100 ml, when background reading of the counter indicated 30 cpm, did not exceed the permissible error of the analysis and was caused by the penetration of a few *Chromatium* cells through the filter pores when the water was prepared for the experiment.

Later on, the purple sulphur bacteria from Lake Belovod were grown in an enrichment culture and their capacity for chemosynthesis was studied in laboratory conditions.

Rhodothece pendens Mol was placed in three 100-ml flasks. One ml of $Na_2C^{14}O_3$ with specific activity of 1.28 μc was introduced into each flask. One flask was left in the light, the other two were placed in the darkness. Aerobic conditions were maintained in one of the dark flasks. The experiment lasted 24 hours. The data in Table VIII show the

Table VII. Intensity of Chemosynthesis in the Water of Lake Belovod. Taken from a Depth of 12.5 m, with and without Purple Sulphur Bacteria

Processes at whose expense carbon dioxide is assimilated	Non-filtered water, Chromatium sp present: cpm/100 ml of water	Non-filtered water, Chromatium sp present: CO_2 mg/l	Filtered water, free from Chromatium sp: cpm/100 ml of water	Filtered water, free from Chromatium sp: CO_2 mg/l
Photosynthesis and chemosynthesis	595	1.14	130	0.25
Chemosynthesis	135	0.26	120	0.23
Photosynthesis (difference)	460	0.88	10	0.019

activity of 10 ml of bacterial culture when filtered through a membrane filter.

The data in Table VIII show that in the dark flasks practically no assimilation of the marked carbon dioxide took place; that is, there was no chemosynthesis, and the presence or absence of air did not change the situation.

These experiments led us to the conclusion that there is no connection between the process of chemosynthesis and the activity of purple sulphur bacteria in Lake Belovod.

Table VIII. The Intensity of Photosynthesis and Chemosynthesis of *Rhodothece Pendens* Enrichment Culture

Conditions of experiment		*cpm/10 ml*
In the light	Anaerobic	2.588
	Anaerobic	18
In the darkness	Aerobic	16

The sulphate $Na_2S^{35}O_4$ labelled with S^{35} was used to study the possible role which the sulphate-reducing bacteria play in the process of hydrogen sulphate reduction. Each of the water samples taken from the depths of 15, 18 and 21 metres was poured into three flasks of 100 ml, and 1 ml of $Na_2S^{35}O_4$ solution with a specific activity of 43,600 cpm was added into each bottle. The first flask was immediately fixed with formalin and served as a control. The second flask was not fixed and indicated the intensity of the process of sulphate reduction. A bubble of gaseous hydrogen was introduced into the third flask, in order to determine the potential possibility of the reduction of sulphates as a result of hydrogen sulphate reduction. After 24 hours of incubation in the lake, sulphides in the experimental flasks were precipitated with cadmium acetate. Cadmium sulphide was filtered on membrane filters and its activity was determined on an end-window counter tube.

All the experiments gave similar results. The activity of the precipitate was equal to zero, which means that hydrogen reduction of sulphates does not take place in the waters of Lake Belovod and consequently, the process of chemosynthesis is carried by some other group of bacteria.

A similar method was employed in the experiments with the surface layer of the bottom deposits of Lake Belovod. During 24-hour exposure of the flasks in the lake, about 1.5 per cent of the introduced marked sulphate was reduced to H_2S, which is another proof of the previously obtained data[11] that in Lake Belovod hydrogen sulphide is formed predominantly in the bottom deposits.

When it became clear that in Lake Belovod the purple sulphur bacteria do not take part in chemosynthesis, it was natural to expect that thionic bacteria participate in this process.

The only argument against this supposition was the fact that in the process of chemosynthesis these organisms utilize the energy of the oxidation of hydrogen sulphide, but in the depth of the lake there is no oxygen which could serve as an oxidizing agent.

The experiment to determine the oxidation of hydrogen sulphide and other sulphides in the water of Lake Belovod was carried in the following manner.

Water for the experiment was taken from the depths 3, 5, 13, 14, 14.5, 18 and 21 metres. Each sample was poured into 2 flasks of 100 ml and 1 ml of Na_2S^{35} solution with the specific activity of 177,000 cpm was introduced into each flask. Then one flask from every pair was fixed with formalin.

After 24-hour exposure in the lake, the remaining flasks were also fixed with formalin, sulphides were precipitated by cadmium acetate, and sulphates, formed at the expense of the oxidation of hydrogen sulphide, were precipitated in the filtrate with benzidine chloride.

The activity of benzidine sulphate precipitated in 1 ml of solution was determined on an end-window counter tube. The difference between the activity of the benzidine sulphate from the experimental flask and from the flask fixed with formalin indicated the intensity of biological oxidation of sulphides in the process of chemosynthesis of thionic bacteria. The results of the analyses are given in Table IX.

The data in Table IX show that chemosynthesis proceeded in the depth of the lake and that the carbon dioxide was fixed most intensively at the depth of 14.5 m. The greatest concentration of sulphides was also observed at this depth. How the hydrogen sulphide is oxidized is not yet clear, but the application of labelled sulphide showed that chemosynthesis proceeds through the activity of thionic bacteria, and that oxidation of hydrogen sulphide serves as a source of energy.

Table IX. The Intensity of the Oxidation of Hydrogen Sulphide by Thionic Bacteria in Lake Belovod. Analysis Made on 1/IV/55

Depth in metres	*Activity of precipitated benzidine sulphate, cpm*	*Activity of chemosynthesic products, cpm/100 ml*	*O_2 mg/l* in lake water	*H_2S mg/l* in lake water	*pH*	*rH_2*
3.0	42	—	6.90	0.0	8.2	23.8
13.0	—	—	2.63	0.0	8.0	20.8
13.5	13	—	2.08	1.26	7.6	22.6
14.0	108	83	0.0	2.67	7.5	14.6
14.5	285	140	—	10.11	7.5	—
18.0	—	81	—	29.81	7.2	8.7
21.0	88	68	—	39.71	7.1	10.2

Table X. The Intensity of Chemosynthesis and Photosynthesis in Lake Glubokoye at the Time of Partial Autumn Circulation (October 24-25, 1953)

Depth, metres	Temperature of water, °C	Fe total mg/l	O_2 mg/l	CO_2 mg/l 24 hr	
				Photosynthesis	Chemosynthesis
0	8.2	0.30	8.16	0.025	0.002
8	8.2	0.30	8.09	—	—
12	8.2	0.30	7.46	0.000	0.001
14	6.6	—	1.24	—	—
15	5.8	0.75	0.00	—	0.0012
20	5.2	1.50	0.00	—	0.000
30	4.9	2.40	0.00	—	0.0014

Considerable amounts of ferrous salts often accumulate in basins of the lake type in which hypolimnion, is well developed. This occurs towards the end of summer and winter stagnation periods.

Such was the case in Lake Glubokoye (Moscow district) in 1953. The oxidation of ferrous compounds to ferric can serve as a source of energy for the development of *Ochrobium tectum, Leptothrix ochracea* and other iron bacteria inhabiting water basins. This was the reason why we attempted to determine the intensity of chemosynthesis in Lake Glubokoye at the time of partial autumn circulation.

The microscopic examination showed that, at that time, the water contained a considerable number of organisms which morphologically resemble *Leptothrix crassa* and whose sheath is impregnated with deposits of ferric oxide. This fact indicated that in the lake there existed conditions favourable for the development of other chemoautotrophic bacteria as well. The results of the analysis are listed in Table X at top of page.

This data in Table X show, however, that in the lake where there is no gas-emission from the bottom and where chemosynthesis might be due to the development of iron bacteria, the daily fixation of carbon dioxide in the process of chemosynthesis did not exceed 0.001–0.002 mg/l.

Evidently the process of nitrification in basins of this type can not exceed these figures because they must be of the order given in the last column of Table X.

CHEMOSYNTHESIS IN BOTTOM DEPOSITS

We considered chemosynthesis in deposits from the point of view of chemoautotrophic assimilation of carbon dioxide as a source of increasing bacterial biomass in deposits. Bottom-inhabiting bacteria constitute the most important component of the food of microbenthic animals.

The intensity of chemosynthesis was determined by the above mentioned method. The results of the determination of chemosynthesis in the deposits of the Rybinsk Reservoir show that the intensity of this process depends to a great extent on the character of bottom deposits.

In grey mud, which is deposited at the mouths of rivers flowing into the reservoir, the daily intensity of chemosynthesis is up to 3–6 mg carbon/kg mud. In peat muds whose organic substance consists mainly of very stable lignin-like substances, which do not disintegrate in anaerobic conditions, chemosynthesis proceeds much slower and its daily value is not more than 1 mg carbon/kg mud.

As in the water, so in the mud, hydrogen and methane formed during anaerobic decomposition of the organic matter, represent the main source of energy for the chemoautotrophic bacteria. To prove this, we carried out experiments showing the influence of aeration on chemosynthesis in the mud. The procedure and results of the experiment with mud from the Rybinsk Reservoir are given in Table XI. The data in the table show that when the products of anaerobic decomposition of organic matter are removed from the mud, the intensity of chemosynthesis is decreased tenfold.

DISCUSSION

Thus, we consider that in the majority of lakes the chemoautotrophic process represents one of the last links in the turnover of organic matter. The chemoautotrophic bacteria complete the processes of decomposition by a new synthesis of bacterial protein from carbon dioxide and simple salts.

Chemosynthesis in lakes should be considered as a secondary process which utilizes the energy of organic matter stored up in the process of photosynthesis. G. G. Winberg[6] was right in asserting this a long time ago. At the present time, his supposition is substantiated by direct analyses in which radioactive isotopes of carbon were used. Chemosynthesis, therefore, enables a more complete utilization of the energy of photosynthesis, stored in the organic matter of lakes.

Table XI. The Influence of Preliminary Aeration of Mud on the Intensity of Chemosynthesis

(The Experiment Continued for 3 Days)

Conditions of experiment	Radioactivity of bacterial biomass washed from 0.5 gm of mud, cpm	Chemosynthesis, %
No preliminary aeration of mud. Experiment carried out in a test tube	3.220	100.0
Aeration of mud for 24 hours. Experiment carried out in a 500 ml flask	320	9.9

CONCLUSIONS

1. The role played by ferrous and nitrifying bacteria in the production of organic matter in lakes is of small importance, and in mesotrophic lakes it does not exceed 0.001 mg CO_2/1/24 hr.

2. In the process of chemosynthesis going on in lakes, the main production of organic substance is due to the activity of bacteria oxidizing hydrogen and methane. In lakes where, in the process of anaerobic decomposition of bottom deposits, these gases are formed, the intensity of chemosynthesis in the water can reach in winter 0.01–0.06 mg CO_2/l/day.

3. Since methane and hydrogen are produced as a result of the anaerobic decomposition of the organic matter formed in the process of photosynthesis, chemosynthesis at the expense of the oxidation of methane and hydrogen cannot be considered as a process of prime production of organic matter. What we have here is only a more economical utilization of the process of photosynthesis.

4. The main role in the basic production of organic matter in lakes is played by the photosynthesis of phytoplankton or bacteria. The production of organic matter in the course of chemosynthesis in lakes is of secondary importance.

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Biosynthesis in C^{14} - Labeled Plants; Their Use in Agricultural and Biological Research

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The use of isotopes as tracer tools is unquestionably one of the most significant peacetime applications of the atom, one which will have particular and continuing utility in agricultural and biological research. Carbon-14, the long-lived isotope of carbon, has proved the most useful tracer isotope in biological research ever since it became readily available with its production in atomic reactors. The full tracer potential of radiocarbon (C^{14}) can be envisioned by simply noting that carbon accounts for 30–40% of the dry weight of living organisms.

The usefulness of any tracer isotope is limited by factors other than its availability in elemental form. The most limiting factor in applying C^{14} as a tracer in biological research is the requirement of incorporating it into the molecules of the particular native organic compound that is to be traced or studied. In many cases this incorporation can be most efficiently accomplished by means of chemical syntheses, in which case it is possible to place the isotope at specific carbon atom positions. In many more cases the mechanism of synthesis of naturally occurring organic compounds is not known and the preparation of a labeled molecule cannot be accomplished. It is only by use of biosynthesis that a radiocarbon-tagged form of all of the diverse organic molecules that occur in living organisms can be prepared.

In biosynthesis an actively metabolizing organism is supplied the carbon-containing substrates or "assimilates" normally utilized in its natural habitat. These substrates are supplied in C^{14}-enriched forms which essentially contain the specific activity required in the particular organic compound desired and which is known to occur in the organism. The initial labeled substrate supplied may be a carbohydrate, in the case of many bacteria, or, with chlorophylous higher plants, it can be carbon dioxide.

This paper is predominantly concerned with detailing the requirements which must be met for efficient biosynthesis of C^{14}-labeled higher plants and their products. The various observations noted are the cumulative result of seven years' investigation of the ways and means of biosynthesizing radiocarbon-labeled plants as well as of their experimental tracer usefulness.[5] The authors wish to acknowledge not only the encouragement which Dr. Willard F. Libby, Department of Chemistry, University of Chicago, gave to the establishment of this particular radiocarbon research program but also the help and advice given during the course of experimentation.

Unless otherwise noted the use of the term "labeled" refers to uniformly labeled plants or their specific products. In such instances a uniformly labeled plant is constituted of organic compounds of identical specific activity of radiocarbon as measured on a gram of carbon basis. Further, the uniformity of labeling is such that the percentage distribution of C^{14} is the same at the various carbon positions in a specific organic compound.

PHYSICAL FACILITIES

A number of diverse criteria were utilized in establishing the final design of the complete physical unit, termed a "biosynthesis chamber" (Fig. 1), required for culture of C^{14}-labeled higher plants. Included were the requirements associated with maintaining a hermetically sealed system and such factors as height and weight of plant species, length of culture period, control of environmental variables such as temperature, humidity and day length, mineral nutrition, and photosynthetic capacity, as

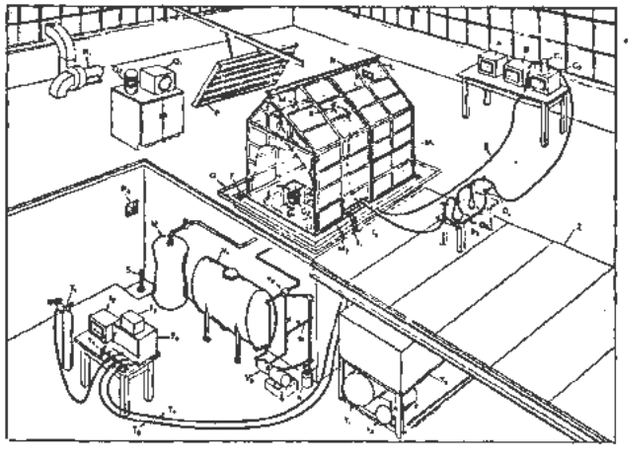

Figure 1. Schematic drawing of a single, complete C^{14} biosynthesis chamber unit. The individual parts, indicated by the letters A–Z, are reviewed in detail in the text

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measured by plant rate of assimilation of carbon dioxide. A two-year period was devoted to the construction and experimental testing of a pilot biosynthesis chamber.[6] The resultant experimental data were used to establish the final design incorporated in each of three independent replicate biosynthesis chamber units. The latter units have been in experimental use for five years.

Each of the various parts of a single, complete biosynthesis chamber unit are schematically designated by letter in Fig. 1 and identified in the text. The three units are located in a conventional greenhouse where plants can be grown with light intensities which substantially exceed those normally available from artificial light sources. Each unit has approximately a 10,000-liter internal free-air capacity when plants are being cultured experimentally under hermetically sealed conditions. The bulk of this volume is contained in the plant growth chamber proper as shown in Fig. 1.

The chamber is constructed of low carbon content steel, with standard commercial window sash continuously welded to the main supporting framework. The chamber dimensions in feet are 7⅔ long, 5 wide and 5½ high to the eave of the roof. The chamber has two removable panels (Fig. 1, M_1, M_2) for use as entrances at harvest times. The panes of standard, double-strength glass are glazed using continuous glazing angles and a carbonate-, sulfide-, and sulfite-free mastic. Continuous with and subtending the base of the chamber is a 21-inch deep stainless steel bed containing the inert substrate (Fig. 1, H) used for nutriculture maintenance of plants.

Normally eight stainless steel, wire mesh baskets (Fig. 1, G_2) are equally spaced in the bed. The baskets are set on the bed-floor, which has a gravel-free, recessed flooding-channel into which the nutrient solution is first pumped in the course of flooding the bed. Each of the baskets has a surface area 10 × 10 inch. They are each contained within a solid, stainless steel retainer (Fig. 1, G_1) open at top and bottom, leaving a ½-inch free air space between the basket and retainer wall. This arrangement facilitates recovery of roots by restricting their growth to the silica sand or quartz gravel substrate volume contained within the baskets.

All plants are supplied a complete nutrient solution which has been experimentally demonstrated to support good growth for the particular species to be cultured. The solution is contained in a nutrient reservoir tank (Fig. 1, V_1); by means of a centrifugal pump (Fig. 1 V_3), activated by a time-clock; this solution is delivered by piping into the bed of the chamber. A solenoid valve (Fig. 1, V_2) permits the solution to drain directly back into the tank without passing through the centrifugal pump. A vent connecting the air in the chamber with that above the solution in the tank avoids pressure change while the solution is pumped to or drained from the bed. If necessary, at the end of an experiment this solution can be pumped via a pipeline (Fig. 1, W) to a hold-up tank. A plastic (Tygon) bag (Fig. 1, U), having an air volume of 200 liters, is also connected by a vent pipe to the air in the growth chamber. This unit serves as an expansion bag, collapsing or expanding in response to change in pressure due either to temperature variation inside the plant biosynthesis unit or to change in outside barometric pressure.

The remaining parts of the hermetic system consist of ¼-inch lumen plastic tubing (Fig. 1, D_1, E, T_6, T_7) connected to a one-liter ionization chamber (Fig. 1, C_1) of a vibrating diaphragm electrometer and to a 20-cm sample-cell of an infra-red, carbon dioxide gas analyzer. The concentration of C^{14} in the growth chamber atmosphere is continuously assayed by use of the electrometer and automatically indicated on a 30-day strip-chart recorder (Fig. 1, C_2) in terms of millivolts. The carbon dioxide detecting unit consists of an infra-red gas analyzer (Fig. 1, T_4) and a time-relay circuit (Fig. 1, T_3) for activating a panel of 5 solenoid valves (Fig. 1, T_5). An hourly assay record (T_2) of the carbon dioxide content is obtained in terms of millivolts in each of two separate biosynthesis chambers. Every hour a 28-minute record of the carbon dioxide in each of the two chambers is obtained. After each assay period the sample-cell of the analyzer is flushed with nitrogen (Fig. 1, T_1), thereby returning the sample to the chamber from which it was drawn. Air pumps located in the chamber continuously supply air samples at a rate of 1.5 liters per minute to both the ionization chamber of the electrometer and the sample-cell of the infra-red gas analyzer.

Additions of labeled carbon dioxide to the chamber are accomplished by forcing a lactic acid solution, using pressure supplied by air pumps, from one flask (Fig. 1, D_2) into a flask (Fig. 1, D_3) containing C^{14}-labeled carbonate. The generated CO_2 is expelled immediately in front of one of two air-circulating fans (Fig. 1, K) in order to effect a quick and uniform distribution of the gas.

The air temperature within the plant growth chamber is regulated to ±2°F over the range of 60–80°F by constantly flowing, temperature-regulated water over the four external chamber surfaces. A modulating thermostat (Fig. 1, I), centrally located inside the chamber, controls the temperature of water flowing out of numerous openings in pipes (Fig. 1, N) located at the top of each external surface-face of the chamber. The thermostat controls a 3-way mixing valve connecting to water supplied from a chiller-tank (Fig. 1, Y_1) associated with a refrigeration compressor (Fig. 1, Y_2) and to unchilled water supplied by a reservoir (Fig. 1, Y_3) which receives all water that has passed over the chamber. Fractional quantities of the two types of water can be mixed, depending on the fractional heat-load recognized by the modulating thermostat. The water flowing off the chamber is caught in a splash-pan (Fig. 1, O) and returned to the unchilled water reservoir by means of a drain (Fig. 1, F). A

multiple-station, continuous recording unit (Fig. 1, B) measures the ambient temperature within the growth chambers as well as that of the greenhouse and outdoor air.

The relative humidity inside the chamber is regulated to ± 5% over the range of 40–60%. This control is obtained by means of a dehumidifying unit (Fig. 1, L) and a humidistat inside the chamber that activates a small compressor (Fig. 1, X) supplying refrigerant gas to the dehumidifier. Condensed water is returned to the nutrient solution.

Two types of supplemental light are available, each independently operable by means of time-clock control. Three 75-watt incandescent, reflector-type lamps (Fig. 1, J) are used to extend the natural daylength when required. Two movable, 18-tube fluorescent luminaires (Fig. 1, P) provide supplemental, high intensity light for growth of plants when natural light is deficient or not available. Light intensity is continuously recorded (Fig. 1, A) in foot-candles as measured by a photocell.

The pit in which the nutrient reservoir tank and other equipment is located is normally completely covered with metal-decking (Fig. 1, Z). An exhaust blower (Fig. 1, R_1) and vent (Fig. 1, R_2) provide continuous flush of the pit atmosphere as a safeguard against accumulation of C^{14}-dioxide in event of its leakage from the various parts of the biosynthesis unit located at this site. A drain, topped by a stand-pipe Fig. 1, S), provides a hold-up reservoir for solution which may leak from the nutrient reservoir. This arrangement was particularly designed for biosynthesis of plants in which radioisotopes such as P^{32}, S^{35}, Zn^{65}, Fe^{59}, etc. are also employed. With removal of the stand-pipe the trapped solution is free to drain to hold-up tanks for further processing. The normal greenhouse atmosphere is continuously monitored for $C^{14}O_2$ which may appear as result of leakage from the growth chamber. A vibrating diaphram electrometer (Fig. 1, Q_2) monitors a 5-liter air sample of the normal greenhouse and records (Fig. 1, Q_1) with a full-scale sensitivity representing 50% of the $C^{14}O_2$ concentration considered safe for constant human exposure.

PERFORMANCE OF FACILITY

Certain of the steps as they occur in a typical C^{14}-biosynthesis experiment are noted in a separate section while consideration is particularly given here to various aspects of the performance of the plant biosynthesis facilities based on total experimental experiences. The facilities have proved to be hermetic, and no measurable loss of $C^{14}O_2$ has occurred during experimental usage. An internal pressure equivalent to that of a 10-inch column of light mineral oil could be maintained. A biosynthesis unit is not sealed until the ambient temperature in the growth chamber is at equilibrium, at which time the plastic breather-bag is partially inflated. When thus sealed, a maximum fluctuation of pressure equivalent to a ½-inch oil column has occurred as measured in an attached manometer. This is predominantly the result of barometric pressure changes. Pressure variations due to temperature variation are insignificant because of the precise control of this environmental factor.

A light intensity maximum of 8000 foot-candles has been observed inside the growth chambers when clear skies prevailed in summer, which is approximately a 25% maximum reduction of outdoor intensities.

In any green plant the rate of growth can be limited by the quantity of "photosynthates" available. In natural habitats and greenhouses the rate of photosynthesis may be limited by more environmental variables than in the plant biosynthesis chamber, where controlled environmental conditions exist. The variable that requires the closest attention in the biosynthesis units is the total quantity and concentration of CO_2. At normal concentrations (approximately 0.03%) of this gas, 3.0 liters are present in the system at 75°F. With 1.5 kilograms of fresh leaf tissue, the quantity accepted as maximal for the chamber under optimum light conditions, several species have assimilated this quantity of CO_2 in one hour. When the natural concentration is increased, still greater rates of fixation occur. Most plant species have not been able to effect a net assimilation of CO_2 from an atmosphere which has been lowered to 0.003%. On the other hand, it is detrimental to some plants if they are maintained for a few weeks with 0.20% CO_2 during winter light intensity and daylength conditions. In such instances alfalfa has been observed to exhibit severe epinastic responses and arrested growth rates.

Irrespective of seasonal period all species used in chamber biosynthesis experiments are cultured with a maximum CO_2 concentration of 0.10%. When assimilation has lowered the concentration to a third of the normal, or 0.010%, it is again raised. With the maximum quantity of leaf tissue noted earlier and with optimal photosynthetic conditions, several species have assimilated 24 liters of carbon dioxide during a single 12-hour photosynthesis period. In this instance three additions or generations of carbon dioxide had to be supplied to the growth chamber.

Usually a biosynthesis experiment which is designed to produce uniformly labeled plants is initiated with seedling stocks. These are then cultured to selected stages of maturity while exposed to atmospheres which are kept in the total carbon dioxide range noted earlier and always at the specific activity desired in the uniformly labeled plant or its particular products. It is maintained in the air phase by readjustment with additional quantities of carbon dioxide of the appropriate specific activity. Frequent readjustments are necessary early in the culture period, particularly if the experiment is started with plants well beyond seedling stage size. As much as a 50-fold increase in the initial seedling carbon pool has been obtained in a four-week culture of plants in the growth chamber. The air in the chamber is circulated at a 3-mph rate by the fans. When

additional quantities of carbon dioxide are introduced the nutrient solution is pumped into the chamber bed to facilitate reaching the equilibrium concentration of the gas. Equilibrium is reached in a 15–20-minute period.

The nutrient reservoir tank contains 750 liters of a complete mineral element solution freshly prepared at the start of an experiment. It is usually pumped into the bed twice a day, requiring 30 minutes per pumping period. A comparable drainage time is required. This large solution volume is necessary to flood completely the two tons of silica sand or quartz gravel substrate in the bed. This volume of nutrient has been found adequate to supply completely all the mineral elements necessary to the development of the plant tissue, irrespective of the length of culture period. No addition of any element has been necessary for any of 15 species of plants cultured in the chamber.

As a safeguard against the effect of overchilling by the temperature-controlling water passing over the chamber, two 1000-kw strip-heaters are controlled by a thermostat and serve as a heat source when the temperature drops several degrees Fahrenheit below the established mean ambient temperature.

With certain biosyntheses it is necessary to enter the chamber relatively frequently. At these times, as well as at final harvest of plants, the C^{14}-activity in the air phase is lowered to a safe health physics level by allowing the plants to reduce the total carbon dioxide concentration photosynthetically to levels (approximately 0.003%) at or near the compensation point, where respiratory loss is equal to the photosynthetic fixation rate of the gas. An activity of 0.001 μc of C^{14} per liter of air is accepted as safe in terms of continuous human exposure. When this concentration level is reached the large access panel is removed. The remaining C^{14} activity is removed from the chamber by a fan and the chamber is then entered. If a partial harvest is required a smaller panel can be removed. When the chamber is resealed after a harvest the activity of the air is then readjusted. If a harvest period is desired during a period of low natural light intensity it is necessary to use the fluorescent light source to expedite the lowering of the chamber atmospheric C^{14} to the accepted health-safety level.

A TYPICAL BIOSYNTHESIS: C^{14} TOBACCO

A biosynthesis was undertaken to produce uniformly tagged tobacco tissue from which tagged organic compounds could be isolated for use in tracer studies intended to clarify the role of the compounds in plant metabolism. The compounds selected for study were the flavonoid pigments and related polyhydroxy phenolic compounds.[8] These compounds are apparently distributed throughout the plant kingdom, in concentrations varying from a few parts per million in some tissues up to as high as 20% of the dry weight of floral parts of some plants. Little is known of the exact role that they play in relation to the over-all metabolism of plants. While these compounds are present in foods used by animals and man our knowledge concerning their metabolic fate is sparse. It is likely that compounds exist in plants that have hitherto escaped isolation and identification because of their exceedingly low concentrations as well as a lack of suitable methods for detecting their presence by chemical, physical or biological tests. The labeled tobacco tissue is being utilized in part to make such a natural product survey. The carbon-14 tag allows the detection of amounts about 10,000 times less than can be detected by the usual chemical methods. Since all the compounds of uniformly tagged material contain carbon-14, the isolated individual compounds can be detected with tracer sensitivity either by counting or by radiographic techniques.

CULTURE OF CARBON-14 TOBACCO PLANTS

Seed of *Nicotiana rustica* was planted in April in soil in the greenhouse and maintained on 18-hour daylength. Six weeks later the soil was carefully washed from the seedling roots which were then transplanted into fine quartz sand contained in two-quart glazed crocks having bottom-drains. At this time the plants were supplied a complete nutrient solution (Table I). Three weeks later eight of the more vigorous seedling plants, which had four small, partially expanded leaves, were carefully transplanted to quartz gravel in the C^{14} biosynthesis chamber.

The complete nutrient solution (Table I) was supplied twice daily to plants. The temperature and humidity, respectively, were maintained at 75°±2°F and 50–55% relative. Two days later the chamber was hermetically sealed and the atmosphere was charged with $C^{12}O_2$ to raise the concentration of CO_2 to 0.060%; at the same time enough $C^{14}O_2$ was released to adjust the specific activity of the air phase to 350 μc/gm C. After 4 hours of photosynthesis the plants had dropped the CO_2 concentration to 0.052% and the specific activity had dropped to 300 μc/gm C. The following morning the CO_2 concentration had risen to 0.062% while the specific activity of the carbon-14 in the air phase had dropped to 200 μc/gm C. These figures reflect the usual dilution effect which plant respiration has upon the

Table I. Composition of Nutrient Solution

Macro-elements	*Molarity*
$Ca(NO_3)_2$	0.0045
$MgSO_4$	0.0023
KH_2PO_4	0.0023
NH_4SO_4	0.0007
Micro-elements	*ppm*
B	0.50
Mn	0.25
Zn	0.05
Cu	0.02
Fe*	0.50
Mo	0.05

* Fe added as chelated iron salt.

specific activity of the atmosphere early in a biosynthesis period. As the biosynthesis period progressed the plants became more uniformly labeled and the specific activity of the air phase was progressively less influenced by respiratory CO_2. Essentially the plants became uniformly labeled at the desired specific activity when it was observed that the activity of the air phase remained constant throughout a dark period.

At intervals, usually at the start of a photosynthesis period, a new charge was introduced into the chamber to raise the CO_2 concentration and, if necessary, to readjust the specific activity to the desired level. It was the intent of this experiment to tag the entire plant at a specific activity of 250 μc/gm C. Therefore in the early stages of the biosynthesis the specific activity of the air phase was adjusted to 350 μc/gm C, after two weeks to 300 μc/gm C, and in a few more days to 275 μc/gm C. Throughout the entire experimental period, the CO_2 level was maintained within the concentration range of 0.100% to 0.010%. When the plants lowered the atmospheric CO_2 concentration to 0.010%, a new charge was introduced to raise the CO_2 to the maximum level and adjust the specific activity.

Figure 2, A and B, respectively, shows tobacco plants after 32 days in the biosynthesis chamber and after 44 days, just previous to the final harvest.

Forty-seven mc were fixed by the plants and about 3.8 kilograms of fresh plant tissue were produced. Four of the eight plants were harvested on the twentieth culture day, two more on the thirty-first day and the final two on the forty-fourth or last day. The plants were divided into leaves, stems, roots and flowers at harvest time and stored either in a deep freeze or in 85% isopropyl alcohol.

When a harvest was to be made the plants were allowed to photosynthesize until they had reached a compensation point; at this point the rate of CO_2 fixation was equivalent to the rate of CO_2 respired. For tobacco at high light intensities (3000–4000 foot candles), the compensation point concentration was 0.003% CO_2. At this time the level of C^{14} activity had dropped below the maximum permissible health physics level for C^{14} in air. The large access panel was removed and the small amount of residual activity blown off before the chamber was entered.

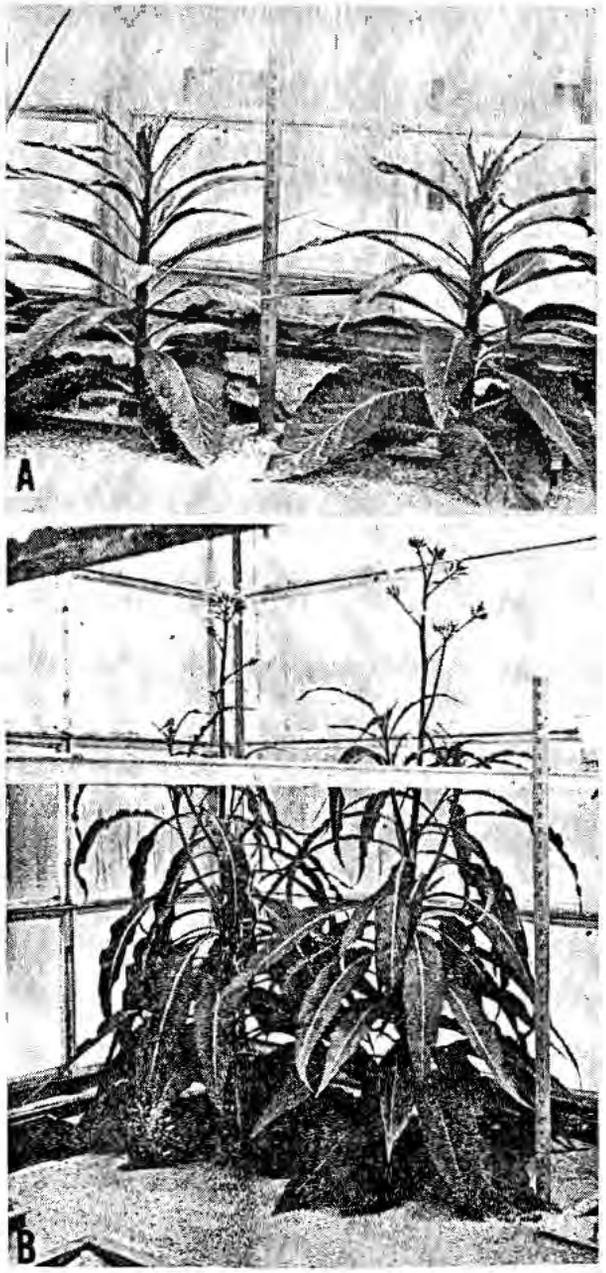

Figure 2. Tobacco plants cultured with $C^{14}O_2$ in biosynthesis chamber for 32 days (A) and 44 days (B)

FIXING AND STORAGE OF PLANT TISSUE

The manner in which the plant tissue is fixed and stored after harvest is determined by its projected use. The most convenient method is drying in a forced-air oven which is vented to the outside atmosphere. It is understood that a drying temperature is employed which does not destroy the desired compounds. Tissue can also be frozen and stored in a deep freeze or it can be fixed by lyophilization (freeze drying). When one is interested in labile materials the tissues can be worked up fresh in suitable solvents like alcohol or boiling water. In the case of the tobacco tissue some was worked up fresh by fixing in boiling isopropyl alcohol and some was placed in plastic bags and stored in the frozen state.

ISOLATION AND IDENTIFICATION OF FLAVONOIDS

The leaf tissue was transferred to boiling 85% isopropyl alcohol and boiled for 5 minutes to destroy enzymatic activity. The tissue was then disintegrated in a Waring blender, transferred quantitatively to a Soxhlet extractor and extracted for 16 hours with 85% isopropyl alcohol. The flavonoids were in the alcohol extract. The alcohol was evaporated from this solution, care being exercised to prevent the solution from going to dryness. The fats, pigments, tannins and other water insolubles were removed by filtration. The flavonoids were in the water filtrate.

They were further purified by extracting with isoamyl alcohol. The isoamyl alcohol was evaporated to dryness under reduced pressure in the presence of nitrogen.

The residue was taken up in a minimum of 80% ethyl alcohol for paper chromatographic analysis. A small aliquot of the 80% ethyl alcohol solution, containing 50–100 μg, was spotted on a large sheet of paper and a two-dimensional chromatogram run. The various components of the mixture separate on the paper because of their different rates of migration in the various solvent systems employed. Since most of the flavonoids and other polyhydroxy phenolic compounds exhibit fluorescences in ultraviolet light, these compounds can be visualized by examination under the ultraviolet lamp provided their concentration is sufficiently great. The minimum amount that can be detected is usually of the order of 5–10 micrograms, and partial identification can be established by the character of the movement of the compound on paper plus its fluorescent color. Colored derivates can also be made of the flavonoids by spraying the chromatographic paper with appropriate chromogenic sprays, such as aluminum chloride or lead acetate, and the colors observed in normal or ultraviolet light. For certain flavonoids as little as 0.5–1 μg can thus be detected.[3] When the paper chromatogram obtained by the above procedures was examined, six spots or compounds were evident. Since all the compounds obtained from the randomly tagged tobacco plants contained C^{14} a radioautograph was made by exposing the paper chromatogram to ordinary no-screen X-ray film. After such exposure and development of the film, additional compounds were detected and the number of compounds was then found to he twenty.

In Fig. 3 is shown the difference which was noted in the detection sensitivity of the chemical (Fig. 3, A) as against the radioautographic (Fig. 3, B) technique with respect to an organic fraction separated by a paper chromatographic method.

Previous to this work three flavonoid compounds had been reported in tobacco leaves, namely, rutin, isoquercitrin and quercetin. By use of the above technique at least four additional flavonoids previously unreported have been shown to occur naturally in tobacco leaves. In order that these compounds be further identified and characterized by classical chemical procedures, much larger quantities than normally employed with paper chromatographic methods have to be processed. Such large-scale separation procedures can be worked out using small amounts of tagged compounds.

The biosynthesis of labeled forms of organic compounds, as well as their isolation and identification, completes the first major step in the application of radiocarbon as a tracer tool.

DISCUSSION

Uniformly C^{14}-labeled intact plants have particular usefulness in the application of radiocarbon as a tracer tool in general agricultural and biological research. They can serve not only as the source of tracer forms of known natural organic constituents of importance in these and other fields, but can be uniquely used in studies designed to isolate and identify previously unknown natural products. It is likely the latter use of radiocarbon plants which can make the more significant contributions.

Figure 3. Chromatogram of compounds separated by paper chromatographic technique. (A), compounds detectable after chemical treatment; (B), compounds detectable when a radioautograph is made of the same chromatogram shown in (A)

A great number of uniformly C^{14}-labeled organic constituents, particularly those classifield as early products of photosynthesis, can be efficiently biosynthesized by use of simple "bell-jar" type facilities.[2] However, it is not possible to biosynthesize uniformly labeled, intact plants with such facilities. This is predominantly true because it is not possible to provide adequately controlled conditions for normal plant growth in small volume systems that are hermetically sealed for extended periods of time. A biosynthesis facility of the type reported here is needed to culture higher plants efficiently under these conditions. It should be noted that the usefulness of this type of facility extends to tracers other than radiocarbon, both stable and unstable types. In addition, the facility is adaptable to a variety of non-tracer, physiological studies concerned with the interaction effects of such environmental variables as carbon dioxide concentration, temperature, mineral nutrition and daylength upon plant growth and development.

Plants of alfalfa, rye, artichoke, *Tradescantia*, onion, *Digitalis*, opium poppy, red kidney bean, *Hevea*, and several varieties of soybean, tobacco, buckwheat and snapdragon have been cultured with $C^{14}O_2$ atmospheres in the plant biosynthesis chambers. The objective of the individual experiments, as well as the duration of the culture periods, varied from species to species. Many of these studies were conducted collaboratively with personnel of other research groups in the course of exploring and applying the usefulness of the biosynthesis facilities and the tracer products synthesized. Certain of these experimental studies are worthy of review for individuals interested in the scope of the research application which can be made with this approach.

The biosynthesis of uniformly labeled sucrose and dextran, a blood volume expander, has been reported.[7] In this study the biosynthesis of a total of 175 mc of uniformly C^{14}-labeled sucrose was accomplished with use of photosynthesis periods which were as short as 5 hours in duration. Of all the $C^{14}O_2$ supplied to excised *Canna* leaves an average of 99% was assimilated and an average of 57% was converted to sucrose. Microbiological degradation of the isolated sucrose indicated that the molecule was uniformly labeled.[7] Comparable efficiencies can be attained in the biosynthesis of a specific organic constituent when intact higher plants are cultured for periods as long as seven weeks. Uniformly C^{14}-labeled soybean oil, tagged at 70 μc per gram of carbon, was biosynthesized for use in tracer animal metabolism studies.[9] Soybean plants were photoperiodically induced by short-day treatment to form microscopic terminal inflorescences at all meristems just previous to placing them in the biosynthesis chamber for a seven-week culture period. Such photoperiodic treatment prevents further meristematic differentiation of leaves in this variety of soybean. At harvest the tagged plants had mature pods which contained 56% of all the assimilated C^{14}. In the seed alone 39.6% of the assimilated C^{14} was present, while 12.6% was present in 12.2 grams of tagged oil isolated from the seed. This biosynthesis points out the efficiency which can be attained in converting C^{14} into either specific plant organs or their products by the physiological regulation of the character of growth of plants.

It is essential to emphasize the major limitation associated with the biosynthesis of C^{14} plants or their products. It is obvious that plants cannot be cultured with tissues which contain concentrations of C^{14} which will induce abnormal growth in response to the beta radiation emitted by this isotope. One of the continuing objectives of the C^{14} program has been to establish the concentrations of this isotope that induce abnormal plant behavior as judged by a number of response indices.

Roots from C^{14}-labeled onion plants cultured in the biosynthesis chamber for periods ranging from 7–14 weeks and tagged with 44–46 μc per gram of dry tissue (absolute activity) exhibited both chromosome and chromatid breaks and fragments in dividing cells, as well as bridging and micronuclei.[1] While no visible developmental anomalies occurred during the chamber culture period, the new growth of harvested dormant sets made in normal atmospheres was arrested and mutant leaf areas (chlorophyll deficiency) occurred. At this C^{14} concentration macroscopic anomalies in this species only occurred after long exposure to the beta radiation.

Several factors must be considered in evaluating the concentration of C^{14}, occurring as constituent body carbon, which can make the biosynthesis of labeled plants or their products either impractical from an efficiency standpoint or an impossibility. The major biolngical factor to be evaluated is the relative radiation sensitivity of plant species, including not only the species sensitivity at various life-cycle stages, bot also the relative sensitivity of the different tissue types that constitute the several organs.

The various organs of uniformly C^{14}-labeled plants are by no means exposed to identical beta-radiation dosages. In all cases a microcurie of C^{14} uniformly distributed in a gram of fresh tissue is calculated to result in approxionately 3 rep (roentgen equivalent physical) per day.[4] This applies to a gram of tissue centrally located in an organ which is labeled at the same C^{14} concentration and whose average diameter exceeds the tissue range of the C^{14} beta particle. While a measurable variation occurs in the quantity of carbon per dry tissue weight the most significant plant composition factor in effecting different radiation dosage is variation in moisture or dry weight. The freshly harvested seed of uniformly C^{14}-labeled mature red kidney bean and soybean plants were found to have radiation dose-rates that were 8 and 9 times greater, respectively, than that occurring in the fresh leaves. While no abnormal tissues appeared on the soybean plants while in the biosynthesis chamber, their seedlings exhibited arrested growth rates and abnormal tissues when grown immediately following harvest of the tagged seed (Fig. 4).

As much as a 10-fold difference in species sensitivity to radiocarbon has been observed in the course of culturing the various plants noted earlier. Red kidney bean has been predominantly used in evaluat-

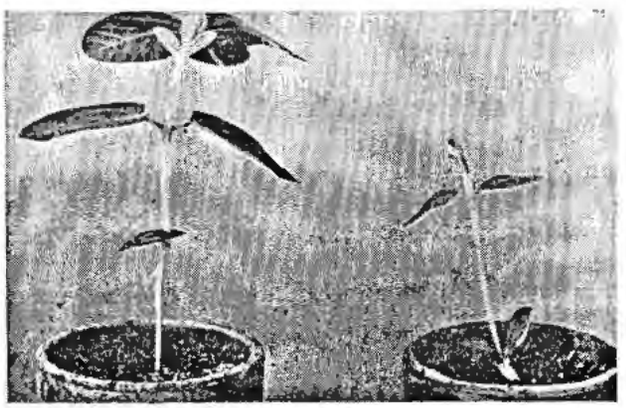

Figure 4. Seedlings of Chief variety soybean grown from control (left) and C^{14}-treated (right) seed

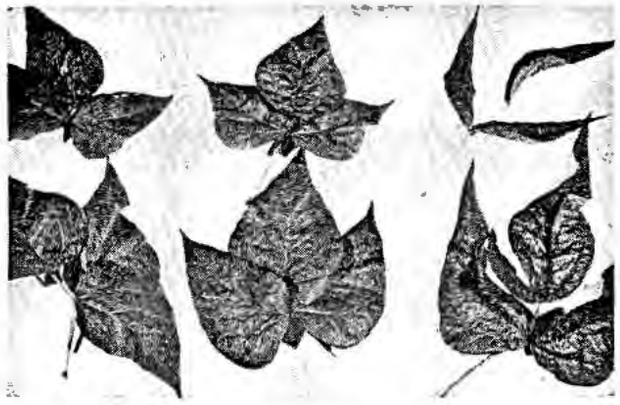

Figure 5. Leaves of red kidney bean plants grown in the biosynthesis chambers for 33 days in atmospheres containing indicated specific activities of $C^{14}O_2$. For each C^{14} concentration the upper young leaf was completely differentiated during the culture period while the bottom mature leaf had just emerged from the terminal bud at start of the experiment

ing the pattern of anomalies which occur in response to toxic concentrations of C^{14}. This species was cultured with atmospheres containing $C^{14}O_2$ with specific activities of 0, 50, 100, 1000 and 2000 μc per gram of carbon. In all cases plants were grown from seedling to adult stages. With the two highest specific activities, radiation-induced types of deformed leaves occurred (Fig. 5). In addition a chlorotic leaf symptom developed which was strikingly similar to that typically induced by nitrogen deficiency. With the 2000-μc level the terminal, main axes meristems were aborted or partially necrotic when observed under the dissection microscope. Obviously in such instances the development of flowers or fruit cannot take place.

An effort was made to relate the "nitrogen deficiency," leaf response noted in the case of plants cultured with specific activities of 1000 and 2000 μc per gram of carbon to possible physiological or biochemical differences in the leaf tissues. Chemical assay for total nitrogen and ascorbic acid of both young and mature leaves of control and C^{14} plants was made. These assays indicated that a significantly decreased level of nitrogen and an increased level of ascorbic acid occurred beginning with plants cultured with the 1000 μc concentration of C^{14}. Although no other chemical constituents were assayed it is likely that additional differences would have been found in the abnormal tissues of these plants and likely can be found in tissues of plants cultured at C^{14} concentrations which induce macroscopically visible growth anomalies.

The plant biosynthesis facility (Fig. 1) has been used in connection with a number of C^{14} tracer physiological studies. Relatively typical of the qualitative experimental data that can result is that recently obtained in the case of *Hevea braziliensis*. This tracer study was concerned with carbon assimilation and rubber formation and turnover in *Hevea*.[10] These studies have determined the rate at which current "photosynthate" is translocated from the leaf to other organ sites, as well as the rate of conversion of "photosynthate" into rubber in latex in the different organs. Using intact young plants it was found that tagged rubber first appeared in leaf latex, suggestive of the possibility that the organic precursor of the rubber molecule is predominantly formed in and supplied from this organ. Trees approximately 3-4 inches in diameter were tapped for latex 3 times a week during the course of a 115-day C^{14} culture period in order to evaluate the possible organic latex fractions which contain substrates that can efficiently serve as substrates for conversion into the rubber molecule. The results of assay of latex solvent fractions showed that a marked correlation existed between the rate at which C^{14} appeared in latex constituents soluble in 80% alcohol and that at which it appeared in isolated rubber (Fig. 6). The isolated, tagged compounds of several of the organic solvent fractions are to be infiltrated into *Hevea* tissues to evaluate their suitability for conversion into rubber.

Other *Hevea* trees containing rubber tagged at a given concentration of C^{14} exhibited approximately a 10-fold loss of rubber activity when defoliated and retained in normal atmospheres. Since no appreciable variation in total rubber concentration occurred in this experimental period the data suggest that rubber, once formed, does turnover at a measureable rate, a point which has been in question for some time.

While the biosynthesis of C^{14} plants and their products is obviously restricted by the biological effects which beta radiation can exert at elevated dosages, this method of tracer synthesis nevertheless

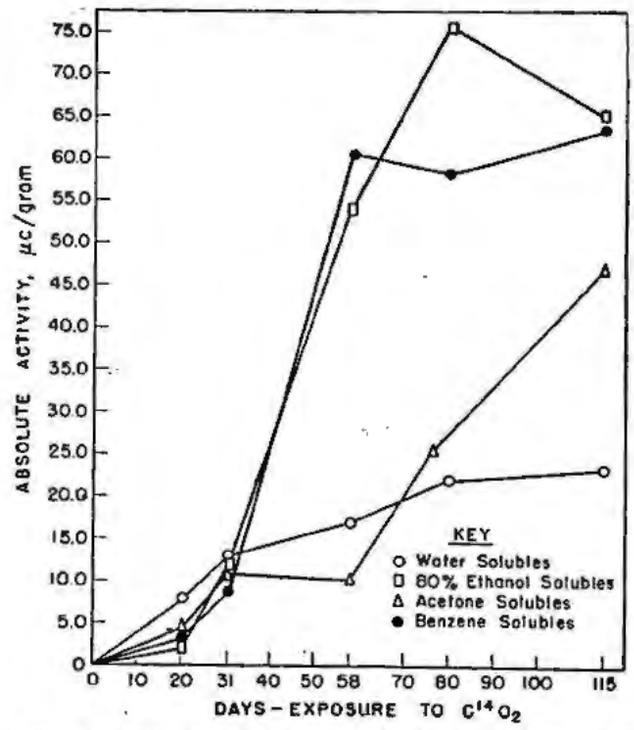

Figure 6. Absolute activities of organic fractions of stock stem latex from Hevea tree cultured in biosynthesis chamber with $C^{14}O_2$ for 115 days. Solvent extraction sequence was water, alcohol, acetone followed by benzene. Benzene soluble fraction represents rubber

offers relatively unlimited opportunity towards the profitable application of radiocarbon as a tracer tool where efforts are being made to obtain a better understanding of organic compounds, whether the concern be in the fields of agriculture, chemistry, biology or medicine. It is hoped that the selected observations noted here will be useful to others as a basis for determining the value which the C^{14} plant biosynthesis facility and its products may be to those who forsee that radiocarbon can serve as a useful tool in their particular areas of research.

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8. This study is being carried out in cooperation with Dr. Simon H. Wender, Department of Chemistry, University of Oklahoma. Details of complete study will be reported elsewhere in a separate report.
9. This study was carried out in cooperation with Dr. Theodore W. Rall and Dr. Gerald H. Becker, US Army Medical Nutrition Laboratory.
10. This study is part of a program being carried out in cooperation with Dr. M. W. Parker, Division of Rubber Plant Investigations, US Department of Agriculture and Dr. Juan C. Montermoso, Rubber Branch, Chemicals and Plastics Division, US Army Quartermaster Corps.

Record of Proceedings of Session 15C

TUESDAY MORNING, 16 AUGUST 1955

Chairman: Mr. A. V. Palladin (Ukrainian SSR)
Vice-Chairman: Mr. E. C. Wassink (The Netherlands)
Scientific Secretaries: Messrs. I. D. Rojanski, E. O. Hughes and C. Polvani

PROGRAMME

15C.1. Animal Physiology

P/90 Radioisotopes in animal physiology and nutrition—mineral metabolism ..... C. L. Comar

P/908 Isotopes in permeability studies ..... H. H. Ussing

DISCUSSION

P/275 Use of I^{131} in the study of the influence of climatic factors on thyroid activity and productivity of livestock ..... C. Blincoe and S. Brody

P/1119 Problems raised by the posterior lobe of the pituitary—and studied by radioactivity ..... A. Jentzer

P/1047 Studies on the metabolism of calcium and phosphorus in the laying hen ..... R. Sasaki

DISCUSSION

15C.2. Plant Biochemistry

P/697 Tracer atoms used to study the products of photosynthesis depending on the conditions under which the process takes place ..... A. A. Nichiporovich

P/259 The photosynthetic cycle ..... M. Calvin and J. A. Bassham

DISCUSSION

P/715 C^{14} in the study of the biosynthesis of chlorophyll ..... T. N. Godnev and A. A. Shlik

P/459 Some aspects of sucrose metabolism in plants ..... H. K. Porter and J. Edelman

DISCUSSION

The CHAIRMAN: Today we pass on to the discussion of questions relating to the utilization of radioactive isotopes in biology. At our previous sessions we heard and discussed papers on the use of radioisotopes in medicine and on the action of ionizing radiations on organisms. Today's session is devoted to papers on the physiology and biochemistry of both animals and plants. The first part of the session will be given over to papers on animal physiology and the second part to papers on plant physiology. In the first part of the session we have papers dealing with the use of radioisotopes in the study of permeability and of the functions of the thyroid gland and the pituitary body and also papers of a more practical character concerning the solution of certain stockbreeding problems. I hope that the papers and the discussion today will prove useful and that our session will make a further contribution to the solution of important problems in biology, physiology and biochemistry.

Mr. C. L. COMAR (USA) presented paper P/90.

Mr. H. H. USSING (Denmark) presented P/908.

DISCUSSION OF P/90 AND P/908

Mr. E. PORIA (Romania): I should like to ask Mr. Comar whether, in his studies on the mineral metabolism of mammals, he took account of vitamin D and parathormone requirements and whether he observed variations in the distribution of calcium, in cases of vitamin or hormone deficiency.

Mr. COMAR (USA): We have always taken these matters into consideration. In the studies reported the animals were on a normal diet with normal vitamin D contents. We have a good many other studies in which we have looked at the effects of parathormone and we find that the method is a very powerful one in showing the effect of parathormone extract in removing these elements from bone, in creating an accumulation of these elements in the kidney, in increasing the urinary excretion of phosphorus.

We find that we get a very good picture with radioisotopes of effects such as you have mentioned.

Mr. B. HASTINGS (USA): I was very much impressed with the results Mr. Ussing had and particularly those concerned with pitressin. I wonder if there is any information on the effect that pH or even other ions such as potassium and calcium ions, might have on the drag force that you mentioned.

Mr. USSING (Denmark): So far we have studied mainly the effect of the hormones only in the presence of this hormone. We have not studied the pH effects very carefully, but there seems to be an optimum range between pH7 and pH9, where the hormone is effective. At low pH there does not seem to be any appreciable effect.

Mr. E. D. BERGMANN (Israel): I have the following question for Mr. Ussing. It has been known for some time that certain triperpenoids, and especially glycorrhyzic acid have a profound effect on the sodium-potassium metabolism in men. Have any experiments been made in the interesting system of the frog skin which might have a bearing on the physiology of the sodium-potassium equilibrium in men under the influence of these substances?

Mr. USSING (Denmark): We have not studied these substances so far. But it certainly is a very good suggestion that they be studied.

Mr. C. BLINCOE (USA) presented paper P/275.

Mr. A. JENTZER (Switzerland) presented P/1119.

Mr. S. MITSUI (Japan) presented paper P/1047.

DISCUSSION OF P/275, P/1119 AND P/1047

Mr. BLINCOE (USA): I have a question for Mr. Jentzer. In normal animals, do you have any information as to the location of I^{131} in the pituitary with regard to the type of cell, and is it associated with any particular structure within the cell?

Mr. JENTZER (Switzerland): I do not quite understand Mr. Blincoe's question. All I can say is that it is not a question of structure, but of cellular localization. The iodine-131 goes into the cells of the thyroid body, where it is converted into thyroxine, which later concentrates in the cells of the posterior pituitary. Is that the reply Mr. Blincoe wanted?

Mr. BLINCOE (USA): I was wondering particularly whether they went into any particular structure within the cell. Is it associated with the membrane, with the nucleus or with the golgi apparatus?

Mr. JENTZER (Switzerland): No. The thyroxine goes into the cell protoplasm, not into the cell nucleus.

Mr. F. LEBORGNE (Uruguay): I should like to know whether the thyroxine has been identified in the pituitary by radiochromatography.

Mr. JENTZER (Switzerland): That is an extremely interesting suggestion. We have not used that method, but it would be very easy to locate the thyroxine by radiochromatography. I am sure my Uruguayan colleague's idea will be confirmed, for when we injected tagged thyroxine it became localized. It will be an additional proof if I can confirm my colleague's suggestion, which, I repeat, is very interesting.

Mr. O. COSTACHEL (Romania): Mr. Jentzer's outstanding paper interested me greatly, as we are also working on artificial hibernation at the Bucharest Institute of Oncology, using a special, drugless method after carbon dioxide narcosis, at an ambient temperature of —25°C. The results of our biochemical investigations confirm Mr. Jentzer's studies in all respects. Tissue respiration, mineral balance and enzymatic activity prove that a shock state is not involved. The biological tests (Thorn, etc.) have given us the same results. I should like to ask Mr. Jentzer whether he has compared the state of artificial hibernation with the compensated and decompensated shock state, and whether he has studied the enzymatic activity of the tissues in the hibernation and shock states by means of radioactive isotopes. This question is of especial interest to us because, in our work, we always compare these three states: narcosis, artificial hibernation and shock state. We have found, I might add, that artificial hibernation is entirely different from shock and from narcosis.

Mr. JENTZER (Switzerland): By the hyperglycemia and other methods we have proved that shock is suppressed in artificial hibernation. I see that Mr. Costachel and I are in agreement on this point.

Mr. COMAR (USA): It was gratifying, Mr. Mitsui, to see that the work of Professor Sasaki paralleled and gave the same over-all picture of much that has been done in other laboratories. I should like particularly to know what you think about the mechanism by means of which the Ca^{45} that you introduced into the albumin found its way into the shell; particularly, whether this represents an accretion of the shell after the egg has been fully formed.

Mr. MITSUI (Japan): I am very sorry that I am not exactly in the same field as Mr. Sasaki and therefore I should like to refrain from replying to your question. However, if you desire, I should be happy to make available to you a series of reprints, which are written in Japanese, but the tables, figures and short summaries are in English.

Mr. G. B. BROWN (USA): Mr. Mitsui, do you know whether Mr. Sasaki did any work on the nucleotides of the yoke or the egg or the hen, and whether or not it was practical to investigate those when the P^{32} studies were being made? I am sure that when the emhryo is developing there is a sufficient amount of soluble nucleotides in order to study them. But in the earlier stages in the egg I do not know how many nucleotides would be there and whether it would be enough to permit any studies.

Mr. MITSUI (Japan): I should have to apologize again for being unable to answer the question. However, I shall be very glad to give you a copy of the reprint.

The CHAIRMAN (Professor E. C. Wassink, Vice-Chairman, took the Chair): Ladies and Gentlemen: We are now starting our session on radioactive isotopes in plant biochemistry. I want first to thank Mr. Palladin for leaving the Chair to me now.

Looking over the papers to be presented, one may state with some surprise that they more or less form an entity related to a restricted sector of the wide field of plant biochemistry, namely, the problem of photosynthesis.

Two of our papers report on studies of the intimate mechanism of the process as such; one paper considers the biosynthesis of the main light absorbing pigment, chlorophyll; whereas the fourth paper is concerned with the conversions of starch and sugars, which are among the main products of photosynthesis.

It seems remarkable that at this congress on the use of atomic energy, the main attention in this plant biochemistry session should be focussed on the process which is foremost in trapping solar energy.

It is useful in this connection to recall to mind a figure that has been presented to us at one of the first sessions of this conference—namely, that from the 29 thousand million megawatt hours of electricity equivalent energy the world produced in 1952 as much as 28.6, or over 98 per cent, were derived from past and present products of photosynthesis if we include natural gas among the biogenic products. Photosynthesis is an attractive energy-yielding process inasmuch as it converts energy income rather than energy capital. This solar energy income is so large that total photosynthesis covers probably less than one per cent, and all our fossil fuel deposits do not equal more than or are perhaps even less than, one year's solar radiation. Any attempt to increase the outcome of plant photosynthesis presupposes an understanding of the characteristics of the process, and the considerations on energy income and capital set up in recent years have certainly helped to keep this process in the focus of scientific interest.

The study of the mechanism of photosynthesis as we know it now started late in the eighteenth century with Priestley's discovery of the oxygen evolution by green leaves, and, closely following, important discoveries by Ingenhousz, Senebier, de Saussure and others. Further milestones were the discovery of the formation of starch in the chloroplasts, the elucidation of the chemistry of chlorophyll, and the formulation of the principle of the limiting factors, which proved extremely useful for kinetic studies of photosynthesis. More recently, kinetic studies have aimed at studying detailed reactions in cell-free preparations or at interconnecting the main features of the process with concurrent phenomena among which the fluorescence of chlorophyll and, to a smaller extent, redox potential observations may be mentioned. At the same time biochemists found a new powerful tool in isotopes for tracing the chemical conversions involved in the various steps, and it is about this type of work that we shall hear this morning.

Mr. A. M. KUZIN (USSR) presented paper P/697.

Mr. M. CALVIN (USA) presented paper P/259, as follows:

The subject which I am about to discuss hardly needs justification to this audience.

The process of photosynthesis is usually defined in terms of the biochemical reaction in this fashion:

$$CO_2 + H_2O \xrightarrow{\text{hv}} (CH_2O)_x + O_2$$

This over-all reaction has been divided into two distinct stages, both chronologically and chemically. The first is the photolysis of water to produce a reducing agent and an oxidizing agent, which in turn leads to molecule O_2:

$$H_2O \xrightarrow{\text{hv}} 2\,[H] + \tfrac{1}{2}\,O_2$$

The second is the reduction of carbon dioxide by the reducing agent:

$$CO_2 + 4\,H \longrightarrow (CH_2O)_x + H_2O\,.$$

It is with this second one that we are most concerned and of which the availability of isotopic carbon from the Oak Ridge piles made possible a detailed study.

As a result of this work, it is now possible to write the complete path of carbon reduction in photosynthesis, with all intermediates and enzymatic reactions, from carbon dioxide to sucrose and starch with a very clear indication of the route to amino acids and lipides as well.

The methods used in studying the path of carbon in photosynthesis are here described briefly. In nearly all cases the initial condition is an actively photosynthesizing plant in which photosynthesis has been maintained long enough to establish a "steady state." In this steady state the concentrations of various intermediate compounds in the pathway from carbon dioxide to sucrose are constant. The plants commonly used in these experiments are the unicellular green algae, *Chlorella* or *Scenedesmus*, but leaves of higher plants have often been used as well.

In the first type of experiment to be discussed, $C^{14}O_2$ is added to the unlabelled CO_2 that the plant has been using. After a measured short period of photosynthesis with $C^{14}O_2$, the plant is killed by sudden treatment with boiling ethanol. All enzymatic processes are thereby quickly halted. Extracts of the plant material are made, concentrated, and then analyzed by two-dimensional paper chromatography and radioautography. See Slide 1 (Fig. 2 of P/259).

The radioautographs obtained from experiments of 60-seconds and 10-seconds photosynthesis with $C^{14}O_2$ are shown in Slide 2 (Fig. 1 of P/259). The 60-second experiment illustrates the importance of various sugar phosphates and acid phosphates in carbon reduction, and one can also see the beginnings of amino acids and other types of material as well.

The lipides are off the scale. The 10-second experiment shows the predominance of phosphoglyceric acid at short times. If the percentage of C^{14} in phosphoglyceric acid (PGA) of the total C^{14} incorporated during photosynthesis for various short periods of time is extrapolated to zero time, it is found that then all the C^{14} is in phosphoglyceric acid. This compound is therefore identified as the first isolatable compound into which carbon dioxide is incorporated in photosynthesis.

Slide 3 (Fig. 3 of P/259) shows the distribution of the labelled carbon in the three carbon atoms of the glyceric acid obtained from the phosphoglyceric acid in a 15-second experiment with barley. From the same experiment some hexose (fructose and glucose) was obtained and degraded. The distribution of radiocarbon in the two 3-carbon halves of the hexose was found to be very much the same as it is in the three carbons of glyceric acid.

This result immediately suggests that the 6-carbon piece is made from the two three's by joining the two carboxyl-carbon atoms. This is simply a reversal of the well-known aldolase split of fructose diphosphate in the glycolytic sequence, a part of which is shown in Slide 4 (Fig. 4 of P/259).

Thus, the two carbon atoms which were originally carboxyl-carbon atoms finally fall in the middle of the hexose chain. It is quite clear that there must be some compound that accepts the carbon dioxide to form the glyceric acid. Furthermore, that compound must be regenerated from the PGA (phosphoglyceric acid), triose phosphates, and hexose phosphates, or some other compound formed from them. It is thus evident that there is a cyclic process involved in the reduction of carbon dioxide.

We must now seek to determine the compounds involved in the cyclic regeneration of the carbon dioxide acceptor. The roles of PGA, triose phosphates and hexose phosphates, leading through a nucleotide carrier (UDPG) to sucrose and starch, have already been identified. The route to some amino acids (glycine, alanine, serine, as partic and glutamic) porphyrins (chlorophyll, cytochromes) and lipids (fats, steroids) all branch off at PGA. Of the compounds labelled by short periods of photosynthesis, there were left *only* the seven- and five-carbon sugar phosphates. These are: sedopheptulose-7-phosphate (SMP), ribose-5-phosphate (RMP), ribulose-5-phosphate (RuMP) and ribulose diphosphate (RuDP).

A detailed analysis of the distribution of radioactivity among the carbons of these sugars is shown in slide 5 (Fig. 8 of P/259). The stars give some indication of the order of appearance of radioactive carbon in these compounds, and it was from an analysis of these data that it became possible to deduce relationships between the various compounds containing three, five, six and seven carbon atoms.

Thus we established the relationship between all the early labelled compounds, excepting the point of entry of CO_2, i.e., the carbon dioxide acceptor.

All the results thus far were obtained with the first type of experiment I have described, in which $C^{14}O_2$ was added to plants for a very short period (1 to 60 seconds) before the plants were killed. A second type of experiment was used for the identification of the CO_2 acceptor. In this case once again the starting condition was an actively photosynthesizing algae suspension in "steady-state" condition. In addition, the intermediate compounds were "saturated" with C^{14}. Under these circumstances, the concentration of each labelled intermediate compound can be determined from the radiocarbon found in that compound on subsequent analysis by chromatography and radioautography.

After this initial C^{14}-saturated steady state was obtained, some environmental condition such as light was suddenly changed. Aliquots of the algae were taken every two or three seconds for about a minute, and then at less frequent intervals. Analysis of these aliquots showed the way in which the concentrations of the various intermediates varies as a result of the environmental change.

In the first such study the light was turned off, and the result is shown in Slide 6 (Fig. 11 of P/259). It was found that the concentration of PGA increased very rapidly while that of ribulose diphosphate (RuDP) decreased rapidly.

These changes in concentrations are accounted for by the reactions shown in Slide 7 (Fig. 12 of P/259). The reduction of PGA to triose and the formation of RuDP are reactions requiring light; while RuDP is converted to PGA via a carboxylation reaction that does not require light.

The series of re-arrangements between triose phosphate and ribulose-5-phosphate are all at the sugar level of oxidation and require no special energy source. The reduction of PGA requires both reducing power—reduced triphosphopyridine nucleotide—and adenosine triphosphate, while the formation of RuDP from RuMP requires only ATP. Both these cofactors are produced at the required rate only when the light is on. Thus, when the light is turned off, the rate of formation of RuDP and the rate of reduction of PGA decrease, but the rate of carboxylation of RuDP to form PGA continues unaffected except by the concentration of RuDP.

From the above scheme it was possible to predict the result if the light were left on but the CO_2 pressure were suddenly decreased. In that event, the carboxylation of RuDP to form PGA should decrease but the formation of RuDP and the reduction of PGA should be unaffected. Consequently the concentration of RuDP should rise while that of PGA should fall.

This experiment was performed and the expected result, shown in Slide 8 (Fig. 13 of P/259), was obtained. These changes provide excellent confirmation for the proposal of the cyclic system. Thus, kinetic *in vitro* evidence is provided for the carboxylation reaction:

$$RuDP + CO_2 + H_2O \longrightarrow 2\ PGA$$

The complete carbon-reduction cycle is shown in Slide 9 (Fig. 14 of P/259). Here are shown all the details, including the intermediate compounds and enzymes required for the various transformations. The net result of three complete turns of the complete cycle is the introduction of three molecules of CO_2 and the carboxylation of three molecules of ribulose diphosphate, leading to the formation of six molecules of phosphoglyceric acid. These six molecules of PGA are then reduced to provide six molecules of triose phosphate. Of these, five are eventually converted to five molecules of ribulose diphosphate, thus completing the cycle, while the sixth finds its way ultimately into sucrose and represents the net gain in reduced carbon per three turns of the cycle. All the enzymes shown had been isolated more or less concurrently with the discovery of their labelled substrates and the relationship between them, except for the carboxylation enzyme which converts CO_2 and ribulose diphosphate to PGA.

Using the tracer method, we sought and found this last predicted enzyme in a cell-free preparation, both from algae and from other green plants. It is capable of catalyzing the production of 2 moles of PGA specifically from ribulose diphosphate (RuDP) and sodium bicarbonate.

Having the cycle in all its details, we know precisely what reagents are required to make the cycle turn. See Slide 10 (Fig. 19 of P/259). A calculation of energetic compounds needed per CO_2 molecule entering will show that the net requirement for the reduction of one molecule of CO_2 to the carbohydrate level is four equivalents of reducing agent, i.e., two molecules of TPNH and three molecules of ATP.

These two required cofactors must be made in the plant ultimately by the light through the conversion of the electromagnetic energy in some way. If we could supply those two things from some other source than the photochemical reaction, we should be able to make this whole sequence of operations function.

Such a system consisting only of the soluble purified or partially purified protein enzymes previously enumerated together with a supply of TPNH and of ATP, has indeed been reconstructed by Racker. This collection of dissolved substances converts CO_2 into hexose by the route here deduced entirely on the basis of isotopic studies with intact plants, and provides an additional confirmation of this new cycle.

A survey examination of $C^{14}O_2$ fixation products in a wide range of photosynthetic organisms from purple bacteria and blue-green algae through the green algae, mosses, grasses and higher plants has revealed the presence of the cycle compounds in all. There are of course wide differences among plants in the relative rates and character of the reactions which remove reduced carbon from the cycle. The physiological condition of a given plant as well as its immediate external environment can also effect the quantitative relation among these reactions branching out at different points of the cycle, as the experiments of Mr. Nichiporovich just described by Mr. Kuzin so gratifyingly confirm.

Among the more obvious questions which now face us is that of determining the chemical route between the oxygen atom of the water (H_2O) and the molecular oxygen (O_2) in which it ultimately appears, and perhaps with this will come a more intimate view of the photoreaction itself in which the electronic excitation of chlorophyll is first converted into chemical potential.

DISCUSSION OF P/697 AND P/259

Mr. Calvin (USA): This question, of course, was written prior to the presentation by Mr. Kuzin, so that part of it is already answered. However, in the text given by Mr. Nichiporovich there is some indication of a certain suggestion, and I should like to ask this question. Is the suggestion made that there may exist a major port of entry of carbon in substantial, not catalytic, amount, into the reduced material of the plant, other than the carboxylation of ribulose-phosphate to PGA, and if so, what is that carboxylation reaction, and under what conditions does it function?

Mr. Kuzin (USSR): Professor Nichiporovich did not concern himself especially with investigations of the very first intermediate products of photosynthesis. He assumes that the fundamental process of carbonic acid fixation is according to the scheme which he cited as the primary one and which is taken from your interesting studies. He assumes, however, that subsequent diversification of the reaction processes is possible and must also be materially affected by light, for his experiments show that under the influence of light of different wave-lengths the necessary distribution of the products of photosynthesis is brought about in the chloroplast.

Mr. Calvin (USA): The question of the distinction between the red and the blue lights is a technical one having to do with the question of whether the number of quanta impinging in the red-light experiment and the blue-light experiment are known. In other words, since we know that the relative rate of the various branchings off from this cycle is dependent upon the total over-all light intensity, the answer to the question should be known, namely, when we compare the difference in the products of the red light and the blue light, are they being compared at the same incident quantum rate, or is perhaps the incident quantum rate of the two colors known?

Mr. Kuzin (USSR): So far as I am aware, in Professor Nichiporovich's experiments the energy conditions were completely equalized, and this was taken into account.

Mr. T. N. Godnev (Byelorussian SSR) presented paper P/715.

Mrs. H. K. PORTER (UK) presented paper P/459, as follows:

The aspects of sucrose metabolism that I want to talk about today are those related to its possible role in polysaccharide synthesis.

The fact that starch formation can be induced in leaves depleted of carbohydrate, by floating them on sugar solutions, is one of the oldest observations on which the view that sugars are intermediates in the synthesis of starch from carbon dioxide is based. The root of this postulated transformation still remains an unanswered question. By the use of sugars labelled with C^{14} this question may now be re-examined, since the metabolism of the hexoses or of the hexose residues combined in sucrose can be followed individually.

The first step required is the preparation of the labelled sugars. Uniformly labelled glucose and fructose have been prepared from $C^{14}O_2$ by photosynthesis using detached tobacco leaves. The hexoses were isolated from aqueous extracts and purified chromatographically. Sucrose labelled with C^{14} in either the glucose or fructose moieties can then be prepared by enzyme transfer reactions. Using sucrose phosphorylase transfer of the glucose moiety of inactive sucrose to uniformly labelled fructose yields sucrose labelled in the fructose moiety. Using the sucrose of *A.oryzae* transfer of the fructose moiety of inactive sucrose to uniformly labelled glucose yields sucrose labelled in the glucose moiety.

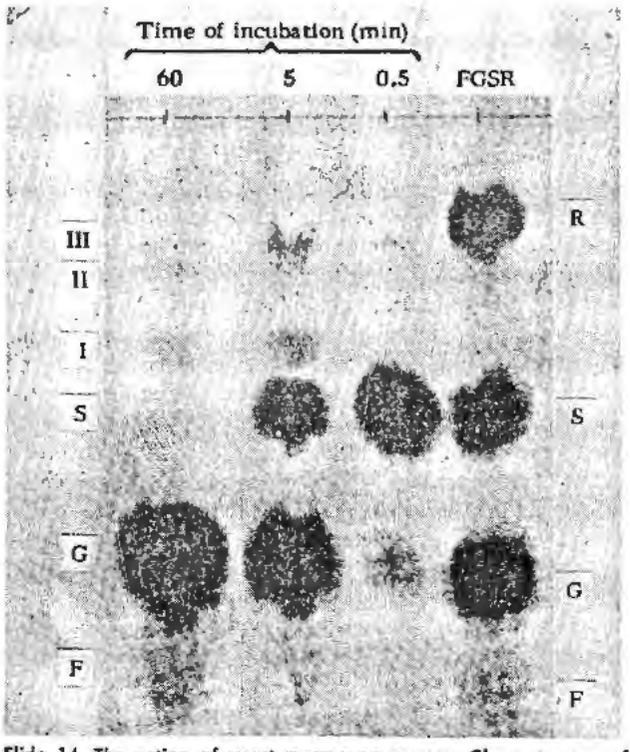
The second step was to supply the purified radio sugars in 5 per cent solution to disks cut from tobacco leaves previously depleted of carbohydrate. Using the Warburg technique so that respiration can be measured, it was found that starch synthesis, carbon dioxide output, and the sugar accumulating within the disks accounted for about 80 per cent of the total sugar lost from the solution, so that from total change in 24 hours a good estimate could be obtained of the amounts of glucose and fructose free or combined in sucrose, which were diverted to respiration and starch synthesis.

The relative specific activities of the starch formed and of the carbon dioxide evolved when sucrose labelled in either the glucose or fructose moieties or invert sugar in which one hexose was labelled, where substrates are shown in Slide 11 (Table I of P/459). The first column shows the mean specific activity of the sugar supplied..

It is evident that the specific activity of the starch formed and of the carbon dioxide evolved were equal to one another, and equal or nearly so to that of the sugar supplied in all four cases. It can be concluded that starch and carbon dioxide arose solely from the sugar supplied, and were themselves derived from a common source. Moreover glucose and fructose whether initially free or combined in sucrose must have contributed equally to this common source, and there could be no question of a preferential use of glucose or fructose for either starch formation or as a substrate for respiration. The sugars entering the cell may therefore be supposed to become incorporated into a metabolic pool, by the formation of derivatives of the hexoses which can be rapidly interconverted.

The distribution of labelling in the sugars accumulating in the disks is shown in Slide 12 (Table II of P/459). When sucrose was supplied the sucrose retained the asymmetry of labelling within the cell and at the same time free hexoses which appeared in considerable quantity had the same activity as the sucrose moieties and are therefore assumed to have been derived from sucrose by inversion. Since there was no exchange of label between the free hexoses, inversion must have occurred at a site removed from that of starch synthesis and respiration. When invert sugar was supplied there was synthesis of sucrose as well as starch and this newly formed sucrose was strongly labelled in both moieties whichever hexose was initially labelled. This new sucrose must therefore also have derived from the metabolic pool after equilibration of the fructose and glucose structures. Free hexoses in this case were strongly but unequally labelled, a circumstance which is thought to arise from mixing of the incoming invert sugar in which only one hexose was labelled with the products of inversion of the sucrose in which both moieties have become labelled, again on the assumption that accumulated inversion takes place out of contact with the interconverting system.

The results of these experiments suggest that



Slide 14. The action of yeast sucrase on sucrose. Chromatogram of 2 μl samples of a reaction mixture at 20° containing 400 mg sucrose, 2.0 ml 0.05 M sodium acetate buffer, pH 5.0, and 0.4 ml vol % yeast sucrase. I, probably a disaccharide; II and III, trisaccharides. I, II and III contain both glucose and fructose

Table 1. The Action of Yeast Sucrase on Sucrose and Radioactive Glucose

	Time of incubation (min)				
	2	6	11	15	60
After development	Counts per minute				
Glucose spot	5900	5820	5470	5720	6060
Sucrose spot	37	29	34	16	23
Spot I	183	227	152	113	68
Spot II	0	8	2	4	1
Spot III	12	11	17	8	5
Total	6132	6095	5675	5861	6157

sucrose is perhaps not on the direct pathway of starch synthesis, but that the link between carbon dioxide and starch is effected through sugar derivatives, probably phosphate esters, which in appropriate circumstances give rise to sucrose too.

To accommodate these results, it would seem necessary to postulate that the first stage in the utilization of free sugar as the substrate for starch synthesis must be the formation of derivatives which could lead to rapid glucose fructose interconversion. The well known need for oxygen in the conversion of sugar to starch in feeding experiments might perhaps be in oxidative phosphorylation to provide adenosine triphosphate for the initial introduction of phosphate into the sugar molecule. By inhibiting oxidative phosphorylation therefore both starch synthesis and respiration should he inhibited if the common precursor requires phosphorylation for its formation. When uniformly labelled sucrose was supplied to tobacco leaf disks in the presence of dinitrophenol as inhibitor, starch formation was progressively prevented as the concentration of the inhibitor was increased, but carbon dioxide output was stimulated.

The radioactivity of the carbon dioxide emitted in such experiments is shown in Slide 13 (Fig. 1 of P/459). At low dinitrophenol concentration the radioactivity of the carbon dioxide was equal or nearly so to that of the original sucrose, indicating that sucrose was the sole respiratory substrate, but as the inhibitor concentration was increased the total activity of the carbon dioxide fell. Increasing output of carbon dioxide was there accompanied by a diminishing use of sucrose and an increasing use of some unlabelled material as substrate. Dinitrophenol thus appeared to restrict sugar utilization both for starch synthesis and for respiration. These preliminary results with this inhibitor of oxidative phosphorylation are thus consistent with the hypothesis that incorporation of free sugar into the metabolic pool does indeed involve phosphorylation, but they also raise the question of the nature of the substrate whose oxidation is stimulated by dinitrophenol. At present the only information is that the respiration quotient fell as the sugar utilization was restricted, so that it is unlikely that it is oxidation of organic acid.

Another aspect of sucrose metabolism is the study of the role which sucrose played. With the discovery that oligosaccharides of greater molecular weight than sucrose can arise as a result of sucrase action, this enzyme activity has assumed a new importance.

This type of reaction is illustrated in Slide 14, which shows the reaction products of the action of yeast sucrase on sucrose after chromatographic separation. Investigation of the structures of these new oligosaccharides has shown that the mode of action of both yeast and mould sucrases was a transfer of fructosyl residues of sucrose to an acceptor, and this conclusion has now received support by the use of radioactive sugars.

The type of reaction which occurs is shown in the following equations:

1. $$G \rightarrow\leftarrow F + E \rightarrow F - E + G$$

2. $$F - E \begin{cases} + \; G \rightarrow\leftarrow F \rightarrow G \rightarrow\leftarrow F \leftarrow F \text{ etc.} \\ + \text{ primary alcohol} \rightarrow \text{fructosyl} - \text{alcohol} + E \\ + \; H_2O \rightarrow F + E \end{cases}$$

3. $$F - E + G \xrightarrow{\text{(mould enzyme)}} G \rightarrow\leftarrow F + E$$

When yeast sucrase acts upon sucrose in the presence of radioactive glucose the activity is rapidly incorporated as combined glucose into the oligosaccharides, but as shown in Table 1 on this page, there is little incorporation of activity into sucrose. The major part goes into a new reducing disaccharide—shown in the table as Spot I—which indicates the position on chromatographic separation above the sucrose spot. The fructosyl residue of sucrose was transferred to the 6 position of the radioactive glucose added.

When, however, mould sucrase acts upon sucrose and radioactive glucose, sucrose is the first oligosaccharide to become labelled, indicating that here fructose is being transferred to the 1 position of glucose (Table 2 overleaf). It is this reaction that was used to prepare sucrose labelled in the glucose moiety in quantity. As the reaction proceeds tri-and tetra-saccharides appear, all containing one labelled glucose component in the molecule, so that it is evident that these compounds are formed by further fructose transfer.

There are some interesting differences in the specificity of the yeast and the mould sucrases although both operate by fructose transfer.

If radioactive fructose is added instead of glucose, labelled fructosyl-fructoses of at least two different

Table 2. The Action of Mould Sucrase on Sucrose and Radioactive Glucose

After development	2 min	Time of incubation (hr) 0.5	1	2	4.25	18	28
		Counts per minute					
Glucose spot	4761	4921	5101	4448	4792	4950	5550
Sucrose spot	33	369	357	485	494	67	21
Spot X	7	5	4	30	48	52	38
Spot α	0	5	10	25	65	47	18
Spot β	—	—	—	—	6	6	8
Total	4801	5300	5472	4988	5405	5122	5635

types are produced, so that free fructose can also act as an acceptor in the system as well as free glucose. However, none of the glucose-containing oligosaccharides became labelled in the presence of radioactive free fructose—a fact which is in accordance with the view that the oligosaccharides are formed by fructose transfer.

The significance of fructose transfer in syntheses of polysaccharides is as yet unknown, but there does appear to be a correlation with the wider occurrence in the plant kingdom of fructose polymers ranging from sucrose, through oligosaccharides, to the polyfructoses. Similar glycosidic linkages are present in the naturally occurring plant fructosans and in the products of mould and yeast sucrases. Fructose-transferring enzymes, therefore, which may possibly be identified with sucrases, are known to occur in plants and we may therefore have to consider sucrases as having a function in polysaccharide syntheses.

DISCUSSION OF P/715 AND P/459

Mr. G. O. Burr (USA): Referring to Mr. Godnev's very interesting calculation of the rate of turnover of chlorophyll in mature, fully-green leaves, I would like to ask about the exact conditions in which the value was found, and further whether or not the value would be highly dependent upon light intensity and other environmental factors?

Mr. Godnev (USSR): Mr. Burr is absolutely right in suggesting that the conditions determine the rate of renewal of chlorophyll. In our experiments the plants were kept under constant conditions at a temperature of 20°C and a light intensity of 10,000 lux. In Mr. Turchin's experiments the rye shoots were in field conditions. In those circumstances renewal took place considerably faster. We are now making a detailed study of the influence of the conditions. We have ascertained, however, that a great deal depends on the variety of plant. Some three years ago I observed the complete destruction of the chlorophyll in a potato in the course of four days. In shade-loving plants, however, the chlorophyll persists for two or three weeks. Development is conditioned in the main by the variety of the plant, but it is, of course, also affected by external conditions.

At lower temperatures renewal proceeds more slowly, and at higher temperatures more rapidly. We are now engaged on a detailed investigation of this process with the help of C^{14}.

Mr. A. L. Kursanov (USSR): (1) I have data available which confirm the findings of Mrs. Porter and Mr. Edelmann that in the synthesis in plants of sucrose from C^{14} tagged glucose or fructose, both sugar moieties of sucrose prove to be radioactive. Pavlinova, when carrying out these experiments in our laboratory on the fibro-vascular bundles of the sugar beet, merely introduced any one radioactive sugar and did not add a second, non-radioactive sugar at all. We discovered that sucrose uniformly labelled in both moieties is rapidly synthesized in the phloem from tagged monosaccharides, taken separately. No second, free radioactive sugar at all is formed, however. This led us to the conclusion that the initial substances used for the synthesis of sucrose in the plant originate in the isomerization of glucose and fructose accompanied by the formation of phosphoric ethers. This conclusion is borne out by the fact that the synthesis of sucrose in the leaves of many plants proceeds as rapidly from glucose or fructose, taken separately, as from mixtures of these sugars, or even more rapidly.

(2) We also have data pointing to the ready conversion of sucrose into starch in plants, although we were studying this reaction in the reverse direction, namely, the utilization in plants of amylose and dextrins with a different number of glucose moieties for the synthesis of sucrose. It was found that glucose moieties linked by 1 : 4α glucoside bonds are more rapidly used than free monosaccharides for the synthesis of sucrose in plant leaves. However, the principal thing was that, in synthesis from amylose, only half as much oxygen was consumed by the leaves to form a unit of sucrose as in synthesis from simple sugars.

Accordingly, the synthesis of sucrose from polysaccharides with a 1 : 4α glucoside bond is, as far as energy is concerned, easier for the plant than its synthesis from free sugars. In order to understand this, we must bear in mind that moieties of simple sugars combined with phosphoric acid or with each other contain an additional supply of free energy and, for that reason, participate in transfer reactions more readily than do free sugars. This is especially true of sucrose, the glucoside-fructoside bond of which is rich in energy, making it, like the phosphoric ethers, an important metabolite in the plant.

Session 16C

RADIOACTIVE ISOTOPES IN PHYSIOLOGY AND BIOCHEMISTRY (*concluded*) - GENERAL BIOCHEMISTRY

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LIST OF PAPERS

(Continued)

Some Recent Applications of Tritium in Biological Research

By R. F. Glascock,* UK

DETERMINATION OF TRITIUM

Because of its very soft β-radiation (maximum energy 0.015 Mev) tritium is best assayed in gaseous form. Other methods such as assay of solid tritiated compounds in internal gas flow counters[1] or assay in solution with scintillation counters[2] have efficiencies of the order of 3%. Gas counters however have efficiencies of 75% or more and other radioisotopes do not interfere because a chemical separation of tritium usually takes place during the preparation of the gas. For example a combustion produces the tritium in the form of water, C^{14} as carbon dioxide and leaves P^{32} behind in the ash.

Tritium has been assayed as water vapour,[3] as hydrogen[4] and as hydrocarbon.[5,6] Water and hydrogen suffer the disadvantage that they contaminate counters through exchange between the tritium in the gas phase and combined or adsorbed hydrogen in the walls of the counter. Although less sensitive, ionisation chambers[7] are to be preferred if hydrogen is used as they are less subject to contamination. Hydrogen also suffers the disadvantage that it is not condensable at the temperature of liquid air and is therefore more difficult to transfer about a vacuum system. Hydrocarbons such as methane have the advantage that the hydrogen combined in them is stably bound and will not exchange causing contamination of counters. Methane, like hydrogen, however is not condensed at the temperature of liquid air and can, furthermore, he used only in proportional counters.[5] *Normal* butane, however, produced by the action of water on a Grignard reagent, is fully condensed at liquid air temperature and can be used in Geiger counters. It is thus readily manipulated in vacuum systems and the minimum of electronic equipment is needed for operating the counters. Counters filled with butane have plateaux 400 volts long and slopes not exceeding 3% per 100 volts.

The technique to be summarised here has been described in detail elsewhere[6,8,9] and involves simply the combustion of 5–10 mg samples of the tritiated compound, the separation of the water and the carbon dioxide so produced and the transference of the water *in vacuo* to a previously prepared tube of dry ether-free *n*-butyl magnesium bromide. The tubes of reagent are prepared in batches of six on a manifold as shown in Fig. 1. The tube to which the water has been added is heated at 120° for 1 hour when, if the reagent is freshly prepared, *n*-butane is produced quantitatively or nearly so. It is measured in the calibrated manometric chamber X_1 and a measured portion taken for counting. This, measured in X_1, or in the McLeod gauge G_1 if smaller in volume than 1 ml, is condensed into the counter D_1, the pressure adjusted to 14 cm with inactive *n*-butane and the counter attached to the electronic equipment where it is operated in the Geiger region

BIOLOGICAL FRACTIONATION OF HYDROGEN ISOTOPES

It can be shown theoretically[10] that for a substance that is biologically synthesised and degraded by a reversible series of reactions in a medium containing water labelled with both deuterium and tritium, the isotopic ratio in the product is given by

$$\frac{TF}{DF} = \frac{k_{1T}}{k_{1D}} \times \frac{k_{2D} + k_{3X}}{k_{2T} + k_{3X}} \times \frac{T}{D}$$

where T/D is the isotopic ratio in the medium and TF/DF is the isotopic ratio in the product, F being

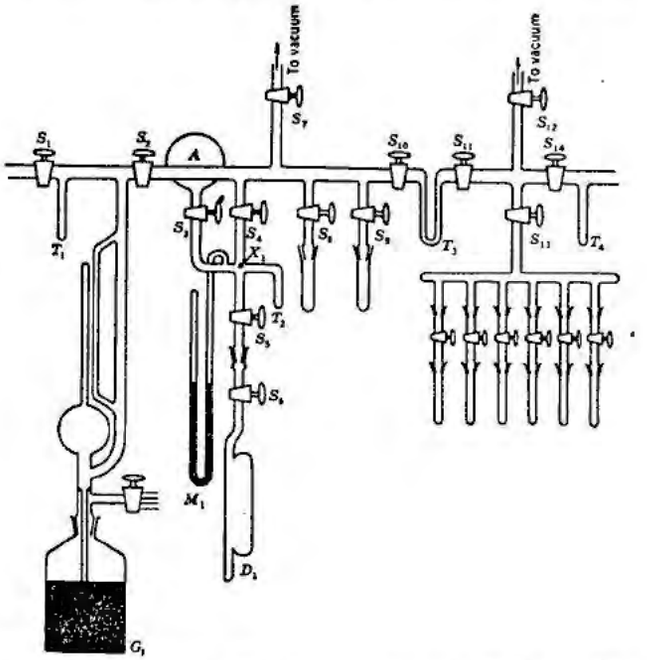

Figure 1. Apparatus for the preparation and measurement of tritiobutane and its introduction into gas counters. A, butane reservoir; D_1, stainless steel cathode gas counter; G_1, McLeod gauge; M_1, manometer; S_1-S_{14}, stopcocks; T_1-T_4, traps and condensation tubes; X_1, manometric chamber. (From "Isotopic Gas Analysis for Biochemists" by R. F. Glascock, Academic Press Inc., 1954; reproduced by permission of the publishers)

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the immediate unlabelled precursor; k_{1T} and k_{1D} are the rate constants for the synthesis of tritium and deuterium-labelled products respectively; similarly k_{2T} and k_{2D} are the rate constants for the *degradation* of tritium and deuterium-labelled products respectively; k_3 is the rate constant for the removal of intact labelled molecules of either species from the site of synthesis; X is the imaginary chemical species formally responsible for the removal of intact labelled molecules (e.g., by secretion) so that when $X = 0$ there is no removal and when $X > 0$ removal does occur.

It can then be shown that under conditions of *turnover* when $X = 0$

$$\frac{TF}{DF} = \frac{T}{D}$$

i.e., the isotopic ratio in the product is the same as that in the substrate water.

It can also be shown that under conditions of net synthesis when $X > 0$ (by definition)

$$\frac{TF}{DF} \text{ tends to } \frac{k_{1T}}{k_{1D}} \times \frac{T}{F}$$

i.e., the isotopic ratio in the product will be different from that in the substrate water by a factor which tends to the ratio of the rate constants for the synthesis of tritium- and deuterium-labelled products. Thus if a substance is undergoing net synthesis at one site and turnover at another in a medium of doubly-labelled water, fractionation of hydrogen isotopes will occur only at the site of *net synthesis*: if there is more net synthesis and less turnover at one site than another then there will be more fractionation at that site than at the other.

There is good evidence that whereas rapid net synthesis of fatty acids occurs in the lactating mammary gland,[11] active degradation and synthesis of fatty acids occur concurrently in liver.[12] It is believed, furthermore, that fat is synthesised and degraded by a reversible series of reactions[13,14] which are therefore of the type to which this argument can be applied.

Isotopic fractionation in liver and mammary gland of rats was therefore investigated. Nursing rats with their litters (6 pups) in three separate groups used at different times were injected with doubly-labelled water so that the body water contained about 1.5–3 at per cent excess deuterium and 0.75–3.0 μc/ml tritium.

A dilution of the same water was given as the only source of drinking water and the rats, fed *ad lib.* maintained on this treatment for 14 days. They were then killed and the following samples taken and analysed for both isotopes: drinking water, blood water, mammary gland fatty acids, liver fatty acids and pup carcass total fatty acids.

Each sample was combusted in duplicate and deuterium determinations performed on the combustion water; duplicate preparations of butane were also prepared from each sample of combustion water and at least two portions of each preparation counted in the tritium determination. Both analyses are accurate to ± 1.5% and probably less and it is therefore believed that the total error in the determination of D/T ratios did not exceed ± 5%. The range of values found for the blood water in which the ratio should be unity indicates that this prediction was correct, only one value (0.94) being more than 5% outside the mean value (1.0).

Table 1. Isotope Ratios (T/D) in Fatty Acids Isolated from Rats Maintained on Doubly-Labelled Water

(1)	Mammary gland fatty acids:
	Mean isotope ratio = 0.7750 ± 0.00930 (7 degrees of freedom)
	Significance of difference of mean value from 1.0 $P < 0.001$
(2)	Liver fatty acids:
	Mean isotope ratio = 0.8712 ± 0.02386 (5 degrees of freedom)
	Significance of difference of mean value from 1.0 $0.01 > P > 0.001$
(3)	Difference between liver and mammary gland fatty acids:
	Paired "t" test using eight rats with complete results (rats 1-3 and 6-10). Difference between mean isotope ratios = 0.1012 + 0.03050 (7 degrees of freedom) $P = 0.0136$ (approx.)

Table 1 shows that the mean ratio in the mammary gland fatty acids was 0.775 compared with 0.871 for liver fatty acids. Both are significantly different from unity and from each other and these results are therefore in accord with the prediction that more fractionation takes place at the site of greater net synthesis.

STUDIES IN FATTY ACID SYNTHESIS

Respiratory experiments[15] having indicated that insulin causes a marked stimulation of fatty acid synthesis *in vitro* by mammary tissue from rat, rabbit and mouse the effect of insulin on the incorporation of both acetate carbon and the hydrogen of water into fatty acids was investigated.[16] Mammary tissue from lactating rats killed on the 14th–15th day of lactation was incubated in bicarbonate saline containing 1-C^{14}-acetate and radioactive water. At the end of the incubation period the fatty acids were extracted, converted to their calcium salts and assayed for both isotopes as described by Glascock.[6,8,9] When insulin was added to the medium it increased the C^{14} content of the fatty acids 1.4–4.5 times and the tritium content 1.3–5.0 times thus suggesting that the effect applies generally to the total synthetic process rather than to the turnover of one particular type of precursor: i.e., both to acetate carbon and to water hydrogen.

This work was followed by a more extensive series of experiments[17] in which 3 isotopes were used: the labelled substrates were 1-C^{13}-2-tritioacetate ($CH_2TC^{13}OONa$) and glucose U-C^{14}. Mammary tissue from sheep and rats (i.e., ruminants and non-ruminants) was incubated in bicarbonate saline containing these substrates. It was thus possible to com-

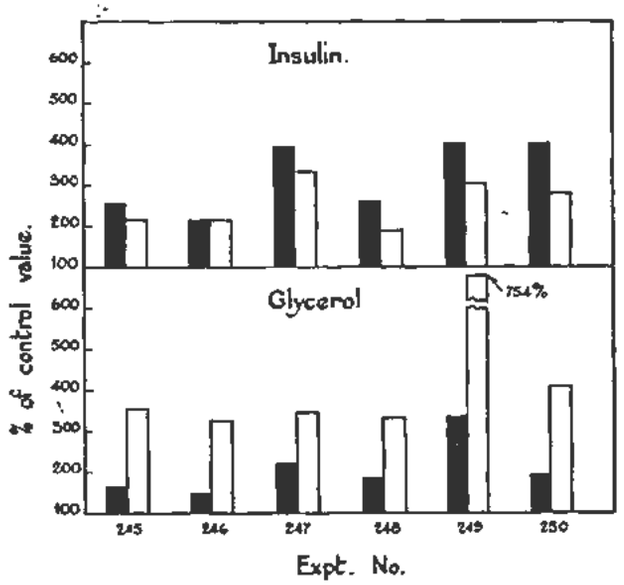


Figure 2. Showing the effect of glycerol and insulin on the *in vitro* incorporation of acetate and glucose carbon into the fatty acids of rat mammary gland. White rectangles, tritium (i.e., acetate carbon); black rectangles, C^{14} (i.e., glucose carbon)

pare in the same sample of tissue in the same flask the relative utilisation of the substrates and to compare the effects of insulin or glycerol. This comparison was desirable because both substances had been shown in respiratory experiments to stimulate fatty acid synthesis by rat mammary tissue[18,19] in a qualitatively similar manner and the theory had been put forward that the effect of insulin might be through its stimulating the formation of glycerol from glucose.[18] That this is not so is shown in the histogram (Fig. 2).

In that figure the base line is the level of isotope in fatty acids from tissue in the control flasks (no insulin or glycerol) and the pairs of rectangles indicate the amount by which insulin or glycerol stimulated incorporation. It will be seen that whereas insulin stimulated the incorporation of both isotopes about equally, glycerol stimulated the incorporation of tritium (white rectangles) much more than it stimulated the incorporation of C^{14} (black rectangles); i.e., it stimulated the incorporation of acetate carbon more than glucose carbon. If insulin exerted its effect by stimulating the production of glycerol which would therefore be the primary stimulating agent the effect of both agents should be qualitatively similar which they are not: hence they must exert their effects by independent mechanisms.

Again using the double labelling technique it has been shown[20] that the incorporation of tritium from acetate and C^{14} from glucose into fatty acids by rat mammary slices is inhibited by cortisone, corticosterone and 11-deoxycorticosterone while cortisol has no inhibitory effect whatsoever. On sheep mammary tissue only corticosterone and 11-deoxycorticosterone are actively inhibitory. Insulin has no stimulating effect on sheep tissue but in rat tissue it neutralises the effect of the corticoids.

THE METABOLISM OF HEXOESTROL

Because its half-life is short (11 years) tritium is available in very high specific activities. Thus 97% tritium would have a specific activity of 2.7 curies/std ml which, in turn, makes possible the preparation of compounds of very high specific activity. This is particularly useful for the tracing of substances which are physiologically active in very small doses, e.g., the sex hormones. Hexoestrol, for example, is active in a rat at a dose of less than 1 μg. Labelling with C^{14} usually involves long and laborious syntheses which yield products of inadequate specific activities. Tritium, on the other hand, can often be introduced into complex molecules by hydrogenation of the appropriate unsaturated compound. Thus tritiohexoestrol itself has been made by the addition of tritium to dienoestrol over a palladium black catalyst.[21] Using a nominally 10% tritium-hydrogen mixture a specific activity has been obtained of 15 μc/μg. A specific activity about ten times as great as this could be obtained by the use of tritium of the highest specific activity available. This specific activity (corresponding to 1.136×10^7 counts/min/μg) however has permitted the tracing of microgram doses in rats. The results of earlier experiments with rabbits using hexoestrol of a lower specific activity have been largely confirmed and it has been found that the major portion of the dose is rapidly excreted. In the rabbit experiments only 60% of the dose was recovered but recoveries of up to 89% in the 32 hours following dosing have been obtained in experiments with rats, radioactivity appearing both in faeces and urine. This is at variance with what has been found by Hanahan *et al.*[22] for C^{14}-stilboestrol. These workers used material of specific activity less than ours by a factor of nearly 1000 (22 μc/mg) though at only 5 times the dose level. They obtained however up to 86% recovery from the faeces virtually no radioactivity appearing in the urine. In our work less than 2% of the dose appeared as water, i.e., less than 2% was oxidized (or exchanged) and less than 0.5% was associated with circulating blood solids. Hanahan *et al.* also found no oxidation. If it is confirmed that whereas stilboestrol is excreted only by way of the faeces, hexoestrol is excreted through faeces and urine about equally, it will be of considerable interest. It is to be noted, however, that earlier work on the excretion of stilboestrol, hexoestrol and dienoestrol[23] showed that glucuronides of all three oestrogens are excreted in the urine after the administration of massive doses, and our findings for the microgram level are thus in agreement with these results.

THE TRANSFER OF DIETARY FAT TO MILK

Tritium also finds an application in work where the cost of a sufficient activity of C^{14}-labelled material would be prohibitively expensive. Thus in experiments with large animals such as cows and goats in our Institute, it has been found necessary to administer doses of up to 5 mc of labelled fatty acid

to goats and about 30 mc to cows. Such experiments would cost very large sums of money if C^{14} were used and, furthermore, only 1- or 2-C^{14}-labelled fatty acids are available commercially. There would then be a danger of losing much of the isotope by β-oxidation leaving an unlabelled residue which could not be traced. By the catalytic addition of tritium to oleic or (better) elaidic acid a labelled stearic acid can be obtained in which the label is not confined to the $C_{(1)}$ or $C_{(2)}$ atoms.[21]

Experiments have been carried out on lactating milch animals using both the free labelled acid dissolved in vegetable oil as carrier and also the labelled acid combined in a triglyceride.[24] In all experiments radioactivity was detected in the milk within a very short time of dosing (Fig. 3) and the specific activity of the milk lipids reached a peak within 24 hours. An example of the results obtained on fractionation of the milk constituents is given in Table 2. In this experiment a total dose of 4×10^9 cpm was administered as stearic acid dissolved in arachis oil. The sample with the peak activity (23 hr) was fractionated. It will be seen that, as was to be expected, most of the activity resided in the long chain fatty acids and that, of these, the saturated fatty acids had a higher specific activity than the unsaturated.

Table 2. Specific Activity of Fatty Acid Fractions and Other Milk Constituents after Administration to a Goat of 4×10^9 cpm of Tritium-Labelled Stearic Acid Dissolved in Arachis Oil

Sample	*Specific activity: cpm/mg combustion water*
Total lipids	11,460
Glyceride fatty acids:	
Non-volatile saturated	14,000
Non-volatile unsaturated	11,030
Steam volatile water soluble	80
Steam volatile water insoluble	206
Total phospholipid fatty acids	13,290
Cholesterol	69
Lactose	150
Glyceride glycerol	346

It is, however, of particular interest, that the unsaturated acids (separated by the lead salt method) had a specific activity as high as they did and it implies extensive conversion of stearic acid to oleic acid—this being the most abundant unsaturated fatty acid in milk fat. This result also had an important bearing on the distribution of isotope in the labelled molecule and led to the investigation described below.

It will also be noticed that the phospholipid fatty acids had about the same specific activity as the glyceride fatty acids implying that they have been drawn from the same pool. The fact that the steam-volatile insoluble glyceride fatty acids (C_8–C_{12}) had only about 1.5% and the volatile soluble fatty acids (C_4–C_8) only 0.5% of the specific activity of the saturated fatty acids shows that degradation of stearic acid, though it obviously occurs to a small extent, is not an important process in the formation of these

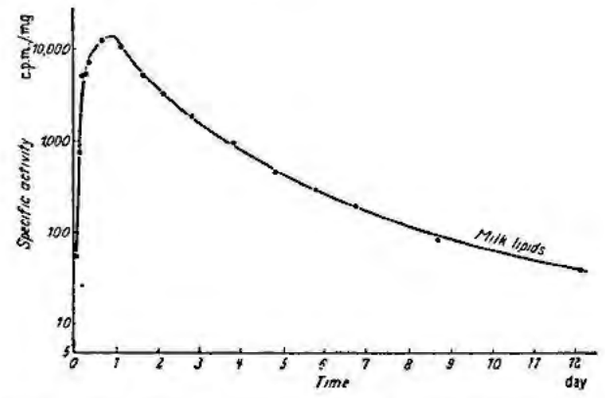
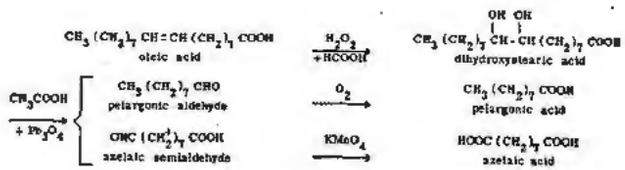


Figure 3. Specific activity-time curve of milk lipids of goat following ingestion of tritium-labelled stearic acid

acids. Equally, the low specific activities of cholesterol, lactose and glyceride glycerol indicate that they are not, to any important degree, derived from stearic acid.

DISTRIBUTION OF TRITIUM IN STEARIC ACID

If the original stearic acid administered to the goat contained tritium attached only to the C_9, C_{10} atoms as might be expected from the mild conditions of catalytic addition then the oleic acid derived from it on biological dehydrogenation should have half the specific activity of the parent stearic acid, provided there was no isotope effect—i.e., provided that hydrogen was not removed preferentially to tritium. We should thus expect, notwithstanding that the saturated and unsaturated fractions as prepared do not consist wholly of stearic and oleic acids, that the unsaturated fraction would have a very much lower specific activity than the saturated fraction—i.e., 50% or less instead of 80% as observed. Because it is very difficult to degrade stearic acid in such a way as to determine the amount of tritium residing on the C_9, C_{10} atoms advantage was taken of the formation of oleic acid in this experiment to carry out the degradation on that compound.[25] The unsaturated fatty acid fractions from two samples of milk collected at different times were mixed with carrier oleic acid to provide a larger amount of experimental material and subjected to the following degradation scheme.



The procedure consists of oxidation of oleic acid to dihydroxystearic acid followed by fission of the molecule at the 9, 10- position to azelaic semialdehyde and pelargonic aldehyde. In the actual experiment no loss of tritium occurred up to this stage although it was found that the specific activity of the pelargonic aldehyde was 1.68 times that of the

azelaic semialdehyde indicating that the tritium in the oleic acid molecule was asymmetrically distributed. On oxidation of the aldehydes to the acids, however, labilisation of the tritium originally attached to the C_9, C_{10} atoms occurs. If all the tritium had been attached in these positions these acids should therefore be non-radioactive; in fact their specific activities were only about 8% and 20% less than that of the aldehydes themselves. It was calculated from this that about 12% of the total tritium in the oleic acid resided on the C_{10} atom and 3% on the C_9 atom. The asymmetry of distribution of tritium in the molecule is largely a matter for conjecture. Whether it arises biologically, the original stearic acid being symmetrically labelled, or whether the tritium becomes asymmetrically distributed along the carbon chain during the catalytic hydrogenation is at present unknown. We are satisfied, however, that it is not an analytical artefact: labilisation of tritium, a necessary prerequisite to redistribution, under the conditions of the experiment would certainly result in loss into the reagents whereas in fact no such loss was observed.

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The Use of Radioactive Isotopes in the Study of Functional Biochemistry of the Brain

By A. V. Palladin and G. E. Vladimirov, Ukrainian SSR

I

Biochemistry of the brain is one of the most interesting but intricate problems of biochemistry. The study of the chemical composition of the brain and of the peculiarities of its metabolism offers considerable difficulties due to the presence of diverse cellular and conducting structures, the extremely complex distribution of the grey and white matter, and the abundance of labile connections in the brain tissue. Particularly great are the difficulties involved in the study of the interrelations between the specific function of the brain and the peculiarities of its metabolism. Yet the task of relating the changes in the cerebral metabolism with its functional state is the main task of the brain biochemistry, which should be functional biochemistry. Of especial interest is the study of metabolism accompanying the principal physiological conditions of the brain, namely stimulation and inhibition of its activity.

These problems have lately been the object of great attention on the part of Soviet physiologists and biochemists.

New great opportunities have been offered to the functional biochemistry of the brain by the use of radioactive isotopes. The tracer method has marked the beginning of a new stage in the general field of biochemical investigations and still more so in that branch of biochemistry which deals with the brain, particularly with the metabolic peculiarities of the brain of a whole, intact animal under various physiological conditions.

Lately, McIlwain[1] and others have widely used the method of electric stimulation of brain sections (i.e., the *in vitro* method) to study the biochemical changes associated with various functional states of the brain in particular with stimulation. It should, however, be borne in mind that section studies may be only of auxiliary significance for functional biochemistry. Apart from the fact that sectioning of the brain may by itself bring about a breakdown of a number of substances which are physiologically very important for the brain, the phenomena in the intact whole brain may follow a quite different course than in sections; the same experimental procedure may produce *in vitro* a different, or even quite an opposite effect than *in vivo*.

In vitro experiments can only disclose the mechanism of the metabolic reaction, and it is only *in vivo* experiments that can illuminate the physiological role of the metabolic processes at issue and their role in the function.

While following up the interrelations between certain functional changes and the respective metabolic processes of the brain, the *in vivo* investigations, i.e., experiments on whole animals, should therefore be preferred, although they involve greater technical difficulties. In this respect the application of radioactive isotopes which has already contributed so much to the study of biochemistry of the brain, is very promising.

The use of radioactive isotopes or labeled molecules made it possible to specify some data on the chemical composition of the brain; to show that some of the brain components formerly believed to be immutable in adult animals, do in fact undergo a continuous breakdown and synthesis; to study individual metabolic processes in the brain and to decipher the influence upon metabolism of various functional conditions.

At present radioactive isotopes are widely used in many scientific institutions of the USSR for investigating biochemistry of the brain. Particularly intense work is carried out at the Institute of Biochemistry of the Ukrainian Academy of Sciences in Kiev (Academician A. V. Palladin), at the Pavlov Institute of Physiology of the USSR Academy of Sciences in Leningrad (Professors G. E. Vladimirov and E. M. Kreps), at the Institute of Biochemistry of the USSR Academy of Sciences in Moscow (Academician V. A. Engelhardt), and at the Institute of Physiology of the Georgian Academy of Sciences at Tbilisi (Professor D. A. Kometiani).

It is the purpose of the present paper to outline the most important findings in the biochemistry of the brain made in the USSR by means of the radioactive isotope method and thus to illustrate the significance of labeled atoms for the solution of problems of functional brain biochemistry.

II

A study of the chemical structure of various divisions of the brain showed that functionally different divisions have a different chemical composition, and that, in particular the functionally most

Original language: Russian.

complex and phylogenetically youngest parts of the central nervous system are most abundant in proteins.

By means of the radioactive isotope method a definite relationship between the functional complexity and intensity of metabolism has been established in various divisions of the brain. Thus, a study of incorporation of labeled methionine (containing S^{35}) by proteins of different divisions of the brain[2] showed that the cerebral cortex and the cerebellum, i.e., functionally the most complex and phylogenetically youngest divisions of the central nervous system, exhibit the highest rate of protein renewal whereas, the lowest renewal rate was found in the spinal cord, i.e., functionally the least complex and phylogenetically the oldest division of the central nervous system. White matter of the cerebral hemispheres approximates the spinal cord in the rate of protein renewal. The medulla, the middle brain and the thalami optici occupy in this respect an intermediate position (Fig. 1).

Similar results, although only for the grey and white matter of the brain, have been obtained by Cohn *et al.*[3] in following up the incorporation of labeled methionine.

The radioactive isotope method has thus made it possible to establish that the functionally most complex and phylogenetically youngest divisions of the central nervous system are characterised not only by the highest content of proteins but by a most intense protein metabolism as well.

The use of phosphorus radioisotopes has made it possible to study the turnover of ribonucleic acid phosphorus, of phosphoproteins and phospholipids in various divisions of the brain.[4] In animals with a well developed cortical function, namely in dogs, the renewal rate of ribonucleic acid and phosphoproteins is the highest in the cerebral hemispheres (both in the cortex and in the white matter), exceeding that in other divisions of the brain. The cortex also ranks first as far as the turnover rate of phospholipids is concerned.

A different picture is to be found in animals with a low level of functional development of the cerebral cortex. It has been shown indeed[5] that the turnover

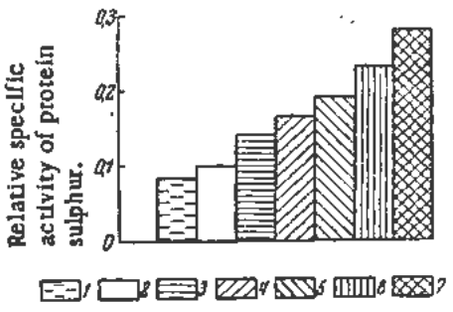

Figure 1. Protein renewal in various divisions of the central nervous system. Relative specific activity of protein sulphur after the administration of methionine containing labeled sulphur. (A. Palladin and N. Vertaimer, 1954). 1, spinal cord; 2, white matter of the cerebral hemispheres; 3, medulla; 4, middle brain; 5, thalami optici; 6, grey matter of the cerebral hemispheres; 7, cerebellum

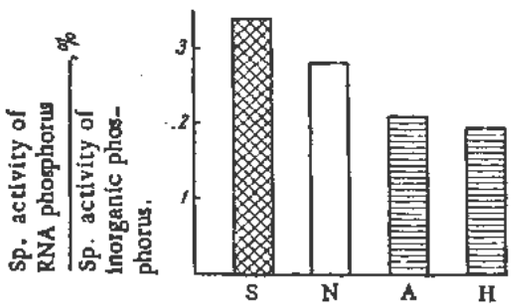

Figure 2. Relative specific activity of ribonucleic acid phosphorus in the rats' brain. Normal, N; stimulated, S; and in sleep caused by hexonastab, H and amytal A. Experiment lasted 3 hours (Vladimirov, T. Ivanova, N. Pravdina, 1954)

rate of ribonucleic acid in the grey matter of the rabbit's brain is lower than in the white matter or the cerebellum. The renewal rate of ribonucleic acid in the spinal cord of rabbits is likewise low (E. Kreps). In the cerebral cortex and cerebellum of rabbits the rate of phosphoprotein renewal is also lower than in the intermediate and midbrain or the medulla. Phospholipids show nearly the same turnover rate in all the divisions of the cerebral cortex of rabbits and a lower rate in the spinal cord.[4]

Thus, the use of radioactive isotopes has led to the establishment of a direct relationship between the level of functional development and the metabolic activity of phosphorus compounds in various divisions of the brain.

Further investigations have shown that various areas of the cerebral cortex of a dog differ in the metabolic activity of phosphorus compounds: in the area of the motor analyser (the parietal and frontal lobes of the cortex) the renewal of ribonucleic acid and phospholipids proceeds at a higher rate than in the area of auditory (temporal lobe), or optic (occipital lobe) analysers.[4]

The method of radioactive phosphorus also showed that, as far as the turnover rate of ribonucleic acid and phosphoproteins is concerned, the white matter of the cerebral hemispheres as well as of the spinal cord are not metabolically inert parts of the brain tissue. Physiologically this is by no means unexpected since the fundamental nervous processes are similar in both the nerve conductors—nervous processes—and in the nerve cells.

The turnover rate of phospholipids in white matter is considerably lower than in grey matter. This is evidently due to the fact that in white matter a considerable proportion of phospholipids belongs to the myelin membranes, while in grey matter it is mostly the lipids of the nerve cells themselves and of their processes that take part in the metabolism.

Thus the problem of chemical topography of the brain, the elaboration of which was begun in the USSR some 30 years ago (A. Palladin), is now being successfully pursued by means of radioactive isotopes, the subject at issue being the metabolic rate and not the composition of various divisions of the brain.

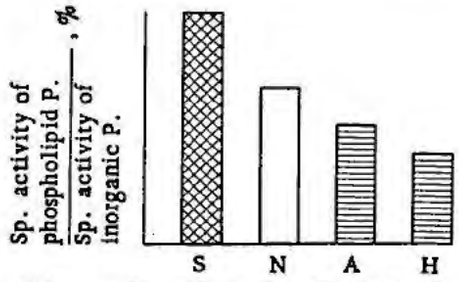

Figure 3. Relative specific activity of phospholipid phosphorus in the rats' brain. Normal, N; stimulated, S; and pharmacological sleep induced by hexanastab, H, and amytal, A. Experiment lasted 3 hours (G. Vladimirov, 1954)

Protein metabolism is likely to furnish a clue to the functional peculiarities of the brain. In this connection of undoubted interest is the protein fraction, known as phosphoproteins and occurring in a number of tissues of the animal body, including nerve tissue. The important functions performed in the brain by phosphoproteins are suggested by the exceedingly high turnover rate of their phosphorus as established with the aid of radiophosphorus.[6,7,8] This far exceeds the turnover rate of nucleic acids and phospholipids.

A high renewal rate of phosphoproteins has also been found in sections of grey matter of the rat's brain.[9] A close relationship has in fact been established between the rate of respiration and of the phosphoprotein renewal rate. The phosphoprotein renewal rate was found to be inhibited as soon as oxidative phosphorylation was disturbed by poisons.

An important part in the nerve tissue is played by carbohydrates which are the main source of energy. There are, however, no great reserves of glycogen or other carbohydrates in the brain. No glycogen has ever been found in the cerebral cortex or the cerebellum in healthy animals. This led to the opinion that glycogen is an inert substance whose content in the brain does not change. This opinion proved, however, inconsistent since the metabolism of glycogen in the brain proceeds at a high rate and varying amounts of it occur in different divisions of the brain.[10]

It was the merit of the radioisotope method to provide crucial evidence disproving the above opinion. Glucose tagged with radioactive carbon has shown indeed that the turnover rate of glycogen in the brain is high, exceeding that in the liver.[11]

III

Another approach to the functional biochemistry of the brain is provided by the comparative-biochemical method, i.e., by studying chemical processes in the brain both in onto- and phylogenesis.

The use of tracer atoms in these investigations likewise proved to be very effective. Thus, a study[5] of nucleic acid metabolism in the brain of rabbits at different stages of ontogenesis (on the 16-20th day and during the last days of embryogenesis, in newborn rabbits and in various periods of postnatal life) showed a correspondence between the changes in the specific activity of nucleic acids and their content. A higher content of ribonucleic acid corresponds indeed with its more intense renewal which is highest at the early stages of embryonic development and then gradually decreases.

The specific activity of labeled phosphatides in the brain of rats is also the highest at early stages of postembryonic development and declines with age.[12,13]

A study of the metabolic rate of phosphorus in the cerebral cortex of rabbits of various ages (4 days of postnatal life up to adults) showed that the incorporation of labeled phosphorus into various phosphoric fractions of the cerebral cortex is greatly reduced during the first weeks of the postembryonic period, the rate of the decrease subsequently declining.[14]

IV

The application of radioactive isotopes proved particularly effective in elucidating the metabolic peculiarities of the brain connected with its functional state, especially with stimulation and inhibition—those fundamental physiological conditions of the central nervous system.

Metabolic studies of stimulation showed first of all that while in a number of cases no change can be detected in the content of various substances and in enzyme activity the radioisotopes reveal metabolic peculiarities characteristic of this state.

Thus, in the brain stimulated by pervitin injection[15] there occur no noticeable changes within three hours after the injection either in the total phospholipid content or in the fraction of saturated and non-saturated phospholipids. It might have been suggested therefore that stimulation does not alter the metabolism of phospholipids.

Radioactive phosphorus, however, revealed some changes in the incorporation of phosphorus into both fractions of phospholipids upon stimulation called forth by the injection of pervitin. Stimulation is therefore associated with certain changes in the phospholipid metabolism.

Mention should be made of a study of the effect of stimulation (produced by a three-hour exposure to electric current of the skin receptors of the rat limbs) on the turnover rate of nucleic acid and of phospholipids.[16] As far as the relative specific activity of phosphorus is concerned, stimulation of the brain is attended with an increased renewal rate of ribonucleic acid and of phospholipids: in the former by 20% and in the latter by 1½ times.

Electric stimulation of rats can be combined with indifferent stimuli (e.g., with definite surroundings in a lit chamber), and in this way a conditioned reflex can be elaborated on the basis of the unconditioned active defence reaction. In these rats conditioned-reflex stimulation increases the metabolic rate of ribonucleic acid (as shown by the experiments with radioactive phosphorus) in response to the formerly indifferent signal.

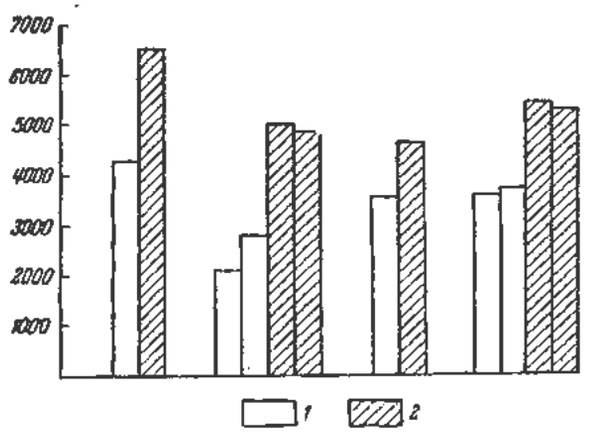

Figure 4. Specific activity of protein sulphur in the rats' brain 1.5 hours after the administration of methionin with labeled sulphur upon stimulation and in amytal-induced sleep. 1, amytal; 2, stimulation. (G. Nechayeva, G. Vladimirov's laboratory, 1955)

A study carried out on dogs with radiophosphorus showed (E. Kreps) that conditioned stimulation combined with feeding reveals an increase in the ribonucleic acid metabolism precisely in that area of the cerebral cortex in which the stimulation is primarily localised, e.g., in case of acoustic stimuli in the auditory area of the cerebral cortex.

Stimulation is also accompanied by an increased incorporation of C^{14} glycine into the brain proteins of rats (A. Urinson, 1955). Similar results (Fig. 4) have been obtained with methionine containing radioactive sulphur (S^{35}), electric stimulation increases the incorporation of radioactive sulphur into the proteins (G. Nechayev, 1955). Thus, the tracer method discloses an increase in protein metabolism called forth by stimulation.

Hence, the use of radioactive isotopes enabled us to establish that stimulation of nervous activity is associated with an increase in the rate of renewal, that is to say, in the metabolic activity of proteins, nucleic acids and phospholipids.

V

The application of radioactive isotopes also proved successful in studying the metabolism accompanying inhibition of nervous activity.

During a 24-hour sleep induced by urethane and medinal, the relative specific activity of ribonucleic acid phosphorus in the brain of rats declines by 27.6%, of phosphoproteins by 19.2%, and of phospholipids by 22.8%.[5] Thus by means of radioactive phosphorus it is possible to ascertain that in narcotic sleep the turnover (or metabolic) rate of ribonucleic acid, phosphoproteins and phospholipids is decreased (Fig. 5).

Similar results have been obtained with hexanastab and amytal[16]. Amytal mostly produced a more profound sleep, and this was associated with more pronounced changes in the turnover rate of phospholipids and ribonucleic acids in the brain of rats.

In this case, too, it is the use of radioactive isotopes that made it possible to establish the actual effect of

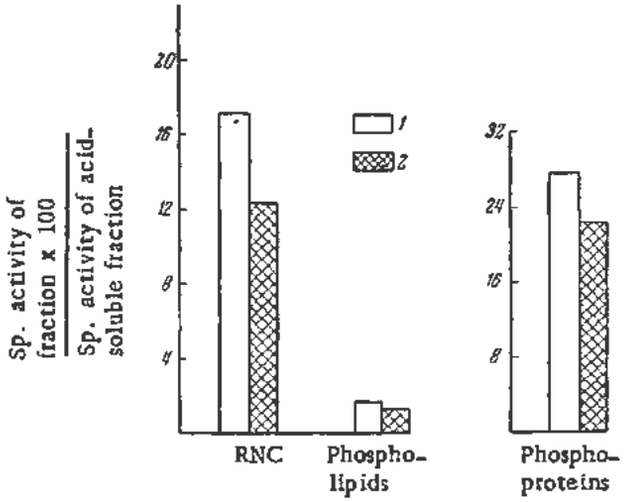

Figure 5. Relative specific activity of ribonucleic acid, phosphoprotein and lipid phosphorus in the brain of rats during 24-hr sleep induced by urethane and medinal, radioactive phosphorus having been administered for 2 hours prior to experiment. 1, normal; 2, narcotic sleep. (E. Skvirskaya and T. Silich, A. Palladin's laboratory, 1954)

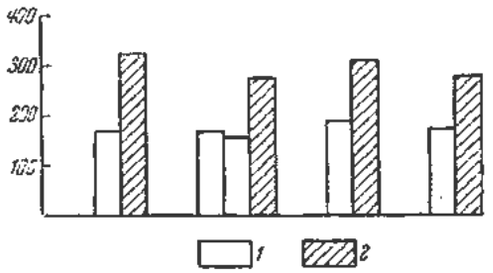

Figure 6. Specific activity of sulphatide sulphur in rats' brain 1.5 hours after the administration of methionine containing labeled sulphur, upon electric stimulation and in amytal-induced sleep. 1, amytal; 2, stimulation. (G. Nechayeva, G. Vladimirov's laboratory, 1955)

sleep on the metabolism of phospholipids, since determinations of the phospholipid content in the brain during artificial sleep did not reveal any change.

The changes induced in the lipid metabolism by stimulation and inhibition have likewise been disclosed (G. Nechayev) by following up the incorporation of radioactive sulphur into the sulphatides of the brain (Fig. 6).

The study of renewal of the phospholipid fraction which accompanies nucleic acids extracted by the Shmidt and Tannhauser method showed (Vladimirov) that the metabolism of this fraction also depends on the functional state of the brain, being increased upon stimulation and decreased in narcotic sleep (Fig. 7).

According to experiments carried out with the use of P^{32} in nembutal narcosis[18] there occurs in the brain of mice an inhibition of synthesis of nucleoproteins and phospholipids.

Inhibition of the nervous system (narcotic sleep) influences protein metabolism as well: sleep induced by amytal slows up the incorporation into the brain proteins of both glycine tagged with radioactive carbon and of methionine containing radioactive sul-

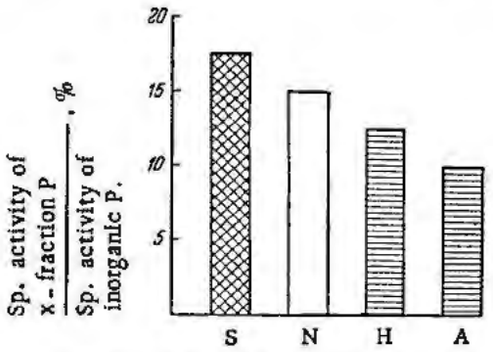

Figure 7. Relative specific activity of lipid phosphorus, forming part of lipoproteins, in the brain. Normal, N; stimulated, S; caused by hexanastab, H, and amytal, A. (G. Vladimirov, 1954)

phur (A. Urinson and G. Nechayev, 1955), (Fig. 4).

It will thus be seen that in artificial sleep the intensity of protein exchange undergoes a decrease.

The changes in metabolism induced by prolonged inhibition have been followed up in hibernating animals, since hibernation is associated with a prolonged inhibition of the central nervous system. Experiments were made on vigilant susliks (*Citellus citillus*), hibernating susliks and on those artificially awakened (four hours before the test).

This study[5] disclosed considerable differences in the rate of radiophosphorus incorporation into ribonucleic acid, phosphoproteins and phospholipids of the brain and spinal cord in vigilant and hibernating animals (while the changes in the content of these substances were much less pronounced): the specific activity of the above phosphorus compounds in the brain and spinal cord of hibernating susliks was several scores times lower than in vigilant animals, and with respect to ribonucleic acid it was practically nil (Figs. 8, 9).

The use of labeled phosphorus showed that during hibernation the penetration of phosphorus from the blood into the tissues is extremely slow. This is evident in the fall of the relative specific activity of the acid-soluble phosphorus in the brain and spinal cord; this activity represents the ratio of the specific activity of acid-soluble phosphorus of the given tissues to the specific activity of acid-soluble phosphorus of the blood plasma (Fig. 10). This has probably some bearing on the decrease in the specific activity of nucleic acid and phospholipid phosphorus. Nevertheless it is quite obvious that hibernation is accompanied by a decrease in the metabolism of nucleic acids and phospholipids. In the artificially awakened animals the turnover rate of nucleic acids and of phospholipids is increased, although being below that of vigilant animals.

Thus the use of radioactive isotopes shows that during inhibition of the nervous activity (in narcotic sleep and hibernation) the renewal rate of proteins, ribonucleic acids and of phospholipids is reduced. In view of the changes caused by inhibition in the content of ATP, glycogen and ammonia, it becomes

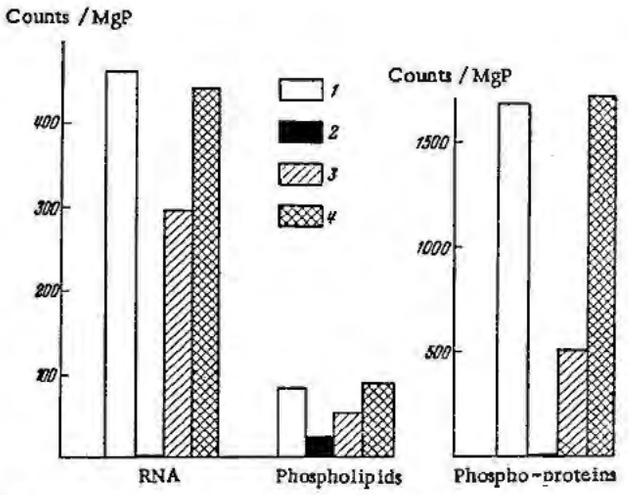

Figure 8. Specific activity of ribonucleic acid, phosphoprotein and phospholipid phosphorus in the brain of vigilant and hibernating susliks treated with radioactive phosphorus 4 hours prior to experiment. 1, normal; 2, hibernating; 3, artificially wakened; 4, narcotic sleep. (E. Skvirskaya and T. Silich, A. Palladin's laboratory, 1954)

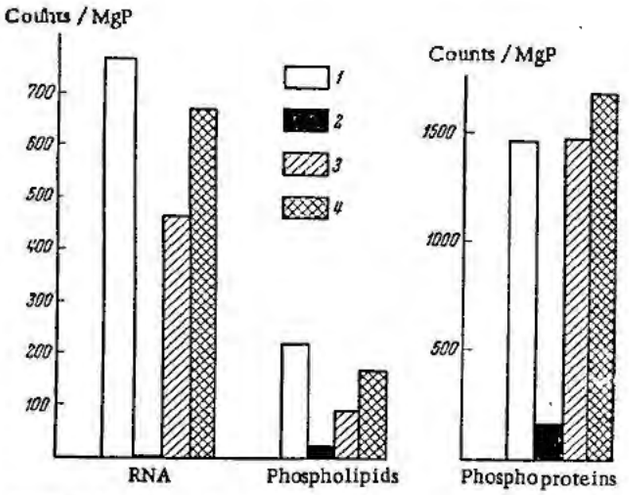

Figure 9. Specific activity of phosphorus of ribonucleic acid, phosphoproteins and phospholipids in the spinal cord of vigilant and hibernating susliks upon injecting radioactive phosphorus 4 hours prior to experiment, 1, normal; 2, hibernating; 3, wakened; 4, narcotic sleep, 24 hours. (E. Skvirskaya and T. Silich, A. Palladin's laboratory, 1954)

clear that during inhibition the breakdown processes are retarded while more favourable conditions are created for the processes of synthesis, which ensures the restoration of the brain efficiency.

VI

An important part in cerebral metabolism is undoubtedly played by the high energy compound, ATP. The study of cerebral metabolism in different functional states includes therefore also a study of ATP, in particular the turnover rate of its phosphorus after administration to the animal of labeled phosphorus. It was found, however, that the turnover of ATP in the brain of warm-blooded animals proceeds at a high rate, which precludes the possibility of determining its renewal rate from the specific

activity values of its phosphorus. In fact it may be renewed several times in one hour. It is impossible therefore to ascertain the effect of various functional states of the nervous system on the incorporation of radioactive phosphorus into the ATP of the brain.

Somewhat different are the relations observed in cold-blooded animals, in particular when the experiments are conducted at low temperatures. Due to the lower rates of metabolic processes the renewal of the ATP phosphorus in these animals may last scores of minutes or several hours.

This may be illustrated by the data obtained (O. Savchenko, 1955) in perfusing at various temperatures a detached carp head (*Cyprinus carpio*) with the Krebs solution containing radioactive phosphorus. The fluid of the brain capillaries was removed by perfusion, prior to the experiment. The determination of the relative specific activity of the ATP phosphorus has shown that a drop in temperature from 16°C to 2°C approximately halves the rate of the ATP breakdown and synthesis (Fig. 11).

In connection with the elaboration of surgical interference methods in the heart it is of great importance to reduce the rate of ATP breakdown whose synthesis is disturbed by cessation of blood supply to the brain of warm-blooded animals and man. During these operations the brain gets into a state of severe oxygen deficiency lasting several minutes. During this period ATP may completely disappear due to intense breakdown which results in irreversible disturbances of the cerebral metabolism. By slowing up the ATP breakdown, hypothermia considerably prolongs the life of the patient with a stopped blood circulation.

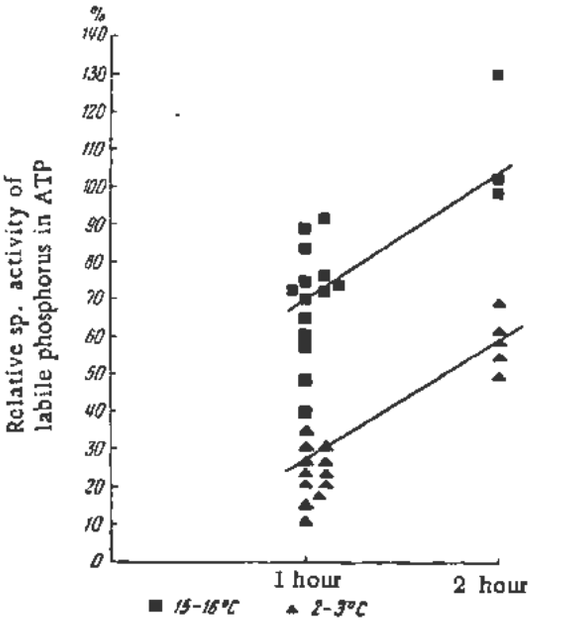

Figure 11. Relative specific activity of ATP phosphorus (determined at 1 hour and 2 hours) in the brain of a perfused carp's head (*Cyprinus carpio*) at various temperatures. (O. Savchenko, G. Vladimirov's laboratory, 1955)

VII

Changes in the functional state of the brain are likewise called forth by avitaminosis, various types of avitaminosis affecting the brain to a different degree. This is borne out by the results of a study of the renewal rate of brain proteins in C- and E-avitaminosis.[2]

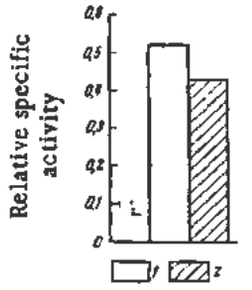

Figure 12. Relative specific activity of proteins of guinea pig brain in C-avitaminosis. 1, normal; 2, C-avitaminosis. (A. Palladin and N. Vertaimer, 1954)

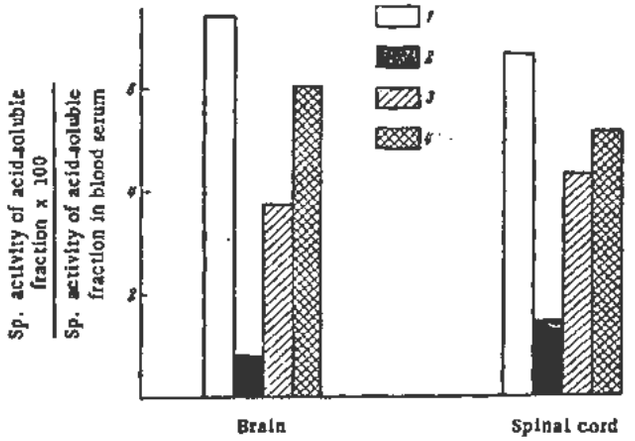

Figure 10. Ratio of specific activity of acid-soluble phosphorus in brain or spinal cord to that in blood plasma of vigilant, hibernating, and artificially awakened, susliks. Radioactive phosphorus administered 4 hours prior to experiment. 1, normal; 2, hibernating; 3, wakened; 4, narcotic sleep, 24 hours. (E. Skvirskaya and T. Silich, A. Palladin's laboratory 1954)

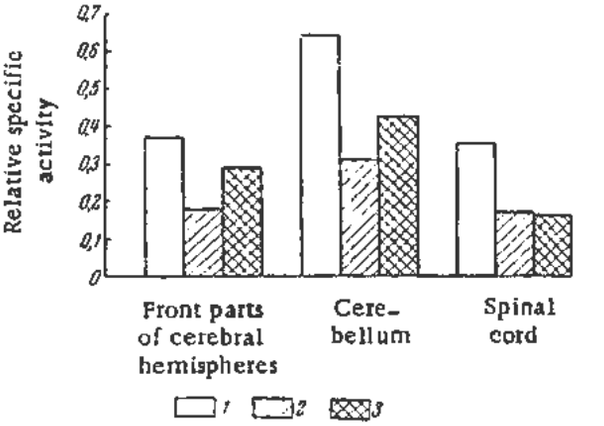

Figure 13. Relative specific activity of proteins of cerebral hemispheres, cerebellum and spinal cord of rabbits in E-avitaminosis. 1, normal; 2, E-avitaminosis; 3, starving. (A. Palladin and N. Vertaimer, 1954)

In C-avitaminosis a slight decrease was found in the renewal rate of proteins in the brain of guinea pigs (Fig. 12), while E-avitaminosis exerts a greater effect on the protein metabolism in the brain: the renewal rate of proteins in the cerebral hemispheres, the cerebellum and the spinal cord of rabbits is considerably lowered by 50% on the average (Fig. 13).

One of the factors greatly affecting the functional state of the central nervous system is the disturbance in the normal oxygen supply of the brain. By means of radioactive isotopes it was shown[4] that in experimental animals (rats) lifted (for 2–4 hours) in a barochamber to an altitude of 9000 to 11,000 metres, the severe hypoxemia thus created is not attended with any marked changes in the metabolic rate of phosphorus-containing proteins of the brain.

However, when both common carotids were ligated in rats thereby disturbing cerebral circulation, which in its turn impeded the oxidative processes, the renewal rate of ribonucleic acid phosphorus, of phosphoproteins and phospholipids was considerably reduced. This effect was particularly distinct in phospholipids.

Thus various methods of inducing cerebral hypoxemia affect the viability of the brain in a different way as evidenced by the presence or absence of shifts in the metabolism of phosphorus-containing compounds and by the scope of these shifts.

VIII

Such are some of the most interesting results obtained by Soviet scientists in studying the biochemistry of the brain by means of radioactive isotopes. These results prove that the method at issue has already contributed to the elucidation of a number of important problems of functional biochemistry of the brain. The application of new isotopes may afford still greater opportunities for studying the chemical basis of cerebral activity. Of particular value is the fact that the method of tracer elements meets one of the major requirements, i.e., it enables one to study cerebral metabolism in the organism as a whole which is not affected by any trauma.

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Some Biochemical Functions of the Cell Surface as Deduced by Isotope Studies

By A. Rothstein,* USA

In recent years there has been a growing interest in the relationship of cellular structure to biochemical function, partly because methods have been developed for isolating certain of the cellular constituents such as mitochondria, nuclei and chloroplasts. In the case of the cell surface, it has not been feasible, except in the red blood cell, to isolate the surface structure. However, the cell surface is in direct contact with the outer environment and it is therefore accessible to experimentation *in vivo,* that is, in the intact living cell. Recent studies have indicated that the peripheral portion of the cell possesses definite biochemical functions in addition to its long estahlished mechanical and physical roles as the limiting membrane of the cell, and as a permeability barrier.

The biochemical functions of the cell surface fall into four categories: (*a*) the digestion of extracellular substances; (*b*) the synthesis of extracellular macromolecules; (*c*) the transport of substances against their activity gradients and (*d*) the synthesis of new cell-surface structural material. The evidence upon which the above conclusions are based, has been reviewed recently in some detail.[1] Certain of the evidence was based on the action of uranyl ion on sugar uptake by yeast cells. It was found that uranyl ion in low concentrations could block the utilization of sugars, though it did not inhibit the metabolism of other substrates such as pyruvate, alcohol, lactate and endogenous stores of carbohydrate.[2,3] The site of action of uranium was the outer surface of the cell, which contained phosphate groups capable of binding uranyl ion in a very stable but reversible complex.[4] Other extracellular cations, particularly potassium, are capable of markedly stimulating the uptake of sugar.[5] It has been suggested on the basis of these and other studies that the glycolytic enzymes in the yeast cell are located in a surface layer and that the action of extracellular cations on sugar uptake may be mediated by action of the cations on the glycolytic reactions.[6]

The present study is concerned primarily with the interactions between physiological ions such as K^+, Na^+, Mg^{++}, Ca^{++}, Mn^{++}, $H_2PO_4^-$, and the surface of the cell. Radioactive isotopes were used in these studies for two reasons: (*a*) they provided a method for measurement of the ions in low concentrations and (*b*) they provided a means for determining the exchanges or absence of exchanges of the ions between intracellular and extracellular compartments. Most of the studies were carried out with Mn^{54}, and a few with Ca^{45}, K^{42}, and P^{32}.

METHOD

Fresh baker's yeast (Standard Brands Inc.) was thoroughly washed and then starved with aeration for 1 to 2 hours. Suspensions were then made up with the appropriate ions, substrates and isotopes. No source of nitrogen was present. After periods of time ranging up to an hour the yeast was centrifuged at high speeds and the supernates were analysed or counted. In some cases, the cells were washed, ashed and counted. In most experiments, the pH was 3.5, with no buffers. Yeast is self buffering at this pH. In a few experiments at other values of pH, inert buffers were used containing triethylamine, succinate and tartrate. All counting was done with a standard thin window mica Geiger-Muller tube.

RESULTS

Yeast cells behave like particles of cation-exchange resin when suspended in solutions containing bivalent cations. There is a rapid and reversible equilibrium established between the cations and binding sites of the cells. The reaction is complete in less than 2 minutes and thereafter there is little further binding of Ca^{45}, Mn^{54}, or $U^{234}O_2$. In the case of uranyl ion, the maximum was attained with concentrations as low as 5×10^{-6} M, but with Mn^{++}, the maximum was not yet attained at 1.2×10^{-3} M (Fig. 1). The tendency to approach a maximum binding indicates that there are a limited number of binding sites for each cell. If it is assumed that the binding of the cations follows the simple equation,

$$M^{++} + Y^{=} \rightleftharpoons MY \qquad (1)$$

where M^{++} is the cation, $Y^{=}$ the binding site of the cell and MY the complex, then according to the mass law,

$$K = \frac{(M)(Y)}{(MY)} \times \frac{fM\ fY}{fMY} \qquad (2)$$

where the fM, fY, and fMY are the activity coefficients. The activity coefficients of the solid phases

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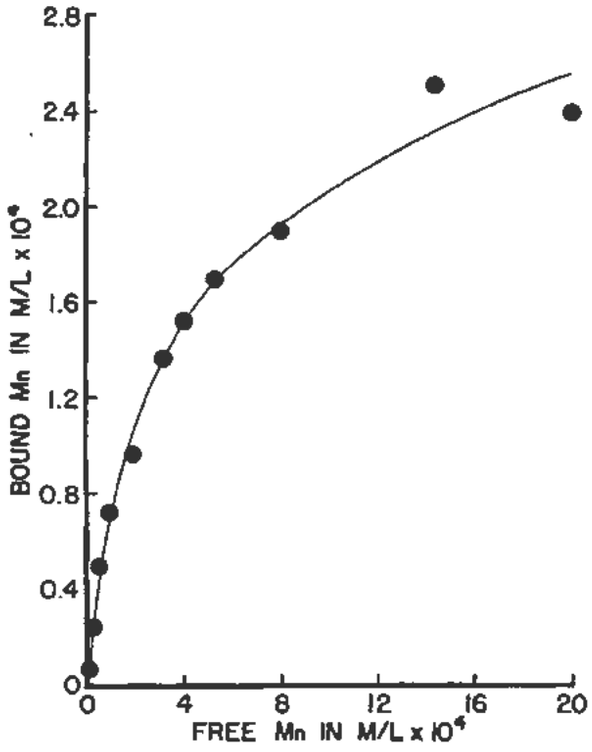

Figure 1. The uptake of Mn^{54} by yeast cells as a function of Mn^{++} concentration

fY and fM are indeterminable, but the ratio fY/fMY is apparently constant under the conditions of the experiment. The mass law equation can be recast in the form

$$\frac{(MY)}{(M)} = \frac{fM\ Yt}{K} - \frac{fM\ (MY)}{K} \qquad (3)$$

where Yt is equal to the concentration of cellular sites $[(Y) + (M)]$. An equation of this form has been used in characterize the binding of ions by proteins.[8] In Fig. 2, $(MY)/(M)$ is plotted against (MY) for Mn^{54} binding. The data fall along two lines suggesting the existence of 2 species of binding sites with different affinities for Mn^{++}. The calculated K in this case is 4×10^{-5}, and the total number of binding sites obtained by extrapolation (dotted line of Fig. 2) amounts to 8×10^{-4} M per kg of cells. The steep slope represents binding by polyphosphate groups (or nucleic acids) on the basis of studies of UO_2^{++} binding.[4] The shallow slope represents binding by carboxyl groups on the basis of preliminary data on the effects of pH.

Other cations both bivalent and monovalent, compete with Mn^{++} for the binding sites of the cell. The competition can most readily be represented by the ability of ions to displace Mn^{54} from the cells. Other bivalent cations tested show an equal or greater affinity for the cell (Table I). UO_2^{++} has an especially high degree of affinity (300 times). Monovalent cations are bound *less* firmly than Mn by a factor of 30 to 50. Large organic cations, such as $(C_2H_5)_3NH^+$ are not appreciably bound. Typical

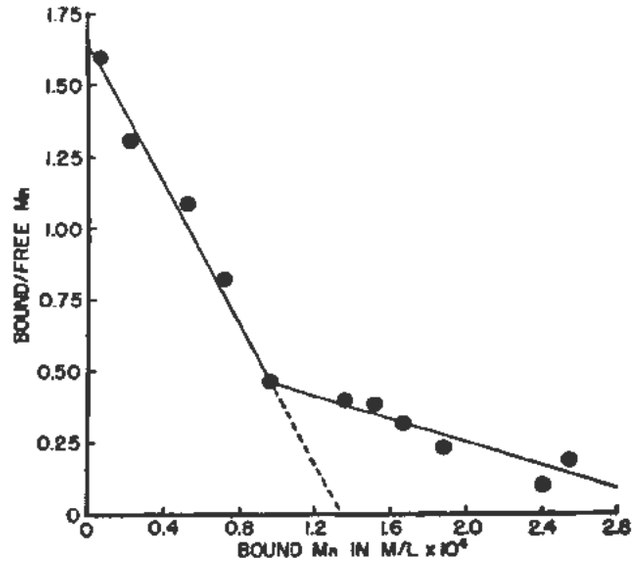

Figure 2. A mass law plot of Mn^{++} uptake by yeast cells

data for competition of K^+ and Mn^{++} are shown in Table II. It is of interest to note that there is a 1 to 1 competition between K^+ and Mn^{++} as indicated by the constancy of the mass law constant calculated on this basis.

Table I. Relative Affinities of Cations for Yeast

UO_2	330	Cu, Hg	1.0
Ba	10	Li, Na	.003
Zn	3	K, Rb, Cs	.005
Co, Mg	1.5	$(C_2H_5)_3NH^+$	<.001 –
Ca, Sr		TRIS	
Mn	1.0		

Table II. Competition of K and Mn

		% of Mn free	Calculated on basis of ionic strength	K_1
1×10^{-4}	Mn	48		7.7×10^{-5}
$+3 \times 10^{-3}$	K	54	50	1.8×10^{-2}
$+1 \times 10^{-2}$	K	62	54	2.0×10^{-2}
$+3 \times 10^{-2}$	K	73	60	2.3×10^{-2}
$+3 \times 10^{-2}$	K	85	72	2.1×10^{-2}

The total cation concentration of the cell is of the order of 0.2 M/kg of cells, of which 0.15 M is K^+ and the remainder, Mg^{++}, Ca^{++}, and Mn^{++}. But the binding of extracellular cations as measured by Mn^{54} or Ca^{45} amounts to only about 1×10^{-3} M/kg of cells. Thus only a small portion of the cell is participating in the ion-binding, about 2% in terms of bivalent cations and 0.5% in terms of total cations. Presumably the ion-binding occurs on the outer layer of the cell, a conclusion which is supported by previous studies[9] with UO_2^{++} and also by the following experiment. If Mn^{++} is allowed to equilibrate with cells, the per cent that is bound depends on the Mn^{++} concentration. Thus in Table III, with 0.4×10^{-4} M Mn^{++}, 52% of Mn^{54} is bound and

Table III. The Back Exchange of Mn^{++} Bound to the Surface Compared to That of Mn^{++} Taken Up with Phosphate

	Mn^{54} uptake in %			
	2 min	45 min	47 min	92 min
Initial Mn 7.2×10^{-4} *M*	22	24	24	25
Initial Mn 0.4×10^{-4} *M*, increased to 7.2×10^{-4} *M* with unlabelled Mn after 45 min	53	52	27	26
Initial Mn, 7.2×10^{-4} *M* with K^+, $H_2PO_4^-$ and glucose. Switched to fresh unlabelled Mn (7.2×10^{-4} *M*) after 45 min	28	100	97	94

with 7.2×10^{-4} *M* Mn^{++}, 24% is bound. If the concentration is raised from 0.4×10^{-4} to 7.2×10^{-4} *M* during the course of the experiment, a re-equilibration occurs. The per cent of bound Mn^{54} shifts immediately from 52% to 24%, by an exchange process. Thus the bound Mn^{++} is in equilibrium with the Mn^{++} concentration of the medium. On the other hand, if Mn^{++} is taken up into the interior of the cell (this occurs during active uptake of orthophosphate[10]), then the Mn^{54} is no longer in equilibrium with the medium and almost no back exchange occurs. Thus bivalent cations in the interior of the cell do not readily exchange with those of the medium, whereas those bound on the cell surface exchange readily.

The cation binding sites of the cell surface are associated with the uptake of glucose. Thus if the binding sites are combined with heavy metal cations such as UO_2^{++} or Hg^{++}, no sugars can be taken up by the cells. However, if the binding sites are combined with Ca^{++} or Mg^{++}, under certain conditions, the sugar uptake may be markedly stimulated (Table IV). In the experiments of Table IV, the buffer cation is triethylamine, which has been shown in other experiments to have no effect on the rate of sugar uptake.

In contrast, the ion binding sites of the cell surface seem to play no role in the ion-transporting systems. For example, yeast cells during metabolism of extracellular substrates, can take up K^+ from the medium against an apparent concentration gradient of as high as 3000 to 1. K^+ is taken up[11,12] in exchange for H^+. The addition of Mg^{++} or UO_2^{++} ions in concentrations sufficiently high to displace essentially all of the K^+ from the binding sites, has only a relatively small effect on the rate of K^+-uptake. It can be concluded either that bivalent cations do not interfere with the K^+-transporting system, or that the system is located below the outer surface in a region inaccessible to bivalent cations of the medium. The latter suggestion seems to be more reasonable on the basis of other experiments. For example, K^+ added to the medium can markedly stimulate the rate of sugar uptake under conditions such that no apparent changes in the cellular content of K^+ occur. Furthermore, the stimulating concentrations can be as low as 1×10^{-3} *M*, whereas the cell contains almost 0.2 *M*. Thus the action of K^+ is apparently confined to an outer part of the cell. Nevertheless, the stimulating effects of K^+ are not influenced by Ca^{++} (Table IV) or by Mg^{++} or Mn^{++} in concentrations sufficiently high to displace essentially all of the K^+ from the binding sites. These data suggest that in addition to the cation-binding sites of the outer surface, there is an underlying compartment into which K^+ from the medium can equilibrate, but into which bivalent cations cannot.

A similar situation exists in regard to H^+. At certain values of extracellular pH, the rate of sugar uptake is remarkably inhibited, despite the fact that the average intracellular pH is relatively constant. Bivalent cations are unable to reverse the inhibiting action of H^+ although they do displace H^+ from the outer binding sites. In contrast, there is a reciprocal relationship between K^+ and H^+, with K^+ counteracting the inhibitory effects of H^+. The K^+ and H^+ equilibrate into an outer compartment of the cell by an exchange reaction, influencing reactions in sugar uptake that take place there. Whether the action of K^+ in reversing the inhibition of H^+ is due to a direct competitive effect at an enzymic site, or whether it is due to the displacement of H^+ from the outer compartment by exchange is difficult to determine.

Table IV. Ions on Rate of Fermentation at pH 6.0

Ions (0.01 *M*)	Rate (μl/mg/hr)
$(C_2H_5)_3NH^+$	14.4
Na	32.6
K	42.3
K + Ca	40.0
Ca	28.3
Mg	22.7
Mn	21.2
UO_2	0.0
Mg	0.0

Some preliminary studies of K^+ distribution in cells, using K^{42} also indicate that there are outer K^+-binding sites for which bivalent cations compete, and in addition an underlying compartment into which K^{42} distributes with no competition from bivalent cations. The latter compartment involves only a few per cent of the total K^+ of the cell. It is separated from the interior of the cell by a membrane which is relatively impermeable to K^+ in the resting

anaerobic cell. However, when glucose is presented to the cell, the permeability of the membrane is markedly increased, with a consequent efflux of K^+ from the regions of high concentration in the interior of the cell. However, there is a simultaneous rapid inward transport of K^+ against its activity gradient. In consequence, the net movement of K^+ is in the inward direction unless the extracellular concentrations are exceedingly low.

Another phenomenon that occurs during the active uptake of sugars is the active transport of phosphate,[13] which proceeds against concentration gradients of over 100 to 1. Phosphate transport in yeast is unusual in that it proceeds without any appreciable back leakage. Thus the resting cell exchanges its phosphate for P^{32} labelled phosphate in the medium only at a very slow rate.[14] The cell is also impermeable to organic phosphates such as ATP or sugar-P as shown by experiments with P^{32}-labelled compounds.[15,16] During sugar uptake, however, there is a rapid inward movement of phosphate, but there is still no appreciable efflux. Consequently, the uptake of phosphate measured chemically and by isotope technique is the same. The process by which phosphate is taken up is essentially irreversible and no continuity or equilibrium exists between cellular inorganic phosphate and extracellular inorganic phosphate. Rather, the phosphate taken up by the cell must be incorporated into an ester of some kind in the surface compartment of the cell. Evidence for formulation of a compound between phosphate and cell constituent is given by data on the kinetics of phosphate uptake. If the phosphate concentration is increased to 0.002 *M* or greater, the rate of uptake reaches a maximal value, indicating saturation of the transport system. It is of some interest that phosphate uptake is associated primarily with the fermentation of sugars and is minimal with other substrates, such as pyruvate and lactate, suggesting that esterification of phosphate at the 3 phosphoglyceraldehyde dehydrogenase reaction may be an important factor. This interpretation is compatible with data presented for phosphate uptake in red blood cells.[18,19]

It has already been pointed out that bivalent cations of the medium do not exchange at an appreciable rate with those of the interior of the cell. However, during periods of phosphate uptake, if bivalent ions are present, they are also carried into the cell. This was demonstrated by a double isotope experiment with Mn^{54} and P^{32}. The samples were counted with a thin window GM tube and then recounted with an aluminum plate inserted between the sample and the counting tube. The latter count gave a measure of Mn^{54} activity by its gamma emission and the former, the total β and gamma activity of Mn^{54} plus the P^{32}. By this technique it could be demonstrated that Mn^{54} is only taken up when P^{32} is taken up, although the reverse is not true. The rate of phosphate uptake is not influenced by Mn^{++}. Furthermore, the maximum Mn^{++} uptake under all conditions is 1 mol for each mol of phosphate uptake. Apparently Mn^{++} is carried into the cell as a complex ion with phosphate.

DISCUSSION AND SUMMARY

The information presented here represents some of the information obtainable with isotopes, concerning the structure of the surface of the cell and the nature of the biochemical events that may occur there. It is apparent that the surface structure is complex, consisting of several compartments and/or membranes, which are in equilibrium with various ionic species of the medium and which are capable, during active metabolism, of transporting ions into the interior of the cell. The dependence of sugar uptake on the ionic composition of the outer compartment of the cell has been interpreted as indicating that the glycolytic machinery of the cell is also located peripherally, providing a source of energy for the active transport mechanisms. The limitations of space prevent a fuller discussion of this concept at the present time, but the supporting evidence has been recently reviewed elsewhere.[1,6,20]

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Use of Isotopes in the Study of Enzyme Mechanisms

By D. E. Koshland, Jr.,* USA

Enzymes are the potent catalysts which control the dynamic processes of biological systems. An explanation of their properties is, therefore, not only of academic interest but also of importance in a rational approach to chemotherapy. Moreover, the detailed understanding of these natural substances might lead to new types of man-made catalysts of significant industrial value. The attainment of this detailed understanding, however, has presented enormous difficulties. The enzyme is a protein containing amino acids whose side chain groups are unexceptional. They contain acids, bases, hydrocarbon chains, etc., i.e., groups which have relatively weak and non-specific powers as catalysts. Evidently the combination acquires properties not present in the individual building blocks. Since these combinations are labile, the most effective single method of investigation is the indirect information obtained in the dynamic action of the enzymes on their suhstrates. In this approach isotopes have proved a powerful new tool and in this paper a few examples of the application of isotopic techniques to the study of enzyme mechanisms will be described.

CARBOXYL ACTIVATION REACTIONS

An important reaction in many physiological processes is the condensation of a carboxylic acid with a second compound to form a carboxylic acid derivative. Such a condensation between amino acids is found necessary in peptide bond formation (Equation 1)

$$H_2NCHRCOOH + H_2NCHR'COOH \rightarrow H_2NCHRCONHCHR'COOH + H_2O \quad (1)$$

and a similar condensation between acetate and the sulfhydryl compound, coenzyme A, is important in fatty acid metabolism (Equation 2).

$$CH_3COOH + HS\text{-}CoA \rightarrow CH_3\overset{\overset{O}{\|}}{C}\text{-}S\text{-}CoA + H_2O \quad (2)$$

As written in their simplest form (Equations 1 and 2) these reactions are energetically unfavorable and would occur to only a minor extent without an outside source of energy. This energy can be provided by adenosinetriphosphate (ATP) which can hydrolyze in either of two ways to liberate large amounts of free energy (Equations 3 and 4).

$$ATP^{-4} + H_2O \rightarrow \text{Adenosine } OPO_3PO_3H^{-2} + HOPO_3^{--} \quad (3)$$

$$ATP^{-4} + H_2O \rightarrow \text{Adenosine-}OPO_3H^{-} + HOPO_3PO_3^{-3} \quad (4)$$

It has been shown that ATP is required in the metabolizing systems to obtain peptide or thioester bonds. Furthermore, individual enzymes have been found which catalyze the formation of particular peptide or thioester bonds. Some examples of these are shown in Equations 5 to 8.[1,2,3,4]

$$\text{glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + ADP + PO_4 \quad (5)$$

$$\text{pantoate} + \text{beta alanine} + ATP \rightarrow \text{pantothenic} + AMP + P_2O^{7} \quad (6)$$

$$\text{acetate} + \text{coenzyme A} + ATP \rightarrow \text{acetylcoenzyme A} + AMP + P_2O^{7} \quad (7)$$

$$\gamma\text{-glutamyl cysteine} + \text{glycine} + ATP \rightarrow \text{glutathione} + ADP + PO_4 \quad (8)$$

Each of these equations represents the sum of the two processes, condensation of the carboxylic acid and hydrolysis of the ATP. They express the evidence that the condensation does not occur unless the hydrolysis occurs simultaneously but they give no indication of the way in which the energy is transferred from the ATP to the carboxylic acid reaction. To aid in clarifying this problem experiments with O^{18} labeled substrates were performed.

Glutamic acid labeled with O^{18} was prepared by exchanging the oxygen of H_2O^{18} with the carboxyl oxygens under acid conditions.[5] The labeled glutamate was then incubated with unlabeled ATP in ordinary water in the presence of the glutamine synthetase enzyme. The products, ADP and inorganic phosphate were isolated and analyzed for isotope by converting to inorganic phosphate and pyrolyzing the KH_2PO_4 to KPO_3 and H_2O. The H_2O so obtained was equilibrated with a known amount of carbon dioxide which was then assayed in a mass spectrometer. From a determination of the mass 46 ($CO^{16}O^{18}$) to the mass 44 ($CO^{16}O^{16}$) ratio, the oxygen-18 contents of the ADP and inorganic phosphate can be calculated. Carbon dioxide rather than H_2O is used in the mass spectrometer because of the serious problem created by adsorption of H_2O to the walls of the apparatus. The unreacted glutamic was assayed by conversion to the pyrollidone-

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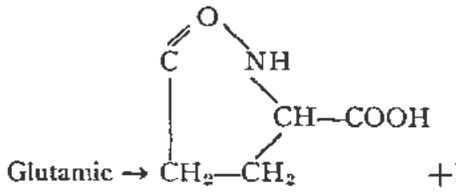
carboxylic acid (Equation 9)



$$\text{Glutamic} \rightarrow \text{CH}_2\text{—CH}_2 \qquad +\text{H}_2\text{O} \quad (9)$$

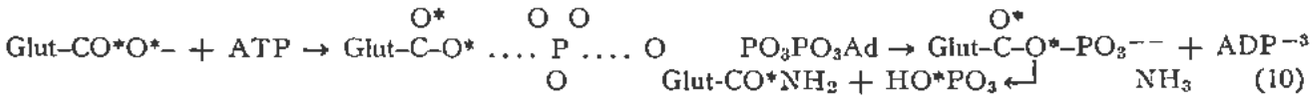
and the water formed was assayed by equilibration with carbon dioxide as described above.

The results of the actual enzymic reaction are shown in Table I. It can be seen that the atom % excess of the O^{18} in the inorganic phosphate produced is approximately one-quarter that of the glutamic acid originally introduced. This is the amount which would be obtained if there is a direct transfer of one oxygen by displacement mechanism.[6]

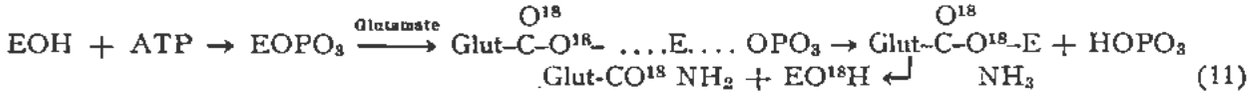
Table I. O^{18} Contents in Glutamine Synthetase Reaction in H_2O^{16}

		Observed atom % excess			
Experiment no.	*Acceptor*	*Glutamic acid used*	*Glutamic acid isolated*	*Inorganic phosphate*	*ΔDP*
1	NH_2OH	—	0.97	0.22	0.01
2	NH_2OH	1.08	1.02	0.24	0.06
3	NH_3	1.08	1.05	0.31	0.03

Possible mechanisms for this reaction are shown in Equation 10



in which a glutamylphosphate intermediate is formed, and in Equation 11:



in which a glutamyl-enzyme is formed.[7] (It might appear in the second mechanism that unlabeled phosphate is produced. However, since O^{18}-labeled enzyme is produced in this step subsequent cycles would yield O^{18} labeled phosphate. The molar amount of enzyme being small, the amount of unlabeled phosphate produced would be undetectable in this case, but could be tested when substrate amounts of enzyme are available.)

Other data[7,8] available in the glutamine synthetase case indicate the mechanisms cannot be quite as simple as depicted but the isotopic results perform two major functions: (*a*) they eliminate a number of alternative mechanisms, and (*b*) they indicate the direction to be followed in the search for intermediates. In this case it is clear that all the intermediates must involve covalently-bonded oxygen and that no exchange of this oxygen and that of the medium can occur. Thus, an indirect activation of the protein molecule which then causes an activation of the absorbed carboxyl is excluded as it would result in exchange with the oxygen of water. Instead the ATP energy is utilized to form a carboxyl-anhydride intermediate. This anhydride intermediate transfer occurs in the oxidation of α-ketoglutarate to form the peptide bond.

The conclusion of direct oxygen transfer through covalently bonded compounds appears to be of general validity for carboxyl condensation reactions. Studies in collaboration with Dr. Werner Maas and Dr. Michael Warmolinsky have shown that direct transfer of oxygen occurs from the carboxyl groups of pantoic acid to the phosphate of adenylic acid in the reaction shown in Equation 6. Independent studies in Dr. Boyer's laboratory have shown similar results in the acetyl kinase reaction, in the citrulline synthesis reaction, and in the glutamine synthetase reaction.[9] Dr. Mildred Cohn has shown that a direct transfer occurs in the oxidation of α-ketoglutarate to succinate with formation of ATP.[10]

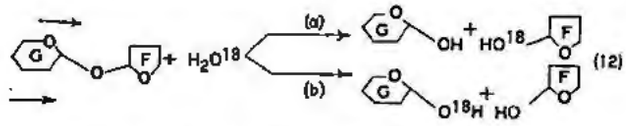
It is of interest to note that two points of attack appear in all of these cases, i.e., attack on the terminal phosphate with formation of a phosphoryl intermediate and attack on the innermost phosphorus with formation of an adenylic intermediate. Other possibilities such as pyrophosphoryl, adenosinediphosphoryl, or triphosphoryl intermediates have not been uncovered so far if they exist in nature. The isotopic results, therefore, indicate there is a direct chemical "gear" transferring the energy of ATP into carboxyl activation and they indicate that either of two main mechanisms are involved in this activation.

CLEAVAGE POINT IN ENZYMATIC REACTIONS

Another important feature of enzyme mechanisms involves the points at which bond breaking and bond formation occur. The point of cleavage is, of course, a fundamental fact which must be known in any particular enzymic reaction. In addition, the patterns of bond splitting for large groups of enzymes can in some cases be correlated on theoretical principles. These patterns frequently give indications of underlying factors which allow the enzyme to rearrange the electrons in molecules undergoing change.

One case in which such a broad correlation has been made involves enzymes catalyzing hydrolytic reactions.[11] When an oxygen bond in the original substrate, GOF, is split, the products GOH and FOH give no indication of the point of bond cleavage.

Performing the reaction in H_2O^{18}, however, allows a distinction between two alternatives as illustrated for the case of sucrose hydrolysis in Equation 12.



In this experiment sucrose was hydrolyzed by invertase in H_2O^{18}. The glucose formed in the reaction was isolated. It was found to contain essentially no O^{18} and, therefore, the alternative (*a*) must be the pathway utilized by invertase.

For this reaction the cleavage point was actually deduced by theoretical arguments[11] based on the assumptions that (*a*) the enzymic reaction proceeded by a displacement mechanism and (*b*) the enzyme required a precise orientation of catalytic groups to be effective. The agreement of theory with experiment was taken as support for the underlying assumptions. It was predicted also that beta glucosidase would cause cleavage between the glucose carbon and C-1 oxygen rather than between the aglucone and the C-1 oxygen. Salicin was hydrolyzed in H_2O^{18} and the O^{18} was found in the glucose rather than in the aromatic compound. This adds further corroboration to the theory.

A practical aspect of the determination of cleavage point is that it suggests the type of enzyme intermediates to be looked for in subsequent studies on the enzyme. Thus in the cleavage of ATP by purified muscle proteins, hydrolysis of ATP in H_2O^{18} showed[13] that the inorganic phosphate produced contained all the O^{18} whereas the adenosinediphosphate contained none (Equation 13).

$$\begin{aligned} &ATP + H_2O^{18} \rightarrow \\ &\quad \text{Adenosine–}OPO_3\ PO_3 + HO^{18}PO_3^{-} \end{aligned} \qquad (13)$$

The results proved that splitting had occurred between the terminal phosphorus and its bridge oxygen atom. Thus, if any covalent enzyme-substrate intermediate was formed it would be a phosphoryl-enzyme.

Isotope exchange reactions of the type shown in Equations 14 and 15 were then performed to give further information about the character of a phosphoryl-enzyme intermediate.

$$\begin{aligned} &HOPO_3^{--} + H_2O^{18} \rightleftharpoons \\ &\qquad HO^{18}PO_3^{--} + H_2O \end{aligned} \qquad (14)$$

$$\begin{aligned} &Ad\text{-}OPO_3PO_3PO_3 + AdOPO_3\overset{*}{P}O_3 \rightarrow \\ &\quad Ad\text{-}OPO_3\overset{*}{P}O_3PO_3 + AdOPO_3\ PO_3 \end{aligned} \qquad (15)$$

The experiments showed no detectable rate of exchange in either case. From this one can conclude that the phosphorylated enzyme must be very transient if it exists at all. Thus the isotopic experiments were able to indicate the type of intermediate which might exist, and also its approximate lifetime.

EXPERIMENTAL PROCEDURES

Two basic types of procedures have been used in our laboratories for the assay of O^{18}-labeled compounds. One involves the splitting of H_2O or of CO_2 from a particular position in the substrate molecule. If H_2O is obtained it must be equilibrated with a known amount of CO_2 before being analyzed in the mass spectrometer. A typical analysis of this type is the liberation of water from glutamic acid by pyrolysis to the pyrollidone carboxylic acid. In this case only the oxygen of the gamma carboxyl group is liberated. This method has the advantage of being pointed to a specific position but requires a new procedure for each compound analyzed. A picture of the apparatus is shown in Fig. 1 in which *A* represents the pyrolysis chamber, *B*, the equilibration chamber, *C*, the receiver into which the equilibrated CO_2 is distilled, and *D*, the chamber of normal CO_2. Chamber *C* is removed from this vacuum line and attached to the mass spectrometer for determination of the $CO^{16}O^{18}$ peaks.

A second procedure uses the complete conversion of all the oxygen of an organic compound to carbon monoxide and the subsequent oxidation of the CO to CO_2 by iodine pentoxide as in the Unterzaucher procedure. Doering and Dorfman[14] have recently shown that the procedure is applicable to the analysis of O^{18}-labeled compounds since the conversion of carbon monoxide to carbon dioxide does not involve exchange of oxygen with the I_2O_5 solid. Thus CO^{18} obtained from the organic molecule can be quantitatively converted to $CO^{18}O^{16}$ by passing over hot I_2O_5. The method is of value since it is applicable directly to any organic compound and to some inorganic compounds. It does not, however, pinpoint the particular position occupied by the O^{18} and hence is in many cases complementary to the previously described analytical method. The apparatus is shown in Fig. 2 in which *A* denotes the purification train, *B* denotes the sample, *C* denotes the 1100°C furnace surrounding the carbon bed used to reduce the gases quantitatively to CO, and *D* denotes the I_2O_5 oxidation chamber. Other traps and bubblers shown are used to purify the gases and pyrolysis products.

CONCLUSION

A thorough understanding of enzyme action requires a picture of the enzyme-substrate intermediate. Because of the transient nature of the intermediate, however, direct observation is rarely possible. Isotopes which provide a permanent record in the products of the pathway taken by the intermediate and allow the study of otherwise unobservable exchange reactions add a powerful tool to the equipment of the enzymologist.

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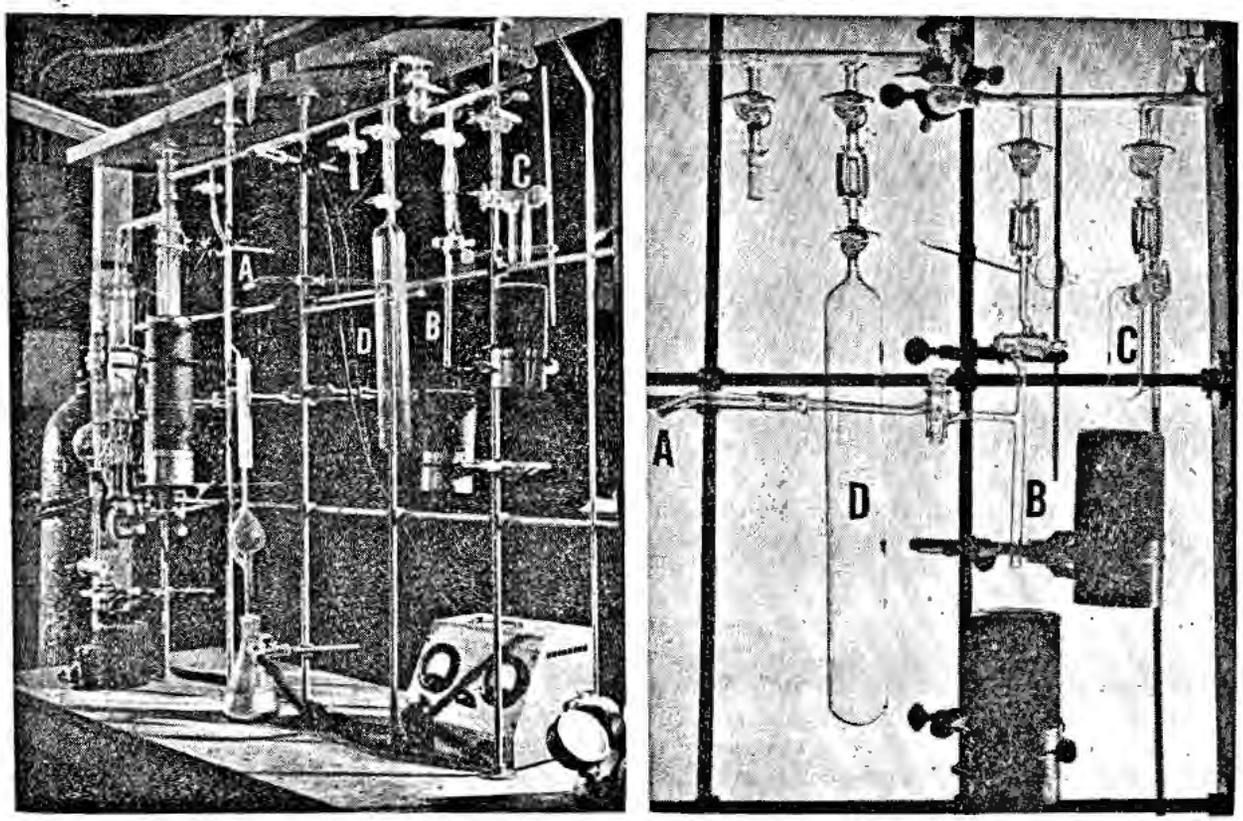

Figure 1. Apparatus for determination of O^{18} in specific positions in organic and inorganic compounds. Left complete line; right, O^{18} reaction section. (Components explained in text)

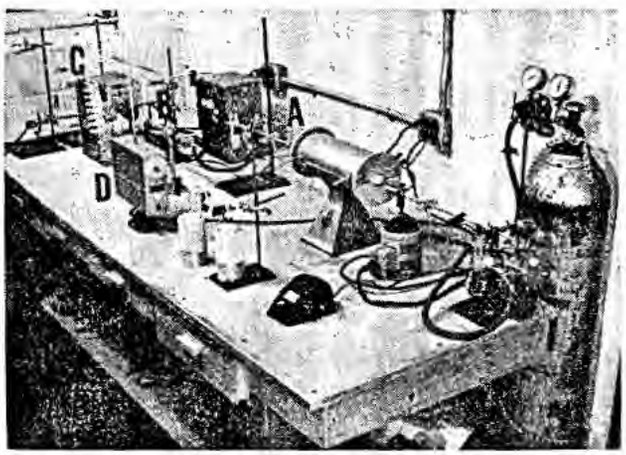

Figure 2. Apparatus for general determination of O^{18} in organic compounds following procedure of Doering and Dorfman. (Components named in text)

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The Role of Radioactive Isotopes in Immunologic Investigation Including Recent Studies on the Rates of Antibody Synthesis

By Frank J. Dixon,* USA

The availability of radioactive isotopes has provided a major stimulus for research in the field of immunology. Radioactive tracer techniques have been employed in virtually all branches of immunologic investigation including studies on:[1-4] the fate of antigens *in vivo*,[5] the synthesis and degradation of antibody,[6-9] the interaction between antigen and antibody *in vivo* especially in relation to hypersensitivity lesions, and[10,11] the physical-chemical aspects of antigen-antibody interaction *in vitro*. In all four of these areas much new knowledge has been gained through the use of isotopes, but many basic questions are still unresolved.

FATE OF ANTIGENS *IN VIVO*

With isotopes it has been possible to obtain quantitative data concerning concentration of labelled antigens in various tissues.[1-4] In addition, the technique of autoradiography has made possible histologic observations concerning the cellular localization of these antigens.[5] The studies dealing with the fate of antigens during the early phases of the immune response have done much to elucidate the handling of antigen during this period. In the later stages of the immune response, however, it is difficult, using labelled antigens, to determine whether the last traces of isotope label that can be detected represent residual antigen or the metabolic reincorporation of the label into the host's own constituents. Observations of these later stages have produced conflicting data, primarily because of the different types of labelled antigens used.

I^{131} trace labelled foreign serum proteins have been used extensively in studies of the fate of antigens. I^{131} is well-suited as an antigen label for *in vivo* work since it can be readily traced in fluid or tissues by virtue of its relatively hard beta and gamma radiations.[4] Addition of approximately one atom of iodine/molecule of protein provides an adequate radioactive label and does not alter detectably the immunologic or physiologic properties of the protein.[6-9] As far as has been determined, the I^{131} label remains attached to the protein until the latter is catabolized beyond the point of immunologic recognition.[10,11] Observations with these antigens indicate that as antibody is formed, antigen is rapidly catabolized and eliminated from blood and tissues.[12] These labelled antigens do not appear to persist in significant amounts in the host following the appearance of circulating free antibody.[4] During the secondary response to these antigens, antibody synthesis appears to persist in the absence of detectable antigen.[13]

A second type of isotopically labelled antigen that has enjoyed considerable usage has been a combination of a haptene bearing an isotope label with a carrier protein.[3,14] Usually C^{14} or S^{35} in the form of anthranilic or sulfanilic acid have been used in these antigen preparations. Following administration of such materials there has been found long term persistence of considerable amounts of isotope in the tissues of the host. It is likely that the persistence nf label indicates at least in part persistence of haptene group, but the fate of the protein component is less certain. However, because of the lack of observations of associated antibody synthesis in these experiments, it has not yet been determined whether the persisting haptene is antigenically active or not.

Studies with labelled antigeus have indicated that in some instances where the label persists in the tissues it appears to be selectively associated with the mitochondria of the cells, but the significance of this association in relation to antibody synthesis is still not clear.[15,16]

A third general type of labelled antigen has been internally labelled; i.e., the isotope label has been metabolically incorporated into the antigen.[3,17] Examples of this are protein antigens naturally synthesized in the presence of isotopically labelled amino acids, viruses grown in a medium containing isotopes, and polysaccharides synthesized in part from labelled simple sugars. The isotopes most frequently used for internal labelling have been S^{35}, C^{14}, and P^{32}. Such antigens are desirable from a chemical point of view since they are labelled without any alteration of the natural composition of the protein or polysaccharide. They are undesirable, however, since the isotopes used are all metabolically very active, and following degradation of the antigen are easily reincorporated into the hosts' own constituents. Thus, it is impossible

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to evaluate the persistence of the isotope label of these antigens in terms of persistence of original antigen.

SYNTHESIS AND DEGRADATION OF ANTIBODY

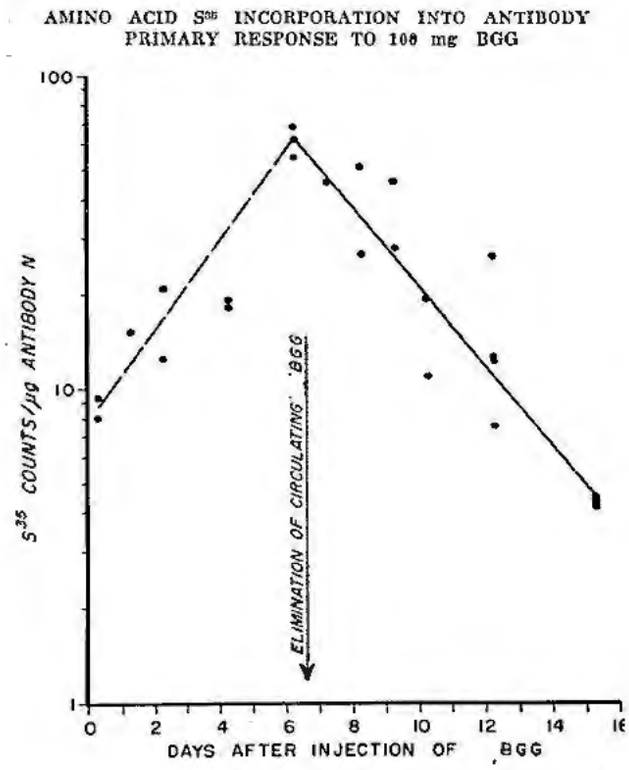
Previous studies of the metabolism of antibody have shown clearly *first*, that isotopically labelled amino acids were incorporated into antibody during active immunization and *second*, such amino acids were not incorporated into passively transferred antibody.[18] On the basis of several experiments it was apparent that antibody was synthesized *de novo* from amino acids and that, once formed, the antibody molecules were catabolized much as are other serum protein molecules.

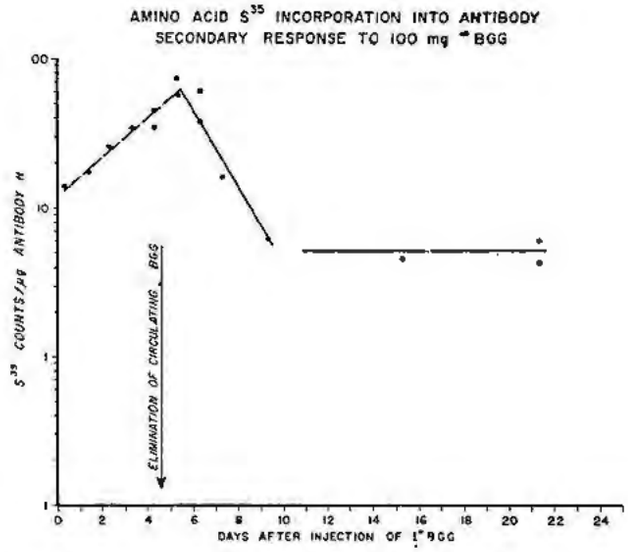
Later studies extending these investigations have been concerned with rates of antibody synthesis and degradation. By using foreign serum protein antigens in rabbits, it was shown that the rates of antibody catabolism were extremely rapid so long as antigen persisted in the circulation.[19] Apparently antibody combined with antigen in this situation, and the complex was rapidly catabolized by the host. However, in the absence of antigen, as with passively transferred antibody or else late in an active immune response, most antibodies were catabolized at the same rate as other serum globulins. This rate of catabolism is probably related to the general metabolic activity of the host and varies in terms of half-life from 1–2 days in the mouse to 5 days in the rabbit, 2 weeks in the human, and 3 weeks in the cow.[20] An exception to this rule is the hemolysin antibody of rabbits which is known to be much larger than the average gamma globulin molecule and which has a half-life of about 3 days.[21]

Recent studies in our laboratories have attempted to define the rates of antibody synthesis throughout the primary and secondary responses of rabbits to serum protein antigens. Such information when added to the extensive available data concerning fate of serum protein antigens in rabbits should give a relatively comprehensive picture of most of the aspects of this particular immunological response. In these studies, at various times after the primary or secondary injection of 100 μg bovine gamma globulin, rabbits were given intravenously a single injection of a standard amount of S^{35} labelled amino acids in the form of a yeast cell hydrolysate. Then S^{35} labelled amino acids are either rapidly incorporated into protein or degraded and/or excreted. Within 7 hours, approximately 90% of the S^{35} remaining in a rabbit is incorporated into protein. In such an experiment following injection of S^{35} labelled amino acids the S^{35} content of the rabbit's protein, including antibody, is a reflection of the rate of synthesis of that protein during a period of several hours following injection of S^{35}. Those rabbits given S^{35} prior to the time of maximum circulating antibody, three days after elimination of antigen, were exsanguinated at the time of maximum circulating antibody. Those receiving S^{35} after the time of maximum circulating antibody were exsanguinated one day after injection of S^{35}. The sera from all rabbits were decomplemented and determinations of the concentrations of S^{35} in antibody and in non-antibody globulin were made as well as determinations of the concentration of antibody in the serum.

From these observations it was possible to calculate the relative rates of antibody synthesis for each day following the elimination of circulating antigen. Minimum rates of synthesis for the period prior to antigen elimination could also be determined but during this period no provision could be made for the antibody which might have combined with circulating antigen and been catabolized prior to the obtaining of serum. The concentrations of S^{35}/μg antibody N obtained at the time of exsanguination are plotted according to the day of S^{35} administration in Fig. 1 for the primary response and Fig. 2 for the secondary response. These points indicate the relative rates of antibody synthesis on the different days throughout both responses. The early points in each response which were obtained with S^{35} injections made prior to elimination of antigen are minimum values uncorrected for loss of antibody by combination with antigen and are indicated by the dotted line. The observations made at the time of, or subsequent to, antigen elimination are indicated by solid lines. It appears that in both responses the maximum rate of antibody synthesis increases until about the time of elimination of circulating antigen as indicated by the vertical arrow. The rate of antibody synthesis then falls rapidly and continuously throughout the period of observation in the primary response. In the secondary response the rate of synthesis falls rapidly for 4–5 days after elimination of antigen and then continues at a constant level for the next 2 weeks.

The observations on rabbits receiving S^{35} subsequent to the elimination of antigen can be more meaningful when they are expressed as antibody S^{35}/ml serum and corrected for catabolism of antibody during the interval between injection of S^{35} and bleeding and also for variations in amounts of antibody formed by different rabbits. The observations expressed in this manner are shown in Fig. 3 for the primary response and Fig. 4 for the secondary response. For orientation the time of antigen elimination is again indicated by arrows and the typical serum antibody concentrations throughout the responses are indicated by fine lines as μg antibody N/ml serum. These graphs show that the rate of antibody synthesis declines, after its maximum at the time of antigen elimination, at a rate which can be approximately described by a straight line. The rate of decline of antibody synthesis in the primary response has a half-life of 1.4 days and can be followed to a level about 1% of the maximum rate of synthesis at which time observations were discontinued. In the secondary response there is a similar rapid decrease in antibody synthesis for 4–5 days following the time of maximum synthesis. This decline in rate of syn-





Figures 1 and 2. Amino acid S^{35} incorporation into antibody. Concentration of S^{35} in antibody is expressed as S^{35} counts/μg antibody N. Each point indicates concentration of S^{35} in antibody according to the day of S^{35} injection. Antibody bleedings were made 10 or more days after injection of bovine gamma globulin. Each point reflects the relative rate of antibody synthesis occurring at the time of S^{35} injection. Vertical arrows indicate time of disappearance of bovine gamma globulin from the blood. Figure 1, primary response to 100 mg BGG; Fig. 2, secondary response to 100 mg BGG

thesis stops abruptly at a level 2–3% of the maximum value and then this rate of synthesis is maintained for the next 2 weeks.

These observations fit well with the antigen tracer findings and quantitative antibody determinations using I^{131} labelled serum protein antigens.[22] The I^{131} antigens disappear from tissues and blood in both primary and secondary responses at the time of the appearance of circulating antibody. The rapid fall in antibody synthesis following the observed elimination of antigen in both responses has been postulated. The persistence of a slow rate of antibody synthesis for a considerable time after elimination of detectable antigen in the secondary response may represent a lasting orientation of gamma globulin synthesis to the specific antigen. That a lesser degree of persisting orientation and antibody synthesis occurs in the primary response cannot be ruled out by the present observations. It may well be, as postulated previously, that rapid antibody synthesis occurs in the presence of detectable amounts of antigen and that a slower rate of antibody synthesis based on a lasting orientation of the gamma globulin synthesic mechanisms may persist independently of antigen.

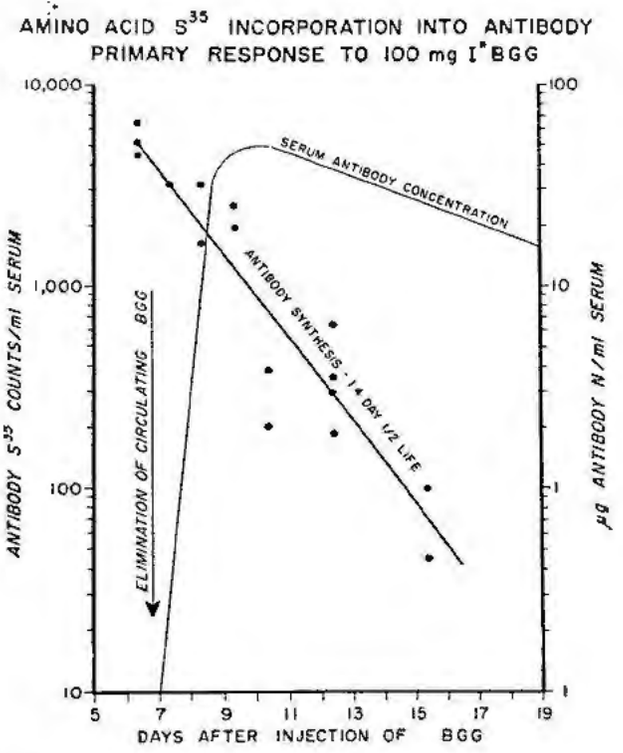
It would seem highly desirable to determine the rates of antibody synthesis in connection with those labelled antigens which have been claimed to persist for long periods in the host. Unless antibody synthesis can be shown to accompany retention of labelled material, it is hazardous to assume that the label is associated with antigenic activity. It may be possible that label bearing fragments of antigen or even masked whole antigen might persist in the host and yet be immunologically inactive. Thus, it seems unwise to postulate a relationship between the presence of antigen and antibody synthesis unless information concerning both aspects of the same immunologic system is available.

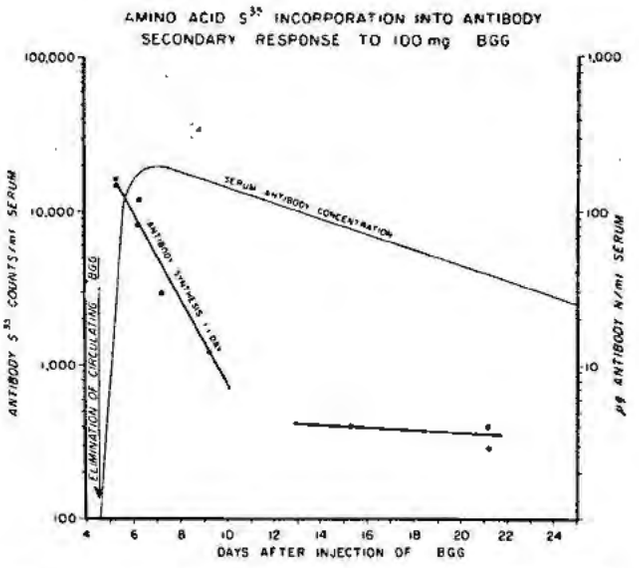
IN VIVO ANTIGEN-ANTIBODY REACTIONS

I^{131} labelled proteins have been used in the study of serum sickness, anaphylaxis, nephrotoxic serum nephritis and immunologic paralysis following injection of pneumococcal polysaccharides. Several investigators have failed to demonstrate any localization or persistence of labelled serum protein antigens in sites of the inflammatory necrotic lesions of serum sickness in rabbits.[4,23]

In anaphylaxis, on the other band, localization of labelled antigen has been demonstrated in the edematous peribronchial tissues of the guinea pig associated with bronchial occlusion.[24] In the rabbit, labelled antigen was found in considerable amounts in large intravascular accellular plugs which distended the pulmonary capillaries.[25] These plugs were in all likelihood made up of antigen-antibody aggregates formed in the presence of large amounts of circulating antibody and filtered out in the lung. It is likely that such an occlusion of pulmonary capillaries would contribute significantly to the resistance to pulmonary circulation which is considered the principal alteration in anaphylaxis in the rabbit.

In glomerulonephritis caused by antikidney antibodies a significant concentration of I^{131} labelled antibodies has been demonstrated in the glomeruli of the injured kidneys.[26] These antibodies apparently persist within the glomeruli for long periods.





Figures 3 and 4. Amino acid S^{35} incorporation into antibody. Points show amount of S^{35} in antibody/ml of serum. These values are based on observations shown in Figs. 1 and 2 but are corrected for catabolism of antibody during interval between injection of S^{35} and bleeding. Slopes of heavy lines indicate the rate of decline of antibody synthesis following maximal values at time of elimination of antigen from blood. Light curves indicate typical serum antibody concentrations in these responses. Figure 3, primary response to 100 mg I* BGG; Fig. 4, secondary response to 100 mg BGG

By tracing I^{131} labelled rabbit anti S 111 transferred to mice immunologically paralyzed by an injection of S 111 one month earlier it was possible to demonstrate that the S 111 was taking up antibody from the circulation and that this antibody was rapidly catabolized by the mouse.[27] This finding suggested the possibility that the immunologic paralysis resulting from injection of large amounts of S 111 might be caused by neutralization of antibody as it is formed and not be a true paralysis of antibody formation as had been postulated earlier.

IN VITRO ANTIGEN-ANTIBODY REACTIONS

One of the most profitable immunologic uses of isotopes has been in the study of *in vitro* reactions of antigens, antibodies, and complement. I^{131} has been used to label antigens, antibodies, or complement for such studies. Analysis of the concentration of these three elements in immunologic precipitates is easily carried out with tracer techniques. In addition, a useful quantitative measure of the antigen precipitating capacity of an antiserum can be readily obtained with the use of labelled antigen.[28,29] In this quantitation of antiserum an I^{131} labelled protein antigen is added to the serum in the region of slight antigen excess. From two or more observations in this region the amount of antigen precipitated at a standard reference point can be calculated. The results are usually expressed as the amount of antigen precipitated at that point where 80% of the antigen added is precipitated per ml of serum. This technique allows quantitation of antisera using relatively small amounts of serum and substituting a simple isotope count for a chemical determination of nitrogen in the precipitate.

Purification of labelled antibody has also been carried out using an I^{131} label.[30] If I^{131} labelled antisera are allowed to react with their homologous antigen and insoluble precipitates form, the I^{131} labelled antibodies can be removed by elution from the precipitate by allowing them to exchange with non-labelled antibodies at appropriate pH and temperature. The eluate from such a procedure contains antibodies which are specifically labelled with I^{131}. Labelled antibodies of this kind should prove useful in both *in vivo* and *in vitro* immunologic studies.

I^{131} labelled complement has been used to advantage in experiments designed to show the behavior of complement in immunologic reactions.[31–33] These studies have shown that the third component of complement, while essential for hemolysis, is not used up in the fixation of complement. Recent studies in our laboratory using primarily I^{131} labelled first components of complement have shown that significant amounts of the first component combine with specific precipitates. It has also been found that this component can be dissociated from specific precipitates with fresh, unlabelled complement. This first component which is active hemolytically can then fix to other specific precipitates or soluble complexes.

Of considerable promise are the studies of incomplete or non-precipitating antibodies in both experimental animals and man using I^{131} labelled antigens. Labelled antigens afford a possible means of quantitating these antibodies which have so far eluded precise detection. One such investigation by Becker and Feinberg[34,35] describes the detection of non-precipitating antibody in rabbit sera. The addition

of I^{131} labelled bovine albumin to rabbit antisera containing both precipitating and non-precipitating antibody to bovine albumin results in a combination of antigen and antibody but virtually no precipitation in far antibody excess. At the equivalence zone, approximately one-half of the labelled antigen was precipitated and one-half was bound to antibody in soluble complexes. The addition of sheep anti-rabbit globulin antibody to the system resulted in the precipitation of the rabbit globulin and the I^{131} labelled bovine albumin with which it was combined, thus providing a measure of the non-precipitating antibody. Further observations indicate that the non-precipitating antibody is more strongly bound to antigen than is precipitating antibody. If measures such as this can be applied to the study of reagins and other non-precipitating antibodies involved in human diseases a potent weapon will be added to our investigative armamentarium.

Another study of non-precipitating (co-precipitating) antibody-antigen complexes carried out in our laboratory has utilized either I^{131} labelled antigen or antibody. The complexes containing either labelled antigen or antibody were precipitated by the addition of excess complement. Radioactivity determinations afforded measures of the amounts of antigen and antibody in such soluble complexes. Analysis of soluble bovine-anti bovine albumin and bovine gamma globulin-anti bovine gamma globulin complexes in far antigen excess revealed a ratio of antibody to antigen molecules of 1 : 2 which conforms to the accepted bivalent nature of antibody.

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The Biosynthesis of Porphyrins

By D. Shemin,* USA

There has been tremendous progress in the last twenty years in the elucidation of the biochemical reactions and transformations which occur in living organisms. Some of the general concepts which have emerged, summarized very briefly, are that the basic reactions in the cells are surprisingly simple, that the cell synthesizes its complex molecules from relatively simple and available substances and that there is a biochemical unity in living matter.

Although these concepts were perhaps not fully appreciated when a study of the biosynthesis of porphyrins was begun ten years ago, the picture which has emerged is rather a good illustration of these basic concepts. The studies have revealed that the complicated looking molecule, protoporphyrin, is synthesized from two simple and available compounds, glycine and succinate, by relatively simple reactions. Furthermore, it has been established that the synthesis of this esoteric looking molecule is intimately related to the citric acid cycle, since the "active" succinate utilized in porphyrin synthesis is produced in these cyclic reactions. Although most of the investigations have been concerned with the biosynthesis of protoporphyrin, it appears that all the porphyrins in nature, including chlorophyll, in all different types of cells are synthesized by the same basic pathway. The different porphyrins merely arise by modifications occurring in the side chains in the β-positions of the pyrrole units.

In 1945 it was found that the nitrogen atom of glycine is the nitrogenous precursor of protoporphyrin in both man and rat.[1,2,3] Although protoporphyrin (Fig. 1) consists of two types of pyrrole units, methyl and vinyl bearing pyrroles and methyl and propionic acid bearing pyrroles, the finding that N^{15}-labeled glycine was equally utilized for these different pyrrole units[4,5] demonstrated that glycine was the nitrogenous precursor of all four pyrrole units and suggested that a common precursor pyrrole is synthesized which is the source of all four pyrrole rings of the porphyrin. This conclusion was well supported by the subsequent experimental results.

It appeared reasonable to expect that since the nitrogen atom of glycine is specifically utilized for porphyrin synthesis, the carbon atoms of this amino acid might also be involved. It was soon found that whereas the α-carbon atom of glycine is indeed utilized for porphyrin synthesis[6,7,8,9], the carboxyl group was not.[6,10] This latter negative finding was an important clue in the elucidation of the mechanism by which glycine and succinate condense. However, on incubation of duck erythrocytes[11] with doubly labeled glycine ($N^{15}H_2$-$C^{14}H_2$—COOH) it was found[7,9] that the dilution for the nitrogen atom was twice that for the α-carbon atom, that is, for every nitrogen atom utilized two carbon atoms from the α-carbon atom of glycine entered the porphyrin molecule. Therefore, it would appear that eight carbon atoms of the porphyrin molecule arise from the α-carbon atom of glycine since the four nitrogen atoms of the porphyrin are derived from glycine. In order to establish definitely that eight carbon atoms of the porphyrin are indeed derived from the α-carbon atom of glycine, and if so to locate the positions of these carbon atoms in the porphyrin molecule, and to gain some insight into the mechanism of porphyrin synthesis we developed a chemical degradation procedure of protoporphyrin whereby each carbon atom from a particular position in the porphyrin could be unequivocally isolated[8,12] (Fig. 2). On degrading protoporphyrin synthesized from glycine-2-C^{14} it was found indeed that eight carbon atoms are derived from the α-carbon atom of glycine and the positions were located; the four methene bridges[8,9] and one in each pyrrole[8] (Fig. 3). It will be noticed that the carbon atoms in the pyrrole rings, derived from the α-carbon atom of glycine, are in the α-position under the vinyl and propionic acid side chains. This finding supported the suggestion of a common precursor pyrrole first being formed and led to the suggestion that the vinyl side chains arose from propionic acid side chains by decarboxylation and dehydrogenation.

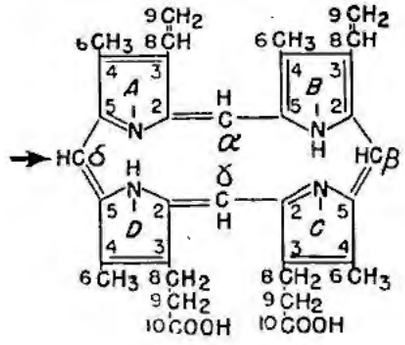

Figure 1. Protoporphyrin IX. The above numbering system is the same as that previously employed [9, 12]

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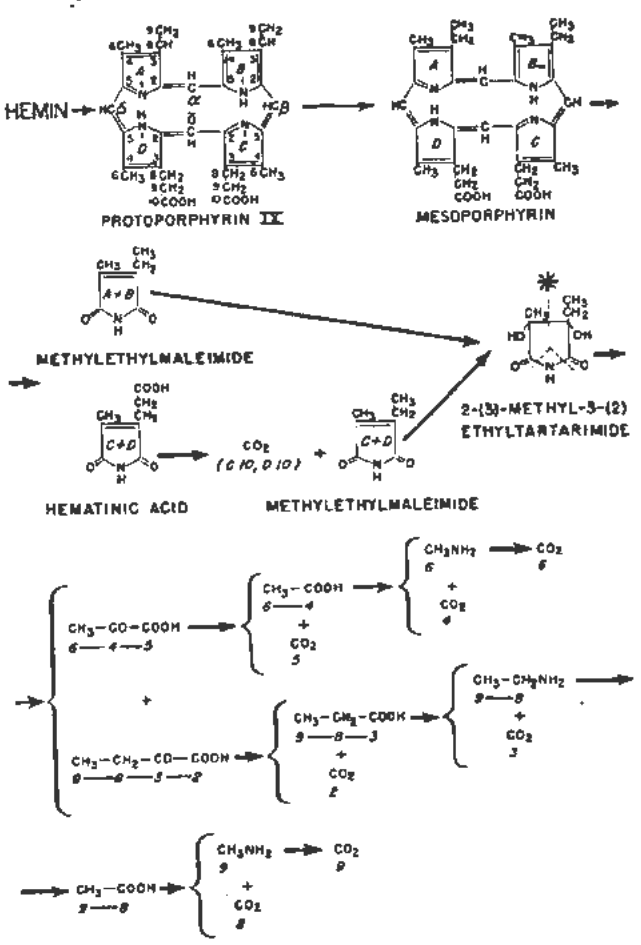

Figure 2. Protoporphyrin degradation. The letters and numbers designate positions of the carbon atoms

Having accounted for eight carbon atoms of protoporphyrin, the origin of the remaining twenty-six carbon atoms remained to be determined. It was found by Bloch and Rittenberg[13] that on administration of deuterioacetic acid (CD_3COOH) to a rat, the hemin isolated contained deuterium. This indicated that some of the side chain carbon atoms, at least, were derived from the methyl group of acetate since these are the only carbon atoms bonded to hydrogen.

In order to determine the extent of utilization of acetate for porphyrin synthesis and to locate all the carbon atoms which may be derived from acetate, duck blood was incubated separately with C^{14} methyl labeled acetate and with C^{14} carboxyl labeled acetate and the resulting C^{14} labeled hemin samples degraded by the method mentioned above. It was found that all the remaining twenty-six carbon atoms were derived from acetate.[12]

The composite C^{14} distribution pattern among all the labeled twenty-six carbon atoms derived from acetate is given in Fig. 4. Since all four pyrrole rings had the same C^{14} distribution pattern, support was obtained for the suggestion that a common precursor pyrrole is first formed. Furthermore, since both the methyl side of the pyrrole units and the vinyl propionic acid sides of the pyrrole units had the

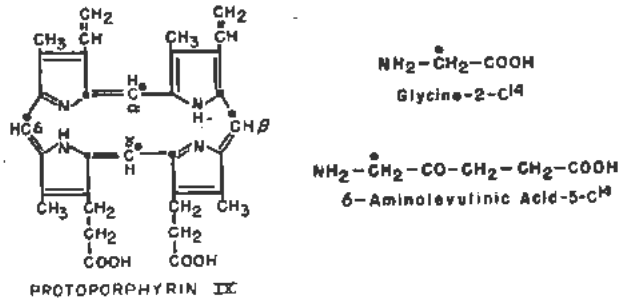

Figure 3. The carbon atoms of protoporphyrin which arise from the α-carbon atom of glycine and from the δ-carbon atom of δ-aminolevulinic acid

same C^{14} distribution pattern, it was concluded that each side of each pyrrole unit is made from the same compound. It would appear that the compound which condenses with glycine to form the pyrrole unit must be either a three or four carbon atom compound. On examination of the structure of protoporphyrin and noting the quantitative distribution of C^{14} among the carbon atoms in the experiments, it can be seen that a three carbon atom compound would satisfy the data as the precursor of the methyl sides of the pyrrole units (carbon atoms 6, 4 and 5) and the same compound would also be consistent with the data as the precursor of the vinyl sides of the pyrrole units (carbon atoms 9, 8 and 3) excluding carbon atom 2, which is derived from the α-carbon atom of glycine. However, it would appear that a four carbon atom compound would be necessary as the precursor for the propionic acid sides (carbon atoms 10, 9, 8 and 3), again exclusive of carbon atom 2. If a three carbon atom compound were utilized, subsequent carboxylations must have occurred to give rise to the propionic acid side chains in pyrrole rings C and D. On the other hand, if a four carbon atom compound were utilized, decarboxylations must have occurred to give rise to the methyl and vinyl groups. It can be decided which of these two alternative mechanisms operates in the synthesis of protoporphyrin by comparing the data obtained in the experiments using

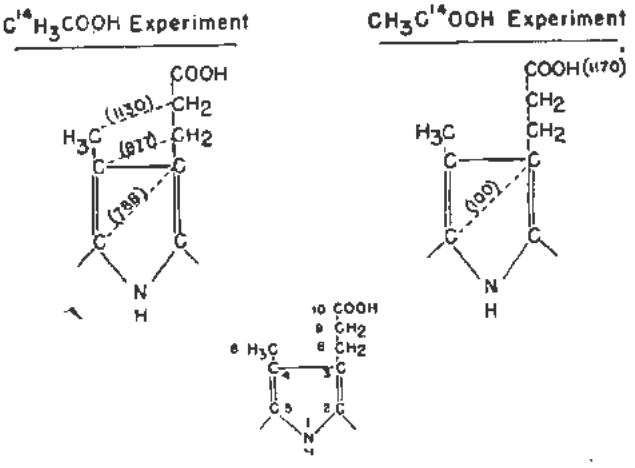

Figure 4. Average activities of comparable carbon atoms in all pyrrole units. The activities are given in parentheses. The pyrrole unit represented contains a carboxyl group which is found only in rings C and D of protoporphyrin

methyl labeled and carboxyl labeled acetate. The C^{14} activities of the carboxyl groups (1170 cpm) in protoporphyrin synthesized from carboxyl labeled acetate are equal to those found in the carbon atoms (1130) adjacent to these groups in the porphyrin synthesized from methyl labeled acetate (Fig. 4). This equality, i.e. the same degree of dilution, demonstrates that the acetic acid enters as a unit and that the utilization of acetic acid for pyrrole formation is via a four carbon atom unsymmetrical compound. Therefore the common precursor pyrrole originally contained acetic and propionic acid side chains in its β-positions and the methyl groups in the porphyrin arose by decarboxylation of the acetic acid side chains and the vinyl groups arose from decarboxylation and dehydrogenation of propionic acid side chains.

The data obtained in these experiments can readily be explained by assuming the participation of the tricarboxylic acid cycle in porphyrin formation. In the light of the relative distribution of the C^{14} activities among the carbon atoms of the porphyrin derived from acetate, it appeared that the acetate was converted to the four carbon unsymmetrical compound via this cycle. The entrance of methyl labeled acetate in the citric acid cycle can give rise to a four carbon atom compound, derived from α-ketoglutarate, which would have a similar relative C^{14} distribution pattern as was found in the porphyrin synthesized from methyl labeled acetate. For example, if one starts with methyl labeled acetate with a relative activity of 10 in the methyl group, the α-ketoglutarate formed on the first turn of the cycle would contain C^{14} activity only in the γ-carbon atom and the relative activity would be 10 (Table I). On formation of symmetrical succinate, the activities of the methylene carbon atoms would be 5 and 5, and those of the oxaloacetate eventually formed would contain half of the C^{14} activity of the γ-carbon atom of α-ketoglutarate. The recycling of this newly formed oxaloacetate with the labeled acetate would now result in a specimen of α-ketoglutarate having the relative activities shown in Table I for the second cycle. In Table I the relative activities found in α-ketoglutarate formed from methyl labeled acetate are given after a number of cycles. It can be seen that a four carbon atom compound arising from

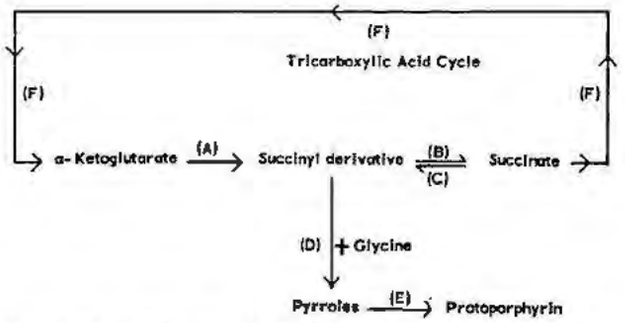

Figure 5. The relationship of the citric acid cycle and protoporphyrin formation

α-ketoglutarate after a finite number of cycles would have the same C^{14} distribution pattern as is found in the four carbon atom unit in the porphyrin synthesized from methyl labeled acetate; three adjacent carbon atoms are radioactive and the one arising from the γ-carbon atom has the highest activity. The relationship between the citric acid cycle and porphyrin formation is shown in Fig. 5.

Direct documentation of these conclusions was obtained by studying the utilization of C^{14}-succinate[14], C^{14}-α-ketoglutarate[15] and C^{14} labeled citrate[15] for porphyrin formation. In each case the predicted carbon atom of the porphyrin molecule contained C^{14}, e.g. α-ketoglutarate-5-C^{14} and primary carboxyl labeled citrate produced the same labeling pattern as was found for carboxyl labeled acetate.

The studies with C^{14} labeled succinate furnished direct evidence for the participation of a four carbon compound for porphyrin formation and also that the succinyl intermediate is formed from succinate as well as from α-ketoglutarate, that is, Reaction C occurs (Fig. 5). In Fig. 6 the labeling pattern which should be found in protoporphyrin synthesized from carboxyl labeled succinate is given. On degradation of protoporphyrin synthesized from carboxyl labeled succinate it was found that the indicated 10 carbon atoms contained the C^{14}. In order to demonstrate that Reaction C occurs a study of the utilization of both methylene labeled and carboxyl labeled succinate for porphyrin formation in the absence and presence of malonate was carried out. Theoretically carboxyl labeled succinate can not produce labeled

Table I. Relative Distribution of C^{14} Activity in Carbon Atoms of α-Ketoglutaric Acid Resulting from Utilization of C^{14}-Labeled Acetate in Tricarboxylic Acid Cycle (Results Expressed in cpm)

	From C^{14}-methyl-labeled acetate (activity of methyl group = 10 cpm)				*From C^{14}-carboxyl-labeled acetate (activity of carboxyl group = 10 cpm)*			
α-ketoglutaric acid	*No. of cycles in tricarboxylic acid cycle*							
	1st	*2nd*	*3rd*	*∞*	*1st*	*2nd*	*3rd*	*∞*
COOH	0	0	0	0	10	10	10	10
CH_2	10	10	10	10	0	0	0	0
CH_2	0	5	7.5	10	0	0	0	0
C=O	0	5	7.5	10	0	0	0	0
COOH	0	0	2.5	5	0	5	5	5

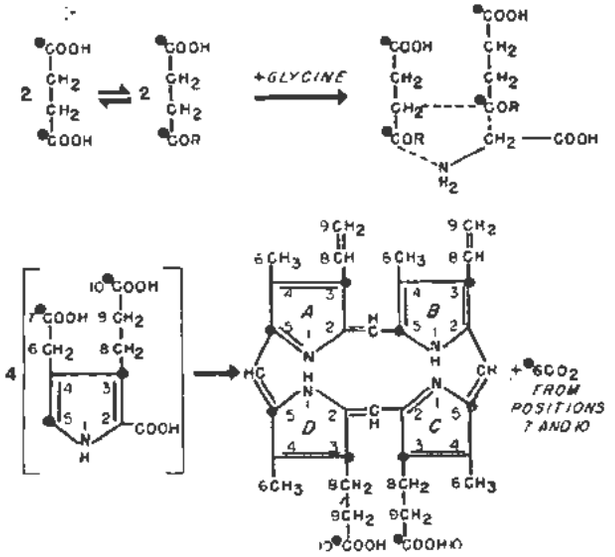

Figure 6. The position of succinate in protoporphyrin and the labeling pattern obtained in protoporphyrin synthesized from succinate-1, 4-C^{14}

porphyrin by entering the oxidative pathway of the citric acid cycle (Reaction F, Fig. 5) for in this direction the four carbon atom compound formed from α-ketoglutarate would no longer contain any of the original carboxyl groups of the labeled succinate. Therefore the formation of labeled porphyrin from carboxyl labeled succinate would be evidence for the occurrence of Reaction C. These theoretical considerations and conclusions can be tested experimentally by blocking Reaction F with malonate. If these considerations are valid and if Reaction C occurs, the degree of labeling found in protoporphyrin from carboxyl labeled succinate should not be influenced by the presence of malonate. On the other hand, methylene labeled succinate can produce labeled porphyrin via Reaction F. In this case the degree of labeling from methylene labeled succinate should be lowered in the presence of malonate. It was found experimentally that the C^{14} activity of the porphyrin synthesized from carboxyl labeled succinate in the presence or absence of malonate was the same, while that of the porphyrin synthesized from methylene labeled succinate was 50–60 per cent lower in the presence of malonate than in its absence.[14] These experiments demonstrated that the succinate can be converted to active succinate via two pathways and the malonate effect is a reflection of the positions in the succinate which contain C^{14}.

It then became of interest to find the mechanism by which the "active" succinate and glycine combine to form the pyrrole unit of the porphyrin. It was realized that in the initial condensation of glycine and succinate the whole molecule of glycine is involved since in all experiments in which glycine-2-C^{14} was the substrate the carbon atom in the pyrrole ring and the methene bridge carbon atom (Fig. 3) had the same C^{14} activity and no derivative of the α-carbon atom of glycine (CH_3OH, H_2CO, HCOOH, CH_3NH_2) could substitute for glycine. These findings led us to the conclusion that the *same* derivative of glycine was utilized for the pyrrole ring carbon atom and for the bridge carbon atom even though the bridge carbon atom was no longer attached to the nitrogen atom of glycine as is the ring atom. On consideration of the possible method of condensation of succinate and glycine, which would give rise to a product from which a pyrrole could reasonably be made, the mode of condensation must also take into consideration a mechanism by which the carboxyl group of glycine is detached from its α-carbon atom, subsequent to the initial condensation, for the carboxyl group of glycine is not utilized for porphyrin synthesis. The condensation of succinate on the α-carbon atom of glycine to form α-amino-β-ketoadipic acid (Fig. 7) would appear to be in agreement with all the experimental findings and conclusions. The compound formed, being a β-keto acid, could then readily decarboxylate and thus provide a mechanism by which the carboxyl group of glycine is detached from its α-carbon atom subsequent to the initial condensation of the whole molecule of glycine with succinate. Further, the product of decarboxylation would be δ-aminolevulinic acid and condensation of two moles of the latter compound, by a Knorr type

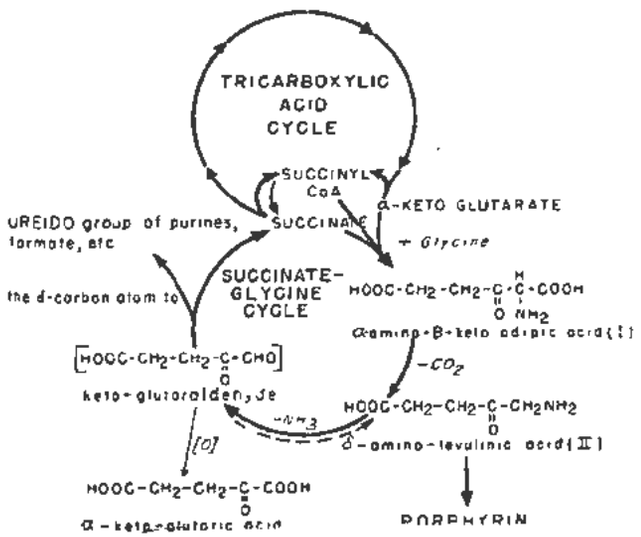

Figure 7. Succinate-glycine cycle: a pathway for the metabolism of glycine

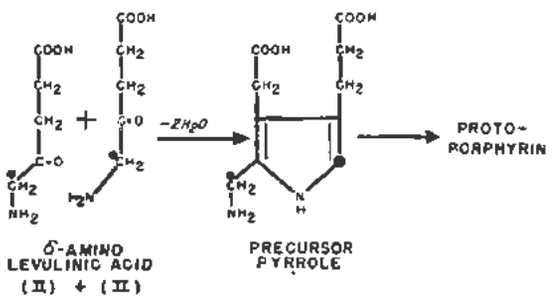

Figure 8. The mechanism for the formation of the monopyrrole, porphobilinogen, by condensation of two moles of δ-aminolevulinic acid. The carbon atoms bearing the closed circles (●) were originally the α-carbon atom of glycine

of condensation (Fig. 8) would give a reasonable mechanism for formation of a pyrrole in which the α-carbon atom of glycine would be distributed in previous position. To test this hypothesis δ-aminolevulinic acid was synthesized and its use for porphyrin synthesis studied.[16,17,18]

In the initial experiments, unlabeled δ-aminolevulinic acid was added to duck red blood cell hemolysates along with either C^{14}-labeled glycine or C^{14}-labeled succinate. The radioactivities of the hemin samples isolated in these experiments were compared with those obtained from controls in which the unlabeled δ-aminolevulinic acid was omitted. The rationale for these dilution-type of experiments is as follows: if δ-aminolevulinic acid is an intermediate formed from the condensation of glycine and succinate, any labeled δ-aminolevulinic acid formed from these labeled substrates will be diluted by the added unlabeled compound, and consequently this should be reflected in the lowered radioactivity of the hemin samples synthesized in the presence of unlabeled δ-aminolevulinic acid. It can be seen from Table II that the hemin samples made in the presence of unlabeled δ-aminolevulinic acid contained less C^{14} than those of the controls made either from C^{14}-labeled glycine or succinate.[17] These results, which are in full agreement with the hypothesis can also be explained, however, by the possibility that δ-aminolevulinic acid is acting not as a diluent but as an inhibitor of heme synthesis. To rule out the latter possibility, the δ-aminolevulinic acid added in Experiment 2 (Table II) was labeled with N^{15}. It can be seen that whereas the incorporation of C^{14} from the glycine was lowered, there was a comparatively large incorporation of N^{15} into the porphyrin, thus demonstrating that the lowered C^{14} activity of the hemin sample was due to dilution rather than inhibition. Further proof that δ-aminolevulinic acid is a result of the condensation of glycine and succinate was obtained by incubating red blood cell hemolysates with glycine-2-C^{14} and unlabeled δ-aminolevulinic acid, and subsequently isolating the δ-carbon atom. In such an experiment it was found that the formaldehyde liberated upon periodate oxidation of a crude fraction containing δ-aminolevulinic acid was highly radioactive.

Table III. Distribution of C^{14} Activity in Protoporphyrin Synthesized from δ-Aminolevulinic Acid-5-C^{14} and from Glycine-2-C^{14}

Fragments of porphyrin	*Molar activity, %, in fragments of porphyrin synthesized from*	
	δ-Aminolevulinic acid-5-C^{14}	*Glycine-2-C^{14}*
Protoporphyrin	100	100
Pyrrole rings A + B (Methylethylmaleimide)	24.5	24.6
Pyrrole rings C + D (Hematinic acid)	25.2	25.3
Pyrrole rings A+B+C+D	49.7	49.9
Methene bridge carbon atoms	50.3	50.1

More rigorous proof that δ-aminolevulinic acid is indeed the precursor for porphyrin synthesis was obtained by degrading a hemin sample synthesized from δ-aminolevulinic acid-5-C^{14} and from δ-aminolevulinic acid-1,4-C^{14}. The δ-carbon atom of the former compound should label the same carbon atoms of protoporphyrin as those which we have previously found to arise from the α-carbon atom of glycine, since according tn the hypothesis the latter carbon atom is the biological source of the δ-carbon atom of δ-aminolevulinic acid. Furthermore, the δ-aminolevulinic acid-1,4-C^{14} should label the same carbon atoms of protoporphyrin found to arise from the carboxyl groups of succinate, since from Fig. 7 these carbon atoms arise from succinate.

It can be seen from Table III that the same C^{14} distribution pattern was found in protoporphyrin synthesized from δ-aminolevulinic acid-5-C^{14} as from glycine-2-C^{14}; 50 per cent of the C^{14} activity resides in the pyrrole rings and 50 per cent in the methene bridge carbon atoms (Fig. 3).[17,18]

Table II. Comparison of C^{14} Activities of Hemin Samples Synthesized from Glycine-2-C^{14} (0.05 mc/mM) or Succinic-2-C^{14} (0.05 mc/mM) in the Presence and Absence of Non-radioactive δ-Aminolevulinic Acid

Experiment	*Substrates*			*Isotope concentration in hemin*	
	C^{14}-labeled	*N^{15}-labeled*	*Unlabeled*	*C^{14}*	*N^{15}*
				cpm	(atom % excess)
1	Glycine—2—C^{14} (0.05 mM)	—	—	125	
	Glycine—2—C^{14} (0.05 mM)	—	δ—Aminolevulinic acid (0.05 mM)	15	
2	Glycine—2—C^{14} (0.05 mM)	—	—	230	
	Glycine—2—C^{14} (0.05 mM)	δ—Aminolevulinic acid (0.05 mM)*	—	48	0.21
		Glycine (0.33 mM)*	—		0.06
3	Succinate—2—C^{14} (0.1 mM)	—	—	660	
	Succinate—2—C^{14} (0.1 mM)	—	δ—Aminolevulinic acid (0.1 mM)	180	

* The isotopic concentrations of these samples were 34 atom per cent excess N^{15}. In each of the experiments the volume of the hemolyzed preparation was 30 ml. Unlabeled succinate (0.1 mM) was added to the flasks in which labeled glycine was the substrate, and unlabeled glycine (0.33 mM) was added to the flasks in which labeled succinate was the substrate. Each flask contained 1 mg of iron (ferric).

Table IV. Distribution of C^{14} Activity in Protoporphyrin Synthesized from δ-Aminolevulinic Acid-1,4-C^{14} and from Succinate-1,4-C^{14}

Fragments of porphyrin	*Molar activity % in fragments of porphyrin synthesized from*	
	δ-Aminolevulinic acid-1, 4-C^{14}	*Succinic acid-1, 4-C^{14}*
Protoporphyrin	100	100
Pyrrole rings A + B (Methylethylmaleimide)	38.0	39.4
Pyrrole rings C + D (Hematinic acid)	61.5	59.5
Pyrrole rings A+B+C+D	99.5	98.5
Carboxyl groups	20.4	20.5

Also it can be seen from Table IV that the same C^{14} distribution pattern was found in protoporphyrin synthesized from δ-aminolevulinic acid-1,4-C^{14} as from succinate-1,4-C^{14}; ten carbon atoms are equally radioactive, 40 per cent of the C^{14} activity resides in pyrrole rings A and B, 60% of the activity resides in pyrrole rings C and D and the carboxyl groups contain 20% of the C^{14} activity (Fig. 6).[19]

Thus all the carbon atoms of protoporphyrin are derived from δ-aminolevulinic acid. The role of δ-aminolevulinic acid in porphyrin synthesis was also actively pursued by Neuberger and Scott,[20] and just subsequent to our initial finding they published a confirmatory paper and further confirmation was published by Dresel and Falk.[21] Furthermore, it may be well to point out that the theoretical formulation of the structure of the precursor pyrrole[16] is the same structure which was determined for porphobilinogen[22] by Cookson and Rimington,[23] a compound excreted in the urine of patients with acute porphyria. These findings make α-amino-β-ketoadipic acid an obligatory intermediate and we have found experimentally that this β-keto is indeed an intermediate. Injection of δ-aminolevulinic acid or the diethyl ester of α-amino-β-ketoadipic acid gives rise to the urinary excretion of porphobilinogen.[24]

The condensation of "active" succinate and glycine to form δ-aminolevulinic acid subsequently, thus far appears to require the partially intact structure of the red blood cell. It has been found that whereas δ-aminolevulinic acid can be converted to protoporphyrin in either an homogenized preparation or in a cell-free extract, the conversion of succinate and glycine to porphyrin takes place only with intact cells or with those cells which have been hemolyzed with water.[18] Homogenized preparations obtained in a blendor are no longer capable of synthesizing protoporphyrin from succinate and glycine. It would appear that on homogenization the functional activity of only those enzymes of the system that are involved in the condensation of succinate and glycine is lost. However, the finding that δ-aminolevulinic acid can be converted to protoporphyrin in a cell-free extract opened up the possibility that soluble enzymes, concerned with each of the steps in this conversion, could be isolated.

Indeed, it was subsequently and independently found in three different laboratories that a highly purified protein fraction from ox liver,[25] duck erythrocytes[26] and from chicken erythrocytes[27] can convert δ-aminolevulinic acid to porphobilinogen. In our laboratory we obtained a highly purified fraction from duck blood which on incubation with δ-aminolevulinic acid-5-C^{14} produced labeled porphobilinogen. Since the porphobilinogen is presumably synthesized from two moles of δ-aminolevulinic acid (Fig. 8), its molar radioactivity should be twice that of the δ-aminolevulinic acid used as the substrate. The molar radioactivities of the substrate, δ-aminolevulinic acid, and of the product, porphobilinogen, were found to be 242×10^3 cpm and 487×10^3 cpm respectively. This finding demonstrates experimentally the utilization of two moles of δ-aminolevulinic acid for porphobilinogen formation. Further evidence that porphobilinogen is an intermediate in protoporphyrin synthesis was obtained by incubating equal volumes of the cell-free extract of duck erythrocytes with equimolar amounts of δ-aminolevulinic acid (0.018 mc/mM) and with the enzymatically synthesized radioactive porphobilinogen (0.036 mc/mM) and subsequently isolating the hemin and determining its radioactivity. The radioactivities of the hemin samples synthesized from δ-aminolevulinic acid and from the porphobilinogen were 92 cpm and 85 cpm respectively, after a two-hour incubation, and 350 and 336 cpm respectively after a fifteen-hour incubation period.[26] This latter result is in agreement with the findings of Falk, Dresel and Rimington[28] and of Bogorad and Granick.[29]

Although no evidence has yet been obtained concerning the biological mechanism of conversion of the monopyrrole to the tetrapyrrole structure, several suggestions have been advanced.[29,30] We would like to suggest still another possibility which may explain the distribution of the α-carbon atom of glycine or the δ-carbon atom of δ-aminolevulinic acid in the porphyrin molecule of the I and III series. This mechanism is based on the synthetic mechanism of dipyrrole and tetrapyrrole formation demonstrated by Corwin and Andrews,[31] and by Andrews, Corwin and Sharp.[32]

Condensation of three moles of the precursor pyrrole (porphobilinogen) or of a closely related derivative, would lead to a tripyrrylmethane compound, as schematically represented in Fig. 9. The tripyrrylmethane then breaks down into a dipyrrylmethane and a monopyrrole. The structure of the dipyrrylmethane is dependent on the place of splitting. An A split would give rise to dipyrryl-

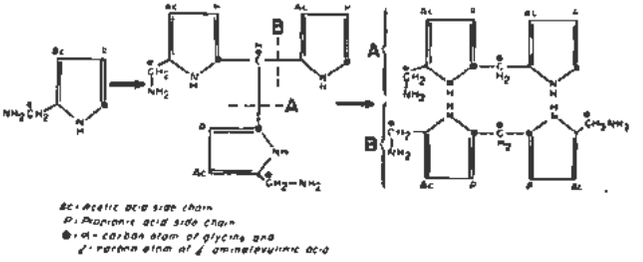

Figure 9. A mechanism of porphyrin formation from the monopyrrole

methane A, and a B split would give rise to dipyrrylmethane B. Condensation of two moles of dipyrrylmethane A would give rise to a porphyrin of the I series, while condensation of a mole of A and a mole of B would give rise to a porphyrin of the III series. In the formation of the porphyrin of the III series it can be seen from Fig. 6 that it is necessary to lose a one-carbon atom compound since there are three aminomethyl side chains and only two are required to condense the two dipyrroles to the porphyrin structure. If the mechanism similar to that outlined in Fig. 6 is concerned with porphyrin synthesis, it would appear that this one-carbon atom compound given off could well be formaldehyde. Consistent with this idea is our finding[17] that on the conversion of porphobilinogen to porphyrins either by heating under acid conditions[22] or by enzymatic conversion in cell-free extracts[18] formaldehyde was formed. This was established by heating or incubating porphobilinogen, labeled with C^{14} in the aminomethyl group, and subsequently isolating radioactive formaldehyde as the dimedon derivative.

It would appear that on conversion of porphobilinogen to porphyrins, formaldehyde from the aminomethyl group is formed and that any postulated mechanism should take this into consideration. It is difficult at present to establish the structure of the intermediate tetrapyrrole compounds which are formed prior to the formation of protoporphyrin. However, we would like to suggest that these intermediate tetrapyrrole compounds may be the more highly reduced state, containing methylene bridge carbon atoms rather than methene bridge carbon atoms, and consequently uroporphyrin and coproporphyrin are oxidized products of the intermediates.

The biosynthetic pathway for porphyrin synthesis given above may, from a more general viewpoint, be looked upon as merely one aspect of glycine metabolism. The α-carbon atom of glycine besides being utilized for porphyrin synthesis is also known to participate in the synthesis of several other compounds: the ureido groups of purines, the β-carbon atom of serine, methyl groups and for formic acid. It would appear that these different compounds and porphyrins may be related via a metabolic pathway of glycine. If indeed these mentioned compounds and porphyrin synthesis are related through a series of reactions occurring with glycine, then an intermediate utilized for porphyrin synthesis may have the same metabolic pattern as is known for glycine. If the succinate-glycine cycle proposed in Fig. 7[16] were the pathway by which all the compounds are related, then specifically the δ-carbon atom of δ-aminolevulinic acid should have the same metabolic spectrum as the α-carbon atom of glycine. In a study carried out in ducks and rats it was found that indeed the δ-carbon atom of this aminoketone is utilized for the ureido groups of purines, for the β-carbon atom of serine, for the methyl group of methionine and is also converted to formic acid. Thus, it has been demonstrated that glycine is metabolized via this pathway.[33]

The condensation of glycine with "active" succinate provides a pathway whereby glycine can be oxidized to carbon dioxide and the intermediates produced in the cycle drawn off for the synthesis of other compounds. This is similar to the citric acid cycle, in which another two-carbon compound is oxidized to carbon dioxide and intermediates are produced which can be drawn off for synthesis. In the succinate-glycine cycle, succinate is the catalyst instead of oxaloacetate.

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Radioactive Oxytetracycline (Terramycin*). I. Mode of Synthesis and Properties of the Radioactive Compound

By J. F. Snell, R. L. Wagner, Jr., and F. A. Hochstein,† USA

Synthesis of a radioactive broad spectrum antibiotic has not been previously reported. The present paper describes the production of radioactive oxytetracycline by fermentation of *S. rimosus* in the presence of C^{14}-labelled materials. The isolation of radiooxytetracycline is described, and position of labelling and properties of the isolated product are summarized.

Previously reported radioactive antibiotics include penicillin and streptomycin. Thus, Howell & Thayer[1] and Rowley *et al.*[2,3,4] synthesized radioactive penicillin containing S^{35} as the label. These preparations were obtained by drastically restricting the sulfur source in the fermentation medium, and incorporating high specific activity sodium sulfate. It was calculated that about 5–20% of the sulfur in the sulfate was converted into penicillin. Although very low yields of penicillin were obtained in the fermentation, the specific activity was very high (0.06–0.18 microcuries/International Unit). Radioactive sulfur-35 labelled penicillin was also synthesized by Johnson and Maass,[5] their products contained about 200 disintegrations/minute/microgram (dpm/μg).

Penicillins K, F and G have been shown to incorporate radioactivity from methyl and carboxyl-labelled acetate, formate, and carboxyl-labelled lactate.[6] Radioactive penicillin G is now commercially available.

Streptomycin labelled with C^{14} has been produced in fermentations of *S. griseus* using radioactive glucose as a precursor.[7]

This study indicated no entry of acetate carbon into the streptomycin molecule.

METHODS

Fermentation Procedure

All radioactive fermentations were carried out in 50 ml Erlenmeyer flasks to the bottom center of which an 8 mm (od) by 27 mm glass well was sealed. The well served as an alkali reservoir, and was filled with 400 microliters (μl) of 20% KOH solution. To the annulus of the flask, 5–10 ml of fermentation media were added. The flasks were sealed with commercial tape, and autoclaved for 15 minutes at 15 pounds. After addition of KOH to the center well and inoculation with a spore suspension of *S. rimosus*, by needle through the tape, the orifice was re-sealed and the flasks placed on a rotary shaker for 96–120 hours at 27°C. Precursor was added by micro-pipette aseptically.

Evaluation of Precursor Efficiency

Following 96–120 hours of fermentation, flasks were removed, and the contents were aseptically filtered. The broth, or an acid-butanol extract of broth, was then chromatographed for oxytetracycline separation in four solvent systems:

System T-1: *n*-butanol saturated with 2*N* HCl. (R_f 0.40.)

System T-2: Ethyl acetate, glacial acetic acid, water (1 : 1 : 1). (R_f 0.80.)

System T-3: Ethyl acetate, pyridine, and water (1 : 1 : 1). (R_f 0.55.)

System T-4: Ethanol, butanol, water (1 : 1 : 1). (R_f 0.50.)

Whatman No. 1 strips, acid washed (2*N* HCl or *N* acetic acid), were suspended in the above systems. A 30 cm solvent rise was permitted. In certain cases, chromatography was performed at 5°C.

Following chromatography, the solvent-free chromatogram was wound on the aluminum cylinder, and placed inside of a methane flow counter (Fig. 3 overleaf).

The operation and calibration of this device (which was developed in cooperation with Dr. R. C. Anderson of the Brookhaven National Laboratories) for the scanning of paper chromatograms will be described in detail elsewhere. The cylinder rotated the paper strip slowly in front of a slit opening to the sensitive counting volume, and the count obtained was recorded on an Esterline-Angus recorder. With proper calibration, an accurate R_f and a semi-quantitative C^{14}-analysis were obtained on the separated spots.[8] The scanned strip was then bioautographed along with oxytetracycline standard strips against *B. subtilis*. Correlation of the bioautogram activity with the position of the radioactivity indicated the position of the oxytetracycline spots. Bioassay of the chromatographed material then allowed the calculation of an approximate specific activity for oxytetracycline which was suitable for precursor screening and for adjustment of optimum precursor conditions.

* Trademark, Chas. Pfizer & Co., Inc., Brooklyn, New York.

† Pfizer Therapeutic Institute, Maywood, New Jersey.

Radiochemical Assay of Final Products

Products isolated in pure form, and degradation products, were assayed for radioactivity by the method of Van Slyke *et al.*[9] in Bernstein-Ballentine tubes.

RESULTS

Table I summarizes the development of a shaker flask medium less rich in carbon than the usual medium; no medium was considered desirable that produced less than 500 micrograms (μg) oxytetracycline/ml. The medium finally developed represented the best compromise between yield and carbon concentration that could be obtained. It was used subsequently for the precursor studies. Constituents of this medium (Medium C, Table I) were (grams/liter): 8.0 $NaNO_3$, 15.0 corn meal, 4.0 beet molasses, and 7.0 ml soybean oil. This was adjusted to pH 6.8 and sterilized at 20 pounds for 15 minutes.

Table I. Oxytetracycline Yield on Reduced Carbon Media

Medium	*Carbon content (gm/l)*	*Oxytetracycline yield (μg/ml)*
A	23.24	805
B	7.49	735
C	7.28	630

Table II presents representative data on the efficiencies of some of the materials as precursors for radioactive oxytetracycline. All materials were added to the fermentation at about 50 hours at an equivalent total activity (20 μc/5 ml fermentation).

Table II. Average Precursor Efficiencies

Precursor	*Spec. act. of carbon of precursor* (μc/mM)*	*Spec. act. of oxytetracycline carbon (μc/mM)*
Sodium acetate 2-C^{14}	500	1.64
	500	2.21
Canna leaf residue (uniformly labelled)	100	1.23
Sodium bicarbonate	2000	1.26
Glucose (uniformly labelled)	167	2.56

* Average initial specific activity of medium carbon was equal to or greater than 6.9 μc/mM.

Table III. Effect of Time of Addition on Acetate Utilization

Time of addition (hr)	*Age of fermentation (hr)*	*Specific activity of oxytetracycline (μc/mM)*	*Efficiency of conversion* (%)*
27	50	79	2.0
	72	190	5.0
	96	73	1.9
	120	120	3.5
43	50	48	1.4
	72	69	2.4
	96	100	3.2
	120	160	5.7

* Total per cent of added C^{14} incorporated into oxytetracycline.

Table IV. Radioactive Oxytetracycline HCl

Properties:

UV absorption, biopotency, paper chromatographic characteristics identical with the non-radioactive compound.

Radiochemical Purification:

Preparation	*Specific activity (μc/mM)*
Crude base	24.3
Crystalline HCl	4.86†
Crystalline base I*	4.82
Crystalline base II	4.73

* Crystalline base preparations I and II were derived from the HCl by dilution 145 × with non-radioactive carrier followed by recrystallization from toluene. Activities are corrected to the original basis.

† This material has one-tenth the specific activity of the oxytetracycline formed in the radioactive fermentation.

At an early stage of the investigation, 2-C^{14} acetate was selected as a potentially usefnl precursor. Evaluation of the precursor was made in terms of the effect of time of addition on the efficiency of utilization for synthesis of oxytetracycline. Data from such experiments are shown in Table III. From these data, the importance of time of addition of precursor emerges. In general, if the precursor were added too early, efficiency of utilization in oxytetracycline was much lower than the best efficiency attained ($\sim 6\%$).

Equipped with such information as appears in Table III, fermentations were set up from which to actually isolate oxytetracycline in pure form. Fermentation procedure is summarized in the Methods section. For this particular fermentation, methyl-labelled acetate (20 μc/5 ml) was added at 50 hr. The flasks were removed for recovery at 96 hours. The general procedure by which these isolations were made has been described.[10] Figure 1 summarizes the

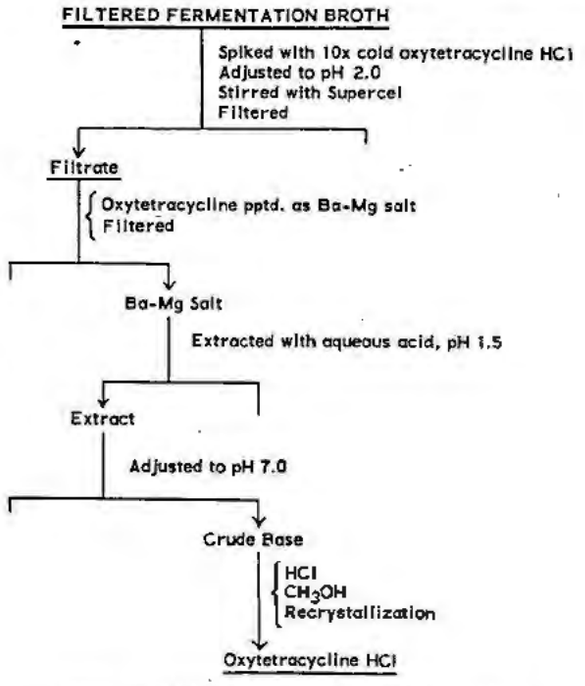

Figure 1. Isolation of radioactive oxytetracycline HCl

method as used in these studies. Table IV summarizes the properties of the crystalline oxytetracycline HCl obtained. Approximately 20 milligrams of this radioactive product were combined with 1.900 grams of carrier oxytetracycline and subjected to alkaline degradation by Fig. 2[11,12] for the purpose of determining roughly the distribution of the label in the molecule. Table V includes the data obtained on the specific activities of the isolated and purified degradation products. Examination of the data indicates a rather generally labelled molecule.

Table V. Specific Activities of Oxytetracycline and Its Alkaline Degradation Products

Compound	*Specific activity (dpm/μM C)*
Oxytetracycline	3.32 ± 0.03
Terracinoic acid	4.00 ± 0.04
Succinic acid	3.78 ± 0.04
Succinic anhydride	3.79 ± 0.04
7-Hydroxy 3-methyl phtalide	3.80 ± 0.20
Dimethylamine HCl	0.668 ± 0.007

DISCUSSION

The carbon content listed for medium *C* (Table I) represents the minimum concentration of carbon in the basal medium that could be achieved without seriously inhibiting oxytetracycline yield. The yields

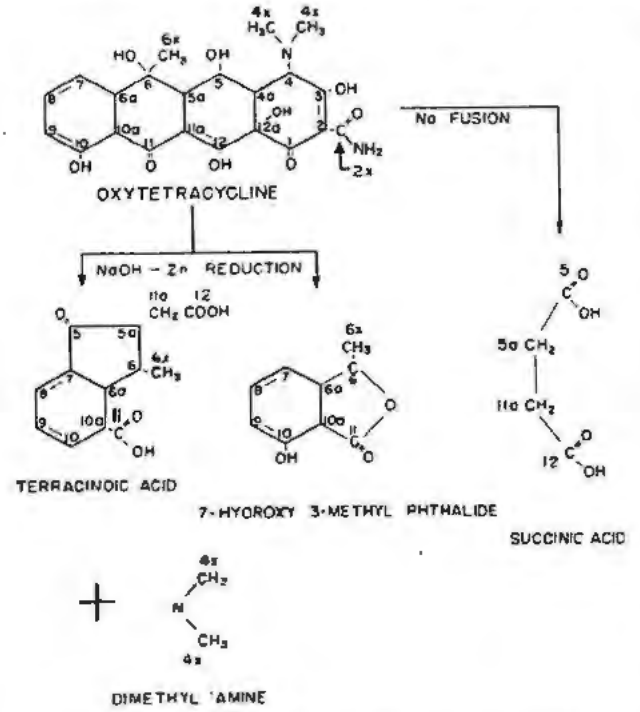

Figure 2. Alkaline degradation of radioactive oxytetracycline

obtained on further sacrifice of carbon content were so small as to preclude isolation of the radiooxytetracycline without the use of large amounts of nonradioactive carrier.

The various precursors listed in Table II were not tried at uniform specific activity. In general, however, judging from the data shown in the Table, the carbon of none of the oxytetracycline preparations

Figure 3. Methane gas flow counter for scanning paper chromatograms

achieved an average specific activity greater than the average specific activity of the medium carbon.

It is apparent that carbon from all of these materials finds its way into oxytetracycline. Canna leaf and other natural residues from radiochemical isolations were tried in hope of obtaining clues to the presence of more highly specific precursors. Glucose and acetate were the best materials screened, and the latter was chosen for further work. Very recent work indicates that acetate will enter oxytetracycline at a higher specific activity than the average specific activity of the medium.

The data shown in Table II do not delineate the possible effect (for any given precursor) of specific activity on the incorporation of radioactivity into oxytetracycline. Experiments have indicated that, for acetate, there is a significant advantage to be gained by using high specific activity material.

Table III summarizes some of the data on the rate of entry of the acetate methyl group into oxytetracycline. These data indicate, as one might expect, that the early addition of acetate gives a product of somewhat higher specific activity during the early hours of the fermentation. However, efficiency of utilization of the precursor was not so great as at later harvest (120 hours), with later precursor addition (43 hours). The later addition also allowed better predictability in oxytetracycline specific activity, since this quantity increased linearly with time over the period from 50 to 120 hours. It is interesting to note the rapid rate at which acetate methyl carbon enters oxytetracycline; for example, when acetate was added at 43 hours, oxytetracycline

achieved about 30% of its maximum specific activity in about 7 hours of fermentation. Furthermore, during the period from 50 to 120 hours, during which specific activity of oxytetracycline increased threefold, net oxytetracycline yield increased only about 25%. It is apparent from this data that a state of dynamic equilibrium existed whereby radioactivity entered oxytetracycline without net increase in yield. These, and additional data will be considered in a separate publication devoted to mechanism of synthesis of oxytetracycline.

The preparation whose properties are summarized in Table IV was obtained for the purpose of studying the extent of C^{14} label in the oxytetracycline molecule. Conditions were chosen for the fermentation synthesis of this material which would be expected (from Table III) to form oxytetracycline of from 50–100 microcuries/millimole (μc/mM). The final purified preparation indicated about 48 μc/mM was obtained in this fermentation. This result was to be expected, since the acetate was added at 50 hours rather than 43 hours; such variation is certainly within the normal range of biological variation occurring in oxytetracycline fermentation. Recrystallization, with conversion to hydrochloride and back to the free base, produced no significant change in specific activity between the crystalline preparations. The usual physical, chemical and biological criteria of purity indicated a preparation identical to standard oxytetracycline. The materials responsible for the large drop in specific activity from crude base to crystalline hydrochloride are being investigated.

A more complete study of oxytetracycline biosynthesis will be presented in conjunction with a full study of this subject. The data presented in Table V indicate the activities obtained for 5 degradation products. Four of the products account for all but seven of the carbon atoms of oxytetracycline (Fig. 2). These seven carbon atoms are those in the 1, 2, 2*x*, 3, 4, 4*a*, and 12*a* positions. From the data of Table V, a calculated average specific activity for these seven carbon atoms may be obtained. The calculation indicates about 3.0 disintegrations per minute/micromole of carbon. Hence, while all four rings contain C^{14}, relatively less appears in the right hand ring.

SUMMARY

1. Reduced carbon media have been developed suitable for production of carbon-14 labelled oxytetracycline.

2. Studies have indicated acetate as a useful precursor.

3. Studies have indicated the importance of time of addition in determining efficiency of acetate utilization for oxytetracycline synthesis.

4. Radioactive oxytetracycline was isolated by the carrier technique; it was highly purified to constant specific activity and specific activity of five of its degradation products were determined. From these studies it was concluded that rather general labelling occurred.

ACKNOWLEDGEMENTS

The authors would like to express their deep appreciation to Mr. E. L. Allen, Mrs. Dorothy Sypowicz, Mrs. Dina Dobrinsky, Miss Marguerite Burke, Miss Jeannine Salvo, and Mrs. R. Paradies for their excellent technical assistance. The encouragement of Dr. Jasper H. Kane, Dr. E. M. Weber, Dr. K. J. Brunings, and Mr. A. C. Finlay is gratefully acknowledged.

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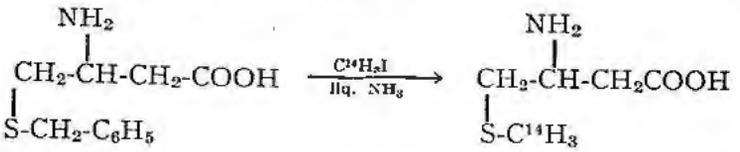
The Synthesis of β-Amino-γ-Methyl-C^{14}-Thiobutyric Acid (β-Methionine-Methyl-C^{14})

By D. Keglević-Brevet,* Yugoslavia

In order to investigate the biological behaviour of β-amino acids, β-amino-γ-methyl-C^{14}-thiobutyric acid (β-methionine-methyl-C^{14}) [II] was synthesized. The methylation of the already described[1] β-amino-γ-benzyl-thiobutyric acid [I] in liquid ammonia gave compound [II] in a good yield:



[I] [II]

The way of preparation was essentially the same as the standard method for the synthesis of α-methionine-methyl-C^{14}, described by Melville, Rachele and Keller.[2]

Biological results will be published elsewhere.

RADIOACTIVITY DETERMINATION

The measurements were carried out on samples of "infinite thickness", mounted on 1 cm^2 "Polythene" discs, as described by Popjak.[3] An argon-heptane filled Geiger-Müller counter having a thin mica window was used.

Specific activities were obtained by comparing the counts of the samples with that of a standardised C^{14}-labelled polymethyl methylacrylate disc of the same size.

PREPARATION OF β-AMINO-γ-METHYL-C^{14}-THIOBUTYRIC ACID

The apparatus used for the preparation has been shown in Fig. 1. 200 mg (0.89 mmole) of β-amino-γ-benzylthiobutyric acid (mp 194–196°) were placed in flask *A*, cooled in a dry ice-acetone mixture, and dry anhydrous ammonia was introduced by distillation through the main arm. A soda lime tube was placed on the side arm of flask *A*. When about 20 ml of ammonia were collected, flask *A* was connected to the apparatus, stopcock *B* closed and dry nitrogen bubbled through the mixture. The minimum amount of sodium wire necessary to produce a permanent blue colour was gradually added through the side arm of flask *A*. Meanwhile vessel *D* was put in liquid air and methyl iodide C^{14} obtained from the Radiochemical Centre, Amersham (2.6 mg—100 μc), plus the carrier CH_3I (126 mg—0.055 ml—0.89 mmole) frozen in it. When the mixture in flask *A* had been dissolved, the stopcock *B* was opened and the three-way stopcock *C* converted to *D*. The break-seal tube was broken by a magnetic hammer and vessel *D* warmed slowly to 70°. In two hours all the methyl iodide evaporated into flask *A*. Flask *A* was then raised from the dry ice-acetone mixture, and the ammonia evaporated in the current of nitrogen.

The white solid residue was dissolved in about 8 ml of water and a drop of hydrobromic acid added to make the solution acid to litmus but alkaline to Congo red. After the filtration, the solution was concentrated to about 0.5 cm^3, warmed, and absolute ethanol added. After standing overnight in the refrigerator, the white crystals of β-amino-γ-methyl-C^{14}-thiobutyric acid were separated by centrifugation, washed with ether and dried. The yield was 93 mg (70%), mp 185–7°. The substance proved to be identical with the radioinactive compound which had been previously prepared by the same method by Balenović and Fleš.[4] After two recrystallizations from ethanol, the radioactivity of the sample remained unchanged giving a specific activity of 0.71 μc/mg. Radiochemical yield was 60%.

In one of our experiments carried out on a larger scale (5 mmole) we tried to purify the condensation product by adsorption on Amberlite IR-100. After the halogen ions were washed out with water, the

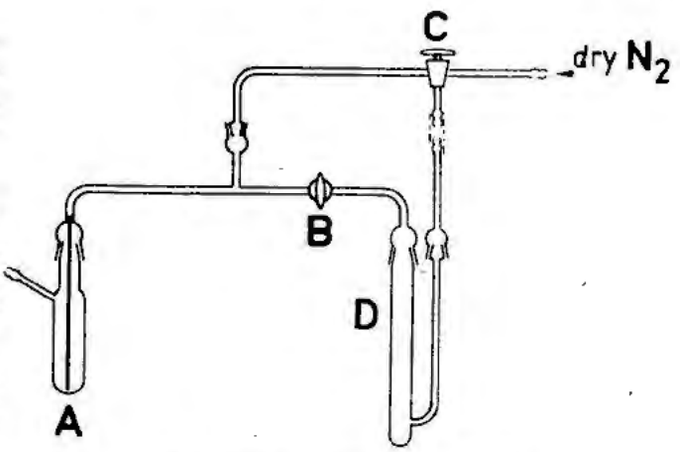


Figure 1. Apparatus used. (Description in text)

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β-amino-γ-methyl-C^{14}-thiobutyric acid was eluted from the column with a 1% pyridine solution. After the fractions had been evaporated, the β-amino-γ-methyl-C^{14}-thiobutyric acid was obtained, having the same melting point as described above.

SUMMARY

The methylation of the already described[1] β-amino-γ-benzyl-thiobutyric acid, by the $C^{14}H_3I$ in liquid ammonia gave in a good yield the β-amino-γ-methyl-C^{14}-thiobutyric acid (β-methionine-methyl-C^{14}). Some modifications of the standard method[2] for the synthesis of α-methionine-methyl-C^{14} were performed.

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The Application of Radioactive Phosphorus in Investigating the Processes of Phosphorylation

By S. E. Severin, USSR

The processes of metabolism, which lead to the generation of energy-rich (macroergic) phosphorus compounds, are of great significance. Functioning of organs and tissues, synthesis of albumen and nucleic acids, forming of intermediate metabolic products with special properties, detoxication, synthesis, and many other processes characteristic of the vital activity of the organism can take place only when living cells contain energy-rich compounds and, in particular, adenosine triphosphoric acid (ATP). Adenosine triphosphoric acid is produced either through anaerobic degradation of carbohydrates—glycolysis, a process rather ineffective as far as energy is concerned—or through the process of respiration, since the phosphorylation is connected with respiration.

The use of radioactive phosphorus, P^{32}, in the investigations of glycolytic and respiratory phosphorylation turned out to be of decisive importance. This method made it possible to follow all the movements of the phosphorus residue as it is transfered from one compound to another, split away from organic compounds in the form of mineral phosphate, and esterified again and returned into organic compounds.

The main object of this report was to give some examples of studies recently carried out in the USSR, which show the effectiveness of the application of radioactive phosphorus isotopes together with conventional, analytical methods of research, in determining the peculiarities of phosphorus metabolism in various cells, organs and tissnes both under normal conditions and in certain pathological states.

RED CORPUSCLES OF BLOOD

Relatively weak respiration and intensive anaerobic, glycolytic degradation of carbohydrates represent a characteristic property of erythrocytes of many mammals.

Human erythrocytes are practically devoid of respiration. It is glycolysis that ensures the formation of macroergic phosphorus compounds in red corpuscles lacking nuclei. The former are required to preserve the structure of these cells, and, consequently, to maintain them as oxygen carriers.

The process of carbohydrate utilization by erythrocytes has some peculiarities. The formation and high concentration of 2,3-diphosphoglyceric acid (2,3-DPGA), a substance found in other cells and tissues of the organism only in the form of traces, is one of these peculiarities.

The research, conducted by G. E. Vladimirov, I. P. Ashmarin and A. P. Urinson,[1] who used radioactive phosphorus, revealed that in 2,3-diphosphoglyceric acid under defibrinated blood incubation occurs an intensive metabolism of the phosphorus residue.

The rate of this metabolism in erythrocytes of different living beings varies greatly. The slowest metabolism of this kind is found in the red blood cells of swine (Fig. 1).

In human erythrocytes phosphorus of diphosphoglyceric acid is renewed with rather great speed, though much slower than phosphorus of adenosine trophosphoric acid (Fig. 2).

The data ohtained show convincingly that diphosphoglyceric acid actively participates in phosphorus metabolism of erythrocytes. These results, however, cannot lead at present to a final conclusion on the mechanism of formation of this compound. Considerably greater speed of renewal of the phosphate residue in adenosine triphosphoric acid than that in diphosphoglyceric acid confirmed the previously stated opinion that the transfer of phosphate groups from adenosine triphosphotie acid into diphosphoglyceric acid takes place in human erythrocytes and their hemolysates. This problem was specially investigated[2] with the use of adenosine triphosphoric acid containing P^{32}. When the acid was added to hemolysates of human erythrocytes, a substantial amount of P^{32} was found in diphosphoglyceric acid. This indicates that adenosine triphosphoric acid can transfer its labile phosphate groups to 2,3-diphosphoglyceric acid. Similar conclusions were reached by Rapoport and Luebering.[3] These authors treated the process of the formation of 2,3-diphosphoglyceric acid as the result of a two-phase reaction:

3-phosphoglyceric acid + adenosine triphosphoric acid $\rightleftarrows$ 1,3-diphosphoglyceric acid + adenosine diphosphoric acid (ADP) (1)

1,3-diphosphoglyceric acid $\overset{\text{mutase of diphosphoglyceric acid}}{\rightleftarrows}$ 2,3-diphosphoglyceric acid (2)

However, the pathways of formation of 2,3-diphosphoglyceric acid in erythrocytes, described above,

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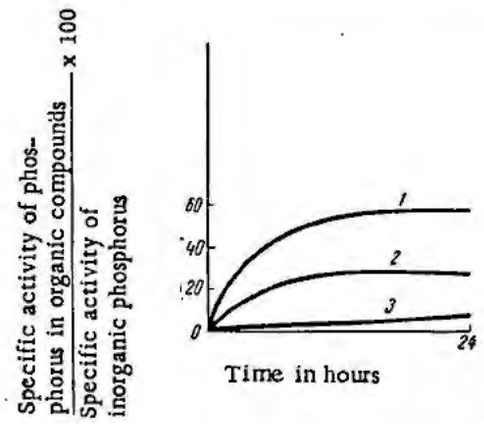

Figure 1. The rate of incorporation of labeled phosphorus into adenosine triphosphoric acid (curve 1), hexosephosphates (curve 2), and diphosphoglyceric acid (curve 3) of swine erythrocytes

could be hardly considered as the only possible, or even as the principle ones. The discovery of diphosphoglyceric acid mutase in erythrocytes makes it more probable that 1,3-diphosphoglyceric acid, formed in glycolytic oxido-reduction, is directly converted into an appropriate 2,3-derivative without participation of the adenylic system.

Cherniak[4] experimentally verified this assumption in her research on dialyzed hemolysates of human erythrocytes, with fructose diphosphate as a substrate. The adding of cozymase resulted in the most pronounced incorporation of labeled phosphate into phosphoglyceric acid fraction, whereas added adenosine triphosphoric acid not only did not increase but on the contrary, decreased the scope of synthesis (Table I). This could be explained by competitive reaction between diphosphoglyceric acid mutase (the transfer of the phosphate residue from 1,3-diphosphoglyceric acid in position "1" to position "2") and phosphokinase reaction (the transfer of the residue from 1,3-diphosphoglyceric acid to the adenylic system).

Employing phosphoglyceric acid as a substrate instead of fructose diphosphate we observed a slight increase in radioactive phosphorus only in the sample

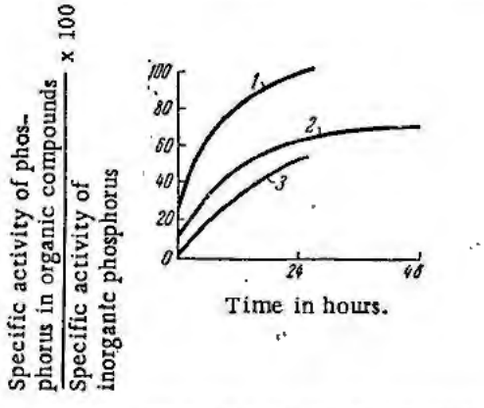

Figure 2. The rate of incorporation of labeled phosphate into adenosine triphospheric acid (curve 1), hexosephosphates (curve 2), and diphosphoglyceric acid (curve 3) of human erythrocytes

Table I. Radioactivity of the Fraction of Phosphoglyceric Acids

	cpm/ml of initial hemolysate	
	Before incubation	After four-hour incubation
Hemolysate + cozymase (DPN)	23	1170
Hemolysate + ATP	6	365
Hemolysate	0	520

Table II

	cpm/ml of initial hemolysate	
	Before incubation	After four-hour incubation
Hemolysate + DPN	0	15
Hemolysate + ATP	3	114
Hemolysate	3	13

to which adenosine triphosphoric acid was added (Table II).

Hence, we may conclude that phosphorylation of 3-phosphoglyceric acid at the expense of labile phosphate group in adenosine triphosphoric acid, leads to the formation of only insignificant amounts of 2,3-diphosphoglyceric acid. And the principle pathway of formation of this substance consists in the conversion of 1,3-diphosphoglyceric acid into 2,3-diphosphoglyceric acid with the participation of mutase. These conclusions were confirmed in Rapoport's recently published paper,[5] in which the author presented the main pathways of phosphoglyceric acid transformation in Fig. 3.

As can be seen, this diagram does not show the formation of 2,3-diphosphoglyceric acid by transfer of the phosphate residue from adenosine triphosphoric acid to 3-phosphoglyceric acid.

Summing up, we may say that the use of the radioactive phosphorus with the conventional methods of biochemical research widened our conception of the characteristic features of carbohydrate metabolism in human erythrocytes. And therefore we were able to find new methods of approach to the problem of blood storage, in particular, to the problem of prolonging storage. This prolongation can be achieved by introducing compounds which prevent the destruction of active enzymes of the glycolytic complex.

OXIDATIVE AND PHOSPHO-CARBOHYDRATE METABOLISM UNDER CONDITION OF EXPERIMENTAL TUBERCULOSIS

The application of radioactive phosphorus in combination with conventional biochemical methods of research for investigating metabolism in the liver under normal conditions and in certain pathological states, proved to be very useful. It is known that the liver plays a very important part in the processes of metabolism, in the synthesis of blood albumens, in the interconversion of some amino acids, in the oxidative degradation and synthesis of high-molecular fatty acids, in the formation of final products of

nitrogen metabolism and so on. All these processes and many other biochemical transformations can occur only if the tissue of the liver possesses sufficient amount of adenosine triphosphoric acid, and if the formation of this acid through glycolytic and especially respiratory phosphorylation proceeds without interruption. The uncoupling of oxidation from phosphorylation underlies, apparently, many pathological states, resulting from the metabolic disturbance.

In the course of studies of the metabolism of parenchymatous organs of guinea pigs under conditions of experimental tuberculosis, a sharp increase in the accumulation of "not fully oxidized" products was noted, though oxygen consumption by the tissues was normal, if not even slightly higher than normal.[6] It was also very characteristic that the oxidation of pyruvic acid, which was added to a sample of minced kiducy cortical layer was seriously disturbed. This

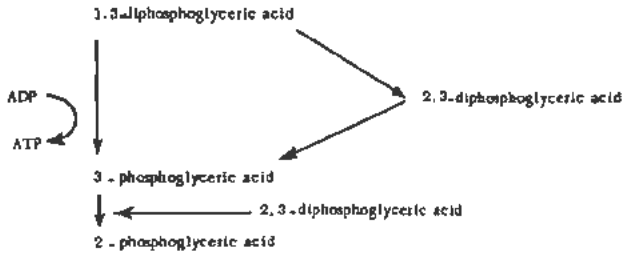

Figure 3. Phosphoglyceric acid transformation

could be considered as the result of qualitative changes of oxidative processes, occurring under conditions of experimental tuberculosis, which are connected with the disturbance of oxidative phosphorylation but do not affect the amount of oxygen consumed by the cells. Special experiments showed that the binding of mineral phosphate in the minced liver of aoimals affected by tuberculosis, under incubation in oxygen was greatly decreased. Findings favouring this point of view were obtained in experiments in which the isotopic labeled phosphates were administered to guinea pigs, infected by tuberculosis. Radioactive phosphate was injected at different time intervals, usually one or two months after the infection, and in quantities corresponding to 3000–3500 counts/gm of the animal's weight. At different time intervals (from 1 hour to 72 hours) after the administration of radioactive phosphate the animals were killed and their livers examined.

It was established that the correlation between mineral phosphate and phosphorus of organic compounds in the livers of infected and normal animals differed radically. The livers of the tuberculosis-infected animals contained almost twice as much mineral phosphate and half as much organic phosphorus compounds, especially fractions of adenosine triphosphoric acid, as those of healthy animals. These correlations are shown on Fig. 4. The curves, indicating time changes of specific activity of the phosphorus fractions, tell of considerable disturbance of metabolism (Fig. 5). These results apparently can be explained by the relatively small amount of acid soluble organic compounds in the liver of tuberculosis-infected guinea pigs, by relatively quick incorporation of radioactive phosphate into these compounds, and by sharply slackened further transformation, in particular, due to a low activity of phosphates.

Thus, the increase of the amount of mineral phosphate and a considerable decrease of acid soluble organic phosphorus compounds, especially adenosine triphosphoric acid, are considered as characteristic changes in the composition of the liver under conditions of experimental tuberculosis. These changes may be estimated as the result of, firstly, impaired oxidative and anaerobic phosphorylation, and, secondly, sharply reduced activities of phosphates, unable to ensure a normal pathway of further conversion of carbohydrate phosphoric esters, typical for the liver tissue.

In cases of intensively developed tuberculosis, morphological changes of the liver were also observed. Such morphological changes alone do not necessarily bring about the disturbance of meta-

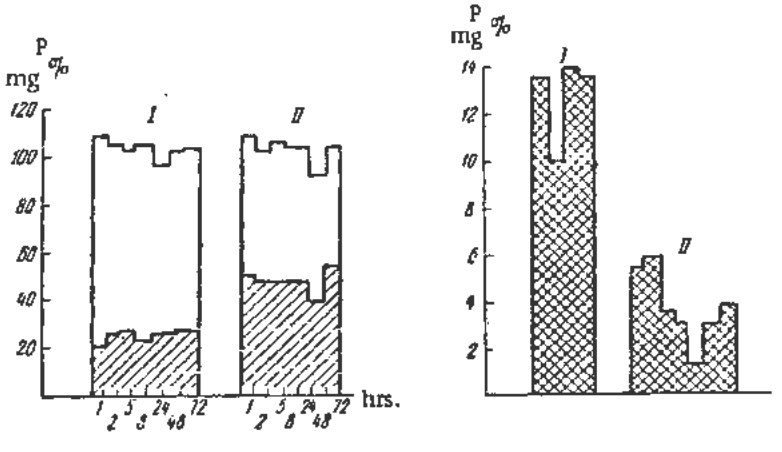

Figure 4. Amount of phosphorus in guinea pig liver. I, under normal conditions; II, under conditions of experimental tuberculosis. Unshaded, acid-soluble organic compounds; shaded, mineral phosphorus; cross-hatched, adenosine phosphoric acid

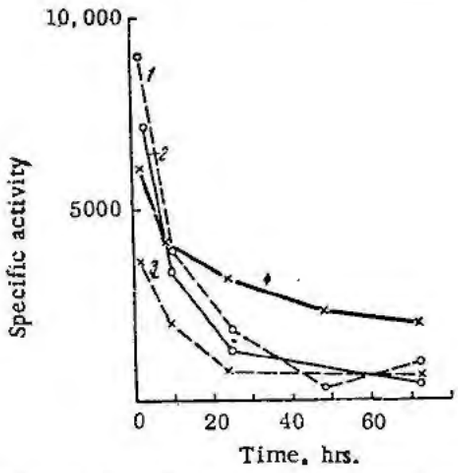

Figure 5. Changes in specific activity of phosphorus in guinea pig liver. Curves 3 and 4, phosphorus in organic compounds; curves 1 and 2, mineral phosphorus. Curves 1 and 3, under normal conditions; curves 2 and 4, under experimental tuberculosis.

bolism. This is proved by the following fact: the infiltration of fats and respective degeneration of the liver, caused by repeated injections of carbon tetrachloride did not give rise to any characteristic changes in the specific activity not only of mineral phosphate, but also of organic phosphorus compounds. These results can lead to the assumption that the disturbance of liver metabolism under conditions of experimental tuberculosis precedes morphological changes in this organ and depends chiefly on the scope of tuberculosis intoxication. The experiments, in which no morphological changes were observed, although the disturbance of oxidative phosphorylation was quite distinct, confirm also this assumption. On the basis of the above data, we may assume that the increasing intoxication of the liver affected by tuberculosis results in a gradual disturbance of the least stable enzyme system, namely, that system which ensures oxidative phosphorylation. The amount of adenosine triphosphoric acid in the liver decreases and appears to be insufficient to provide with energy the highly varied processes occurring in this organ.

PHOSPHORUS COMPOUNDS OF THE HEART MUSCLE UNDER CONDITIONS OF STIMULATION OF THE ACCELERATOR NERVE

The heart muscle is a tissue characterized by intensive oxidative processes and by intensive phosphorylation coupled with them. Various disturbances of metabolism, such as those caused by hyperthyroidism or by general starvation do not affect normal processes in the tissue of the heart muscle when the uncoupling of respiration from phosphorylation takes place, for instance in the liver. Even diphtherial intoxication, which causes certain morphological changes in the myocardium did not disturb the processes of oxidation and phosphorylation in the minced heart ventricular tissue. A series of investigations was carried out by Raiskina in order to determine the rate of renewal of macroergic phosphorus compounds of phosphocreatine and adenosine triphosphoric acid in the heart muscle under normal physiological conditions and also under conditions of stimulating the "accelerator" nerve, which increases the amplitude of systoles and raises blood pressure. The experiments were performed on dogs. It was found that even within the first minutes after the injection of P^{32} into the blood there took place a rapid penetration of the substance into the heart muscle and subsequent incorporation into macroergic phosphorus compounds, first of all, into adenosine triphosphoric acid, and then into phosphocreatine (Fig. 6).

The experiments with the stimulation of Pavlov's accelerator nerve were carried out as stated above, only the apex of the heart was removed within 15 minutes after the injection of P^{32} into the blood. The amount of mineral phosphate, phosphocreatine and adenosine triphosphoric acid in the heart muscle practically remained unchanged. The specific activity of each of these fractions, however, appeared to be considerably higher than that in control tests. The data obtained indicate that, if the accelerator nerve is stimulated, the amplitude of systoles increases (the rhythm does not change) and the metabolism of phosphorus compound is considerably accelerated. The analysis was made at the stage which is characterized by the growth of specific activity of phosphocreatine, by sufficient stability of the specific activity of adenosine triphosphoric acid, and by sharp decline in the specific activity of mineral phosphate, (Fig. 6).

The mechanism of the above-mentioned changes in the metabolism of macroergic phosphorus compounds of the heart muscle is still obscure. However, the fact that the stimulation of the accelerator nerve considerably accelerates incorporation of injected mineral phosphate into energy-rich phosphorus compounds of the heart muscle attracts our attention and requires further investigation. These investigations

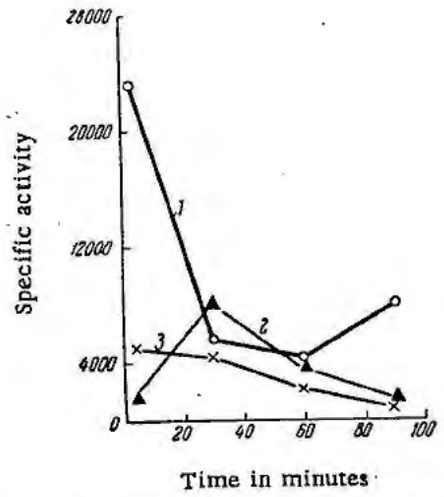

Figure 6. Changes in phosphorus specific activity of mineral phosphorus (curve 1), of phosphocreatine (curve 2), and of adenosine triphosphoric acid in the heart muscle (curve 3)

must throw more light on the peculiarities of metabolism in the heart muscle (respiration, phosphorylation quotient) while the accelerator nerve is stimulated.

The work must be carried out under the normal conditions of biochemical experiments and it must supply the material lacking before a complete analysis of the phenomena described is possible.

Only the combining of conventional methods of biochemical research, employed for the determination of metabolic peculiarities, with methods based on the application of radioactive and heavy isotopes, can ensure fruitful solution of the problems pertaining to dynamic and functional biochemistry in normal and pathological conditions.

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The Measurement of Phosphorylating Oxidation by Means of Radioactive Phosphorus in the Liver Cell

By J. Frei, A. Vannotti and P. Lerch,* Switzerland

In 1953, Krebs *et al.*[1] developed a most elegant technique, making use of inorganic phosphate tagged with P^{32}, for the determination of the phosphorylating oxidation quotient, namely, the phosphorylating power of an animal tissue homogenate. This method, based on the dynamic equilibrium between adenosine triphosphate (ATP) and inorganic phosphate, actually uses the competitive action of the phosphorylating oxidation, on the one hand, and that of the phosphatases, on the other, according to the following scheme:

$$\text{ADP} + \text{P inorg.} \underset{\text{"ATP'ase"}}{\overset{\text{Ox} \quad \text{Ph}}{\rightleftharpoons}} \text{ATP}$$

This dynamic equilibrium is achieved by incubating inorganic and labile phosphates at 20° and at a pH of 6.8–6.9 in Warburg flasks, in the form of ATP (and ADP), in the presence of an oxidation substrate (citrate, α-ketoglutarate, succinate, etc.) and a homogenate of an animal tissue (liver, kidney, muscle, etc.) in a saline medium, according to Krebs.[1] If P^{32} tagged inorganic phosphate is added to the inorganic phosphate, the ATP rapidly becomes radioactive. Using a method which permits the determination of P^{32} in the ATP, it becomes possible to follow the incorporation of the isotope into the adenosine triphosphate. In order to determine the incorporation of P^{32} into ATP, it first was necessary to separate the latter from inorganic phosphate. For this purpose, several fractional precipitation methods, such as the one used by Hersey and Samuel,[2] have been described. These methods are fairly long of execution and of relatively low specificity, since the radioactive precipitate nearly always is contaminated by the supernatant, which also is radioactive; hence the need for washing the precipitate, with attendant losses. Far superior is separation by paper chromatography, which permits very accurate differentiation between the various constituents. Eggleston and Hems[3] have developed a technique inspired by that of Hanes and Isherwood,[4] which makes possible the very accurate separation of inorganic phosphate from the adenylic acids in a single operation on Whatman 1 paper. In a second chromatography on the same strip, using a different solvent, the adenylic acids can be resolved into 5-phosphate-adenylic acid and the di- and triphosphate of adenosine. Since only the incorporation of P^{32} in the adenylic system is of interest to us, we limited ourselves to the first chromatography. In order to determine the amount of P^{32} in the two fractions, Krebs[1] carried out elution and determined the activity in these solutions, by means of a β-counter tube (liquid type) of Veall. This method, in spite of its great accuracy, was not suitable for us, for continued research and semi-routine dosages.

We preferred to measure radioactivity directly on the chromatograms by passing these automatically under a Geiger counter connected to a Tracerlab precision ratemeter and to a recording Trub and Tauber milliammeter. The chromatogram is automatically driven by friction by means of a drum which is on the extension of the drive shaft of the recorder. Since the diameter of the drum which pulls the chromatographic paper is the same as that which causes progress of the recording paper, the linear speeds are the same. By suitable positioning we can superimpose the chromatogram on the recorded curves.

Figures 1 and 2 show, respectively, the general appearance of the device used and some details. They give curves such as those represented on Fig. 3. Planimetry of the various surfaces gives us the distribution of P^{32} in the several phosphates.

We were able, in developing this equipment, to profit by the experience of one of us[5] who already had built a similar system for the measurement of the radioactivity of the substances separated by chromatography or by paper electrophoresis.

We were able to show, in this fashion[6,7], that the phosphorylating power of the liver homogenates taken from rats suffering from beri-beri was markedly reduced. Figure 4 strikingly illustrates the results achieved, which are statistically guaranteed with a safety margin of 99% even with only three minutes of incubation.

We have used α-ketoglutarate here as an oxiding substrate and we can see that, after only 10 minutes of incubation, the dynamic equilibrium is broken in favor of the ATP'ase of the liver homogenates of the vitamin deficient rats. After 50 minutes of incubation, no ATP is left in the deficient system.

Original language: French.

* Medical Clinic, Lausanne; Director: Professor A. Vannotti.

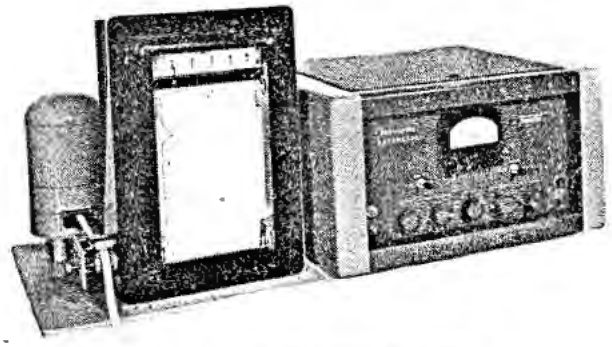

Figure 1. Ratemeter and recording milliammeter

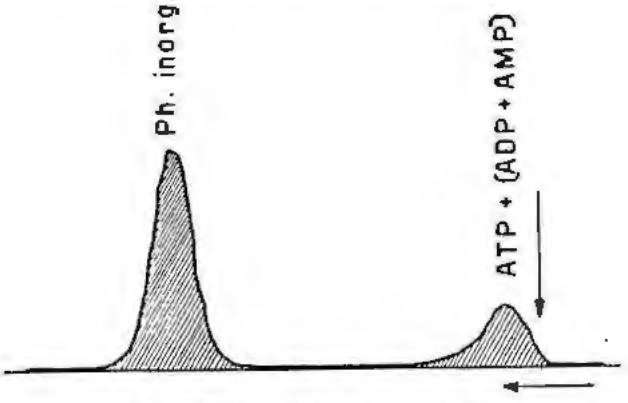

Figure 3. Typical record from apparatus of Fig. 1

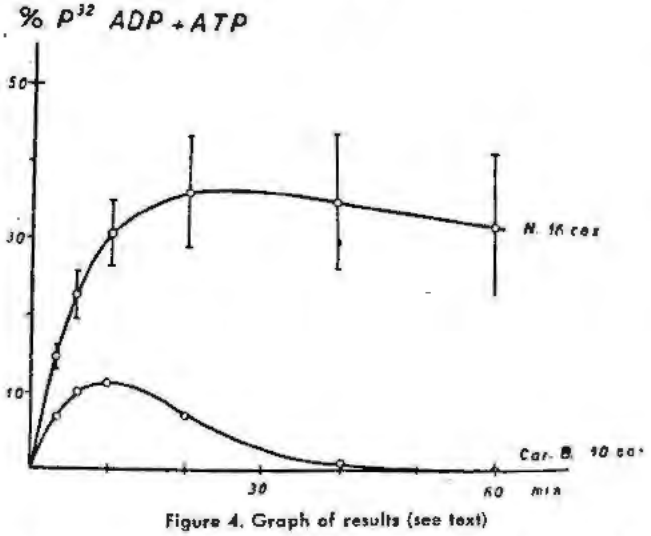

Figure 4. Graph of results (see text)

We obtained very similar results by replacing the α-ketoglutarate, either by citrate, or by succinate. By determining the respiration of the homogenates and the level of the inorganic and labile phosphates, it is possible to establish the phosphorylating oxidation quotient (P/O) by means of these measurements, of the radioactivity of the various chromatographic fractions, using a formula indicated by Krebs[1] and Stewart.[8]

The determinations just mentioned are very important in clinical biochemistry, for the energy produced by the catabolism of the food not degraded into heat is captured by the adenylic system at the level of the "cyclophorase", where it is then transformed into pyrophosphate links of adenosine triphosphate, which are rich in free energy (12–14 kcal/mol of ATP). This energy then is used for anabolic reactions, i.e., for most of the syntheses which take place in the system.

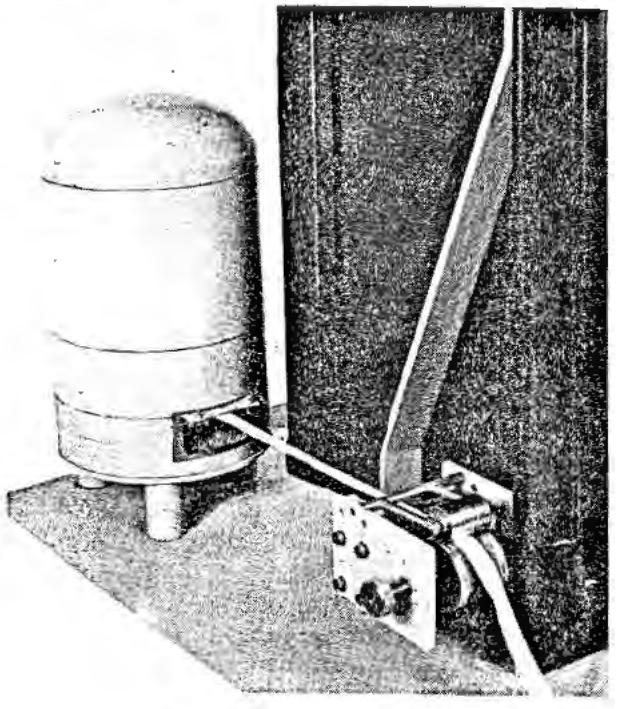

Figure 2. Chromatographic paper

ACKNOWLEDGEMENTS

We wish to extend our warm thanks to the National Fund for Scientific Research, which subsidized this work, as well as to the house of Hoffman-LaRoche, of Bâle, which was kind enough to make available the normal and vitamin deficient animals.

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The Study of Certain Phases of Cell Dynamic States with Short-Lived Isotopes as Exemplified by Mn^{56} Partition Studies in Organs and Intracellular Organelles

By G. C. Cotzias and L. S. Maynard,* USA

Our studies of the kinetics of manganese-56 led us to postulate the existence in the body of a mechanism that quickly diverts the metal in transit, entraps it in a site of high affinity, and then releases it gradually. Such a postulate would explain the discrepant reports by earlier workers who had to use larger than physiological amounts of manganese, under conditions which did not permit them to differentiate between primary partition and secondary redistribution.[1,2,3,4]

The short half-life of Mn^{56} permits easy preparation of the isotope in very high specific activity; thus readily measurable concentrations are found in various animal tissues, after administration of Mn^{56} in amounts well within the physiological limits. Even more important is the fact that the short half-life of this isotope permitted us to focus our observations on the early phases of the partition process when reabsorption, recirculation and redistribution were at a minimum.

From our partition studies in various organs, a correlation appeared between the richness in mitochondria of a given organ and its concentration of manganous salts. The intracellular organelles were studied, therefore, to ascertain whether they constitute a site of entrapment of the metal in transit.

It was considered of additional interest to discover means by which the distribution of the metal in the body could be predictably altered, so that its accumulation in some organs or that its stay in the blood could be eventually controlled at will.

EXPERIMENTAL

Preparation of Mn^{56}

Manganous sulfate powder was bombarded by thermal neutrons in the Brookhaven reactor to yield 2.5 microcuries per microgram Mn^{56}. (Mn^{55} $[n,\gamma]$ $Mn^{56} \rightarrow Fe^{56}$).

Partition of Mn^{56} among Organs

In one human and several rats, the element disappeared rapidly and extensively from the blood stream after intravenous injection, as illustrated in Fig. 1.†

The organ distribution of the metal was tested as follows: A series of 8 Sherman rats weighing 200 ± 30 gm were injected intraperitoneally with 100 μc (38 γ Mn^{++}) dissolved in 0.5 ml of saline. One half of the animals were sacrificed after 15 minutes and the rest after 60 minutes, by exsanguination. Selected organs were removed, weighed, and weighed portions were placed in glass counting vials. They were then counted in a well type scintillation counter (NaI(Tl) crystal) filtered to record only γ-radiation. These counts were compared with dilutions of the injected dose, after correcting for radioactive decay. The counting geometry was determined to be constant between 0 and 2 ml sample volumes. Results are tabulated in Table I.† Employing the intracardiac rather the intraperitoneal route did not seem to affect any of the results presented in this communication. Liver, pancreas and kidney showed the highest concentration of isotope, while skeletal muscle and brain were lowest. The cells of the tissues with highest isotope take-up are known to be rich in

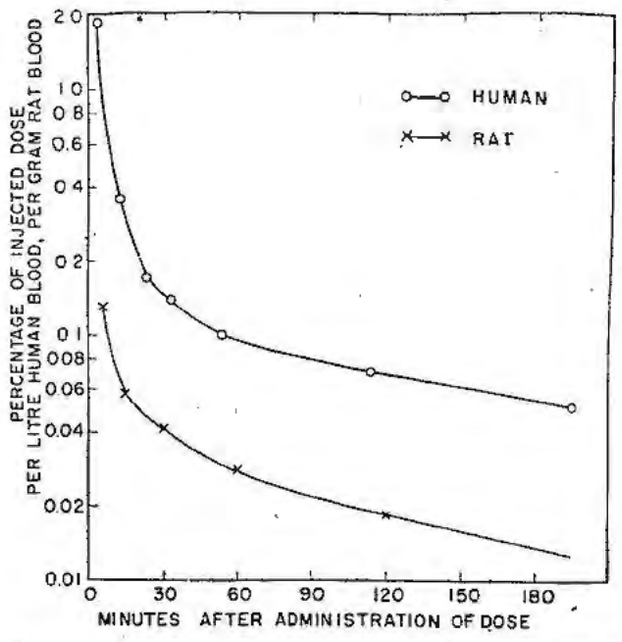

Figure 1. The rate of disappearance of Mn^{56} from the blood of human and rat

* Brookhaven National Laboratory.

† Data in Fig. 1 and in Tables I and II have been accepted for publication by the Journal of Biological Chemistry.

Table I. Uptake of Manganese-56 by Various Organs, Expressed As % of Dose per gm Tissue

Organ	Fifteen minutes after injection		One hour after injection	
	Mean %	Standard deviation	Mean %	Standard deviation
Liver	4.052	1.6691	3.059	0.708
Pancreas	3.300	0.6135	2.701	1.109
Kidney	1.231	0.3314	1.432	0.329
Thyroid	0.386	0.1711	0.395	0.138
Blood†	0.0578	0.01918	0.0278	0.0105
Heart	0.412	0.1166	0.495	0.1466
Muscle	0.062	0.0286	0.065	0.01709
Bone*	0.153	0.0338	0.222	0.0539
Brain Stem*	0.0325	0.00922	0.0457	0.00447
Brain Hemis*	0.028	0.00625	0.037	0.00283

* Difference between means is statistically significant within 5% confidence limits.
† Difference between means is statistically significant within 1% confidence limits.

mitochondria.[6,7] In order to establish whether these bodies were responsible for each organ's uptake of manganese, their *in vivo* enrichment by the isotope was studied.

Partition of Mn^{56} among Cell Organelles

Six rats, similar to the ones used previously, were injected with the same dosage of Mn^{56} and sacrificed 30 minutes later. Weighed portions of the livers were homogenized in cold 0.25 *M* sucrose and subjected to differential centrifugation.[8] Radioactivity was determined on samples of the homogenates, and of the "nuclear", mitochondrial, microsomal and supernatant fractions. Nitrogen was determined by a micro-kjeldahl procedure[9] to estimate the amount of tissue in each fraction.

The effect of repeatedly washing the particulate material (which was a necessary step in these experiments) was tested first: the liver homogenate of an injected animal was centrifuged at 80,000 g for 45 minutes and the supernatant was removed. The sediment was resuspended and recentrifuged. The supernatant solutions from repeated washings showed that an ever-increasing amount of manganese was removed (in excess of the extracted protein) by each successive wash. If the Mn^{56}: N ratio was 1.00 for the initial supernatant liquid, the succeeding washes gave 3.49, 3.79, 9.70, 7.12 and 11.51. This showed that our fractionation experiments were to yield minimal values for the organelles and maximal for the supernatant fractions. Conversely it was shown by appropriate incubation, that passive adsorption of Mn^{56} on nonmetabolizing liver mitochondria *in vitro* was negligible.

These control experiments made for considerable confidence in the data summarized in Table II.† On the basis of nitrogen content, the mitochondria contained almost twice the concentration of radiomanganese as compared to whole liver. Two thirds of the total radioactivity of the liver was found in the isolated mitochondria. All other fractions showed lower concentrations than the unfractionated homogenate. This intracellular distribution remained unaffected when rats were sacrificed 6 or 120 minutes after injection, although the total liver content of manganese was altered.

† Data in Fig. 1 and in Tables I and II have been accepted for publication by the Journal of Biological Chemistry.

Table II. Uptake of Manganese-56 by the Intracellular Components of the Fractionated Rat Liver Cell, One Half Hour after Injection

Fraction	Uptake by fractions as % of whole liver uptake*		Uptake of fractions per micromole of tissue N; expressed as whole liver activity = 1.000†	
	Mean %	Standard deviation	Mean activity	Standard deviation
Nuclei	16.43	3.57	0.949	0.164
Mitochondria	41.25	15.02	1.965	0.564
Microsomes	15.03	2.39	0.768	0.138
Supernatant	28.92	6.55	0.543	0.155

Mean liver uptake one half hour after injection = 3.237% of injected dose per gram liver—standard deviation = 1.141
Mean total liver uptake one half hour after injection = 28.102% of injected dose standard deviation = 5.523.
Mean blood uptake one half hour after injection = 0.041% of injected dose per gram blood—standard deviation = 0.0148
* F ratio = 10.49; $d^1 = 3$, $d^2 = 20$. 1% value is 4.94.
† F ratio = 20.24; $d^1 = 3$, $d^2 = 20$. 1% value is 4.94.

Means of Altering Mn^{56} Partition in the Body

In these preliminary experiments on mice, the possibility of increasing or decreasing at will the isotope uptake by the liver was explored. Prior to injection, Mn^{56} was made up to $4 \times 10^{-3}M$ by addition of $1 \times 10^{-2}M$ solution of various agents, known to possess variable chelating strength towards this metal.[10] The bile solution was a 1 : 500 dilution of dog bile. After bringing them to neutral pH, 50 μc in 0.5 ml of the solutions were injected intraperitoneally into mice weighing about 30 gm. Thirty minutes later the animals were exsanguinated and weighed samples of blood and liver were obtained and analyzed for radioactivity as outlined above. The percentage of the total dose taken up per gram of these tissues was calculated. The ligands used to chelate the manganese are shown in Table III in the order of increasing chelating power. The use of the strongest chelating agent, versene, resulted in high accumulation of manganese in the blood and low uptake by the liver. Conversely, the weaker ligands in this series favored accumulation of the metal in the liver. A measure of the total shift in distribution is given by the ratio of the radioactivity of the liver to that of the blood.

DISCUSSION

Toxic effects of manganese, as well as saturation of the metal's transport mechanism, were avoided in these experiments by means of keeping the administered dose within an estimated 5% of the animals' daily dietary intake.

Examination of the disappearance rate of Mn^{56} from the blood shows that the curves (Fig. 1) can

Table III. Shift of Blood and Liver Distribution of Mn^{56} in Mice by Means of Chelation

No.	Chelating agent	Mn^{56}/ gm liver	Mn^{56}/ gm blood	Liver/Blood ratio	L/B ratio DLS % control
1	No ligand	23.6%	0.13%	182	100
2	Versenate	13.6%	1.49%	9.1	5.0
3	Pyrophosphate*	4.5%	0.01%	450	247
4	Citrate	28.8%	0.07%	411	226
5	Maleate	33.2%	0.07%	415	228
6	Bile	27.1%	0.08%	339	186

* Low values reflect low rate of absorption from the peritoneal cavity of the mice.

be graphically analyzed into at least three components. This speaks for the existence in the body of multiple compartments, one with high and others with lesser concentration of the metal. The organ distribution studies did not bring forward an obvious correlation with either metabolic activity or rate of blood flow of the tissues analyzed. Skeletal muscle, for example, is rich in manganese activated enzymes,[11] and is also luxuriously perfused with blood. Neither one of these properties were reflected in the manganese uptake of this tissue. Conversely, the highest uptake of manganese was exhibited by organs known to be rich in mitochondria. The exception shown by brain tissue can be easily ascribed to a blood-brain barrier for this metal. Thus the mitochondria emerged as a probable site of manganese influx and led to the demonstration of impressive accumulation of the isotope in these organelles. That these mitochondrial concentrations are only minimal estimates is demonstrated by the facts that (*a*) Mn^{56} was easily extracted from these bodies by one of the steps of fractionation, and (*b*) that passive adsorption of Mn^{56} onto these organelles *in vitro* was negligible.

The most characteristic property of mitochondria is that they contain most of the enzyme systems responsible for cell respiration. This consideration suggests a highly probable role of manganese as a respiratory co-factor in the cell's economy.

From the practical viewpoint, these studies pointed to radiomanganese as a possible therapeutic agent in malignant disease of liver and pancreas. This short-lived isotope is capable of delivering irradiation at high dose rates to these organs, where it concentrates to a high degree. That this accumulation can be further enhanced is suggested by the effects of prior artificial chelation of the metal on its distribution in the body (Table III). On the same basis, the already rapid and profound disappearance of radiomanganese from the bloodstream can be further accelerated, in order to minimize the undesirable effects of irradiation on the blood-forming organs. In preliminary therapeutic efforts, we have treated patients suffering from either primary or metastatic cancer of the liver with this isotope. The results were encouraging. They are to be reported elsewhere.

SUMMARY

1. Parenteral injection of radiomanganese (Mn^{56}) is rapidly partitioned through the body and concentrates in mitochondria-rich organs.

2. Liver cell fractionation revealed the mitochondria as the main site of intracellular manganese uptake.

3. This distribution of manganese is compatible with its functioning as a respiratory co-factor.

4. An approach is suggested to predictable modification of the metal's distribution in the body following injection.

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The Incorporation of S^{35} in the Form of Sodium Sulfate in Various Animal Tissues

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The incorporation of S^{35} in animal tissue has been related by autoradiographic methods to the existence of certain substances in cartillage, connective tissue and mucous epithelia.[1,2] However the low microscopic resolution of the methods in common use made it impossible to establish precise correlations between the localization of this radioisotope and the cellular and intercellular structures of the tissues just mentioned. To his extent, the Pelc[3] method (stripping film) amply satisfies the requirements of a truly cytological method. In addition, there has been insufficient clarification of the part which the cells might play in the mechanism of incorporation, particularly in the case of connective tissue. This is why, in this preliminary work, we have pursued the following objectives:

(*a*) A study of the incorporation of S^{35}, by means of the method mentioned, on a variety of connective tissues, mucous epithelia and other parenchymatous tissues.

(*b*) A study of the mechanism of this incorporation with special reference to the cells, by investigating developing embryonic tissue and cultures of animal cells.

(*c*) An attempt at clarifying, particularly in the case of connective tissue, the problem, currently under discussion, of the role of the fibroblasts in the production of some intercellulary substances.

MATERIALS AND METHODS

Use was made of three series of adult rats of the C57 B. L. strain, weighing approximately 270 grams each, of both sexes. Each series was made up of four animals. In addition, 8-day chick embryos also were used. Tissue cultures were on 8- and 10-day chick embryo heart transplants by the hanging drop method. The culture medium was made up of dilute horse serum, and chick emhryo extract. Culture times were 24 to 72 hours.

S^{35} in the form of sodium sulfate was administered or added:

(*a*) In the case of the rats, by the intraperitoneal route, 450 μc per animal, diluted in 1 cm^3 of normal saline. The animals were sacrificed 3, 6, 12, 24, 48 and 72 hours, respectively, after administration. The following organs then were taken out: brain, eyes, trachea and thyroid, ears, liver, spleen, kidney, small intestine, testicles, ovaries, uterus, tubes, stomach, muscles and tendons, sternum, heart and adjacent vascular system, skin of the back and pancreas. These organs were fixed in Bouin's fluid, embedded in paraffin, and sliced in sections 4 to 6 microns thick.

(*b*) In the case of the chick embryos, by chorio-allantoid injection of 100 μc of S^{35} diluted in 0.5 cm^3 of normal saline. They were sacrificed after 24 hours. Each embryo was fixed in Bouin's fluid, and cut into various pieces, going through regions of the head, trunk and limbs. The pieces were embedded in paraffin, and sectioned to a thickness of 3 to 5 microns.

(*c*) In the case of the fibroblast cultures varying doses of S^{35} were added to the culture medium, from 4 to 30 μc per culture. These doses were added after 24 hours of incubation *in vitro*. After 3, 6, 12, 24, 48 and 72 hours, the cultures were fixed in Bouin's fluid, hydrated with alcohol and water, and thus prepared for subsequent autoradiographic treatment, which was carried out with the whole culture.

In every case, the autoradiographs were prepared by the "stripping film" technique of Pelc.[3] Exposure times were approximately 40 days, at the end of which they were developed and fixed according to the same technique. In some cases, the background was stained with Harris hematoxylin, and in other cases the observation was carried out, without staining, under phase contrast and ordinary microscopy.

RESULTS

A description is presented of the results achieved with the various materials used, allowing for variations in S^{35} incorporation over the various time periods used.

Rats

Brain

In the three hour preparations, no positive autoradiograph was obtained with any of the structures tried (meninges, white matter and nervous cells). After 6 hours, there was only a slight incorporation in the connective tissue of the arachnoid. At the end of 34 hours, there was a slight incorporation in the white matter, and the localization in the meninges persisted without any changes. After 57 hours, the former pictures stood unchanged.

Original language: Spanish.

* Comisión Nacional de la Energía Atómica.

Eye

In the cornea, incorporation took place selectively in the stroma, and it persisted with the same intensity from 3 hours after the beginning to the end of the experiment. No appreciable autoradiography of the corneal epithelium (Fig. 21). In the connective tissue of the sclerotic, the findings are similar, with an intensification of the rate of incorporation of S^{35} at the limbus corneae. In the lens, the fixation is slight and uniform throughout, persisting without any change throughout the experiment. In the retina, the same finding applies to all layers. In the vitreous humor, there is diffuse and moderate incorporation, particularly in the preretinal spaces (Fig. 22).

Trachea

In the stratified epithelium, moderate incorporation is noted after 3 hours, specifically in the ciliated part (Fig. 1). The heaviest concentration is noted in the secretory cells. After 6 hours, there is a substantial reduction in the autoradiographs observed in those structures, which disappear after 12 hours. In the sub-epithelial connective tissue, after 3 hours, there was diffuse and moderate incorporation, which persisted until the end of the experiment. On the other hand, the epithelium of the mucous secretory acini, revealed intense fixation which persisted up to 57 hours (Figs. 1 and 3). Fixation was most intense in the cytoplasm of the mastocytes present in that structure (Figs. 4 and 5). In the lumen of the tracheal duct, a slight autoradiograph shows up, between 48 and 57 hours, at the level of the epithelial secretion products (Fig. 2). The hyaline cartilage rings show, after 3 hours, a picture of intra-and pericellular incorporation, which is very intense, with the latter localized in the chondrocyte capsules (Fig. 6). In addition, there is a moderate load in the intercellular matrix. From 6 hours on, until 57 hours, cellular incorporation persists and, at the same time, there is an intensification at the level of the intercellular matrix (Fig. 8). The deep layers of the perichondrium reveal moderate absorption by the fibroblasts, particularly after 12 hours (Fig. 7).

The intercartilagenous muscle showed a slight degree of incorporation from the very first hours of the experiment.

Ears

In the cutaneous epithelium, there was some slight incorporation which persisted, unchanged, until the end of the experiment. There was moderate and diffuse fixation in the connective tissue layers over the whole length of the middle and superficial corium. This was intensified from the very first hours and persisted, unchanged, until 57 hours had elapsed, at the level of the connective tissue of the outer root sheath of the hair follicles (Fig. 23) and of the perivascular mastocytes of that region. At the level of the narrow band of elastic cartilage, findings were similar, as to the intensity and timing of incorporation, to those observed for the hyaline cartilage of the trachea.

Skin of the Back

Both in the epithelium and the connective tissue and its annexes, the autoradiograph showed no major differences from those found in the skin of the ear.

Liver

There is some slight incorporation by liver cells, which persists, unchanged, for the duration of the experiment. The intertrabecular stroma, the walls of vessels and bile ducts show moderate incorporation from the first hours, which remains unchanged.

Spleen

There is no perceptible autoradiograph of the cells of the lymphatic follicles. The same applies to the cells of the red pulp. On the other hand there is a moderate degree of fixation by the connective tissue of the vessel walls and the capsule, which persists throughout the experiment.

After 3 hours, there is a slight and uniformly distributed incorporation in the cells of the follicles and the red pulp, which is stronger at the level of the capsule and of the connective walls. Starting after 6 hours, there is moderate intensification of this incorporation, particularly in the trabeculae and the vessel walls, which persists, unchanged, until the end of the experiment.

Kidney

Three hours after the beginning of the experiment, there is very slight incorporation by the cells of the renal tubules, which persists until 6 hours. After 12 hours, there is intensified incorporation, particularly in the proximal and distal convoluted tubules. The results are negative for the glomeruli. After 57 hours, the autoradiograph is much weaker and shows a tendency to disappear. In the intertuhular stroma, particularly after 6 hours, there is some slight localization, which is a little more intense at the level of the intertubular conhective tissue of the medullary zone. This is markedly appreciable in the sub-epithelial connective tissue of the renal papillae and persists, unchanged, until the end of the experiment (Fig. 20). A moderate load also was observed in the middle and adventitious layers of the capsules and walls of the blood vessels, after a six-hour period. No perceptible autoradiograph was observed at the level of the lining cells of the renal tubules.

Stomach

After 3 hours, there is very active incorporation by the cells of the neck of the fundic and pyloric glands (Figs. 34, 25 and 26). This picture is maintained until 48 hours have elapsed from the injection and, in addition, a positive autoradiograph appears, of a band which covers the surface of the mucous epithelium. Beginning 48 hours after injection, there is a reduction on the fixation of S^{35} by the structures mentioned. In addition there is moderate incorporation in the connective stroma of the mucosa, submucosa and serosa, which becomes marked starting 6 hours after injection, and then persists, unchanged, until the end of the experiment.

Small Intestine

S^{35} is incorporated at the level of the goblet cells, in a cycle very similar to that of the gastric glands. In addition, there was moderate incorporation at the level of the epithelium of the villi, which disappeared after 24 hours. In various areas of the sections which were examined, it was possible to see a few intra- and extracellular localizations of S^{35} in the goblet cells, which seemed to represent various secretory phases of the mucin of these cells, toward the lumen of the intestine (Figs. 27, 28, 29, 30, 31, 32). The connective tissue of the stroma of this organ shows localization pictures similar to those seen in the stomach.

Pancreas

A positive autoradiograph, of slight intensity, uniformly distributed all over the parenchyma, and somewhat more intense in the islands of Langerhans than elsewhere, also was observed. The incorporation of S^{35} was more marked in the connective tissue of the capsule and the walls which separate the lobuli as well as in the walls of the vessels of medium and small diameter. This picture, particularly in evidence after 6 hours, was not appreciably modified until 57 hours had elapsed.

Thymus

At the level of the lymphatic parenchyma and that of the stroma, the fixation pictures for S^{35}, were similar to those seen in the spleen.

Testicle

From three hours on, there is a positive weak autoradiograph in the germ epithelium and interstitial cells, persisting without change until 57 hours. On the other hand, from the very start, the incorporation of S^{35} by the connective tissue of the albuginea and of the intercellulary stroma was more intense, increasing progressively until 24 hours, at which point it continues unchanged, particularly at the level of the first of the formations named, until the end of the experiment.

Tendon and Striated Muscles

After 3 hours no autoradiograph of the muscle cells could be observed. On the other hand, slight and diffuse incorporation of S^{35} was noted in the fasciculated parts of the tendons. These pictures became sharper after 6 hours, and particularly marked until 24 hours, at the level of the tendon. In the muscle, a very slight autoradiograph appeared, at some time between the twelfth and twenty-fourth hours. Both pictures, in these structures, persisted from that moment on, without any changes, until the end of the experiment (Fig. 33).

Heart Muscle

Between 3 and 6 hours, no autoradiograph of the muscle cells was in evidence. From 24 to 57 hours there appeared very slight and uniform incorporation on the whole surface of this structure (Fig. 15).

Aortic and Pulmonary Artesy Walls

From 3 hours on, there was moderate incorporation of S^{35} throughout the thickness of the vascular wall, mostly in the media. From 12 to 24 hours there was an increase in this incorporation, which persisted, unchanged, until 57 hours had elapsed (Figs. 12, 13, 14).

Atrioventricular Valves

There was moderately intense incorporation from the very first hour in the stroma of the valves and their annuli. This picture persisted, unchanged, until 57 hours had elapsed (Figs. 15, 16, 17).

Auricles

The incorporation picture, in the stroma of these structures is very similar to that observed in the atrioventricular valves. The zone of delimitation between the connective stroma of these structures, and the adjacent cardiac muscle, in the zone of their implantation, (Figs. 18 and 19), showed up quite visibly.

Sternum

The zone of ossification of this structure was studied with particular attention. From the very first hours, there was moderate incorporation of S^{35} by the cells and the capsule of the elastic cartilage. Incorporation was slight in the intercellular matrix (Fig. 10). Starting from that time and until 57 hours have elapsed, there is intensification of both pictures, particularly at the level of the intercellular matrix, in which the accumulation increases progressively (Fig. 11). In the area of the metaphysis, the most marked degree of incorporation takes place at the level of the seriate cartilage (Fig. 9). Localization is notable in the cells and the capsule of that structure. A less intense autoradiograph was observed in hypertrophic cartilage and the trabeculae of osteoid cartilage. In the medullary cavity, some foci of cellular conglomerates also show moderate captation of S^{35}. These pictures persisted without any major modifications until 57 hours.

Ovary

The observation carried out only at the end of 24 hours showed a moderate load of S^{35} at the level of the cells of the granulosa. Similarly, in follicles which are developing, with the formation of intercellular Koll-Exner vacuoles, there is a slightly moderately intense accumulation of S^{35}. No autoradiograph is obtained at the level of the ovum or pellucida. Conversely, there was moderate fixation in the connective sheath of the ova and the media of the vessels of the hilum, (Figs. 36 and 37). At the level of the cells of the corpora lutea in the process of formation, incorporation is of slight intensity.

Uterus

A load of moderate intensity was localized at the margin of the epithelium of this organ. There was

also some slight incorporation in the cytoplasm of the same cells. On the other hand, the cells of the stroma of the chorion show an image of moderate cytoplasm incorporation (Figs. 34 and 35). This observation was carried out only after 24 hours.

Fallopian Tubes

After 24 hours, there is moderately intense accumulation, particularly in the margin of the epithelium. It is much more intense in the cells of the sub-epithelial stroma (Fig. 38).

Lymphatic Nodes

The autoradiographic images examined, were always similar to those of such other lymphatic organs as the thymus and spleen. Occasionally, very intense fixation was observed only in the cytoplasm of the mastocytes located in the stroma of the lymph sinuses (Fig. 39).

Chick Embryo

From the first hours, very intense and progressive fixation of S^{35} was observed, in a diffuse fashion, throughout the cartilaginous skeleton, less intense in the cells and intercellular spaces of the mesenchyme. In those regions where the mesenchyme is developing into various kinds of connective tissue, muscle or cartilage, the extent of incorporation increases and tends to become peri- and intracellular. In the parenchymata (liver, nervous tissue, heart) incorporation took place later, and was of moderate intensity. In the epithelia of the respiratory and intestinal tracts, fixation also was very intense from the very first hours at the levels of the glands in development, and of the goblet cells, as well indeed as in some stratified epithelia, e.g., cutaneous epithelium (Figs. 40, 41, 42, 43, 45 and 46).

Cultures of the Chick Embryo Heart

The addition to the medium of S^{35}, (6 μl/culture) which was shown to be the minimal useful dosage, showed intense incorporation in the cytoplasm of the fibroblasts, which was maintained for the duration of the experiment. This picture increased progressively, coinciding with the production of a moderately clear autoradiograph in the vicinity of the cells, which became lighter at some distance from the cells. On the whole, there was marked fixation in the cellular conglomerate of the transplant, which was somewhat lighter in the zone of intense growth close to the transplant and much lighter in the marginal areas of isolated migration of the fibroblasts (Fig. 44).

DISCUSSION

From the findings just presented, it is apparent that there are two main types of structures which actively incorporate S^{35}. They are the connective tissue in its different forms, and the mucous epithelium with its goblet cells.

In connective tissue, the intensity of sulfur fixation varies according to the particular kind of tissue involved. Thus, for instance, in the dense connective tissue which makes up the tendons, the capsules of various organs and trabeculae, incorporation was more intense than in the loose intercellular tissue found in the stroma of many organs. As a special case of fixation by a dense variety of such a tissue, we might mention the cases of the stroma of the cornea and sclera. However, the highest rate of S^{35} fixation was observed at the level of various cartilaginous structures, in other words, in the cells, the capsule and the intercellular matrix of these cartilages. Our results, with respect to connective tissue and cartilage, confirm the findings of other authors, who used S^{35} as a tracer for a study of various metabolic aspects of these tissues.

In secretory epithelia, such as of the stomach, intestine, trachea and mucous glands, it can be noted that there is selective incorporation by the mucin producing cells, as well as in the products of their secretion. The method we used enabled us to analyse the fixation processes at a truly cytological level. In this respect, the muciparous cells proved exceedingly interesting since we could follow, from the outside, distinct stages in the production and secretion of their products, by reason of the incorporation of S^{35} in the latter.

In addition, the observation of the gastric glands proved rewarding: only the cellular part of their neck could incorporate S^{35}, while the cells of the glandular fundus could not do it. This is interesting since it constitutes a contribution of the autoradiographic method to the clarification of the mechanism of formation and nature of the secretion products. In other words, it makes it possible to show that the sulfur containing chemical compound is present in these products only in the portion of the gland just mentioned.

The epithelium of the endometrium and uterine tubes also gave an autoradiographic picture at the margin of the apical edge and outside this edge, showing the formation and excretion of a sulfur containing product in that portion of the cells, which we think worthy of more detailed analysis, particularly in relation with the various functional stages of the female genital tract.

Let us make special mention of the cells of the epithelium of the granulosa of the developing ovarian follicle. We discovered a certain relationship between the development of the follicle, that of the granulosa, the formation of intercellular vacuoles and the incorporation of S^{35}, first in an intracellular, then in an extracellular fashion, in various lacunae. In this sense, we also believe that it would be most interesting to go somewhat deeper into the relationship between the formation of this sulfur-containing substance and the histophysiology of the ovarian follicle.

As regards the timing of the incorporation of S^{35}, from the third to the fifty-seventh hour following the beginning of the experiment, we found two major points of comparison between connective tissue and mucous epithelia. Thus, for instance, everything seems to indicate that the metabolic cycle of sulfur

incorporation in the secretory cells would take place in an average time of 48 hours, since the autoradiographs were intracellular during this time, and progressively extracellular toward the end of it, in addition to which the secretion products—which are already extracellular—were the only ones showing clear autoradiographs. In this respect, our results coincide with those published before by other authors[7] who, using autoradiographic methods with S^{35}, reached the same conclusion. On the other hand, in the varieties of the connective tissue already mentioned, we observed progressive incorporation from the first to the twenty-fourth hour, from which time on it remained in the tissues without any major alterations until the end of the experiments. In this sense, the observations of other authors[8] showed that the presence of S^{35} in fibrous varieties of connective tissue, and in cartilage, persisted for some 10 days.

Our observations have enabled us, in addition, and in this they are different from those made by other authors, to note a participation of the cells in the incorporation of this radioisotope in connective tissue. Thus, in hyaline or elastic cartilage, incorporation was predominantly by the chondrocytes in the first hours, thereafter to be followed by autoradiographic images in the pericellular zones and in the intracellular matrix. However, the fact was not so plain in the case of the other varieties of connective tissue we analyzed, in which the cellular incorporation phases were more difficult to interpret, since we observed, on the contrary, what was more like a uniform distribution over the whole extent of these structures. However, it should be stressed that, in some cases, cellular accumulation was evident, as in the case, for instance of the sub-epithelial stroma of some organs (uterus, renal calyx, tracheal chorion). All this would seem to show that, similarly to what happens in the muciparous epithelia, the metabolism of S^{35} by connective tissue would also include a cellular stage before appearing in the intercellular substance. At any rate, and on the whole, this metabolism would take place at a slow rate, and consume much more time than what happens in the mucous epithelia. On the other hand, other authors[9] have made the same observation with respect to hyaline cartilage and using the same autoradiographic technique.

In corroboration of the cellular participation already mentioned, our observations, particularly in cultures of fibroblasts and of the chick embryo mesenchyme, show very active incorporation by the histiocytes and immature fibroblasts. This would indicate that, in this first stage of the production and structural formation of the intercellular substances of the connective tissue, the function of these cells, with respect to the metabolism of S^{35}, would be of fundamental importance. On the other hand, in the varieties of connective tissue already mentioned which have reached maturity and thus their final form, the role of the fibroblasts, under normal conditions, would be much smaller. This shows the important contribution of the autoradiographic methods to the clarification of the problem of the origin of some of the substances which make up connective tissue.

As regards the nature of the substance which accounts for the incorporation of S^{35}, earlier work[4,5] has shown that the acid and sulfur bearing polysaccharides, of the chondroitin sulfuric acid type, play an important part in its mechanism. This has been checked by the extraction of this sulfur bearing radical with barium salts. However, there are other substances which can fix S^{35} although much less intensely, such as methionine, cystine, glutathione and the sulfur bearing lipids.[10] Our results confirm that the epithelial mucins of the mucoitin sulfuric acid present in the goblet cells and mucous glands take an active part in the incorporation of inorganic sulfur. In the connective tissue, the fact already is recognized that a mucopolysaccharide of the chondroitin sulfuric acid type contains the so-called interfibrillar "amorphous substance."[11,12] In this sense our observations on dense varieties of connective tissue show uniform incorporation of S^{35} by that intercellular substance. However, no accurate localization between the fibers proved possible in this kind of connective tissue. This would indicate, either an artefact in the histological technique that preserves the specific location of this substance or that the substance could be found not only between the fibres but also around them. In this respect, when working on such varieties of fibrous connective tissue, the histochemical techniques used with the mucopolysaccharides also showed that there was a positive inter-and perifibrillar action. The situation is different where this mucopolysaccharide is predominant in its interfibrillar location. Such is the case of the wall of the aorta, in which the amorphous substance appears between the elastic and muscular fibres of the media, in which S^{35} autoradiographs also show interfibrillary distribution. The fact that the same situation is observed with respect to the stroma of the atrioventricular valves and the auricles supports this interpretation.

The finding of diffuse incorporation of S^{35} at the level of the amorphous mass of the vitreous humor also is of great interest. In this case, the chemical dosages have not shown the existence of any sulfur bearing polysaccharides, but that of hyaluronic acid, which, in this structure, does not contain any sulfuric radicals.[13]

The postulate of some authors[3] to the effect that some basal membranes might incorporate S^{35} due to the existence of some polysaccharides has not been confirmed in our studies, since the analysis of the basal membranes of the renal tubules, of skin, the testicles and other parenchymatous tissues failed to show any perceptible autoradiograph. It should be remembered, in this sense, that the chemical and histochemical techniques applied to this structure until now only show the presence of non-sulfur bearing, protein bound, acid polysaccharides.[14] Finally, one should not forget the slight incorporation of S^{35} in some parenchymatous tissues, such as the liver,

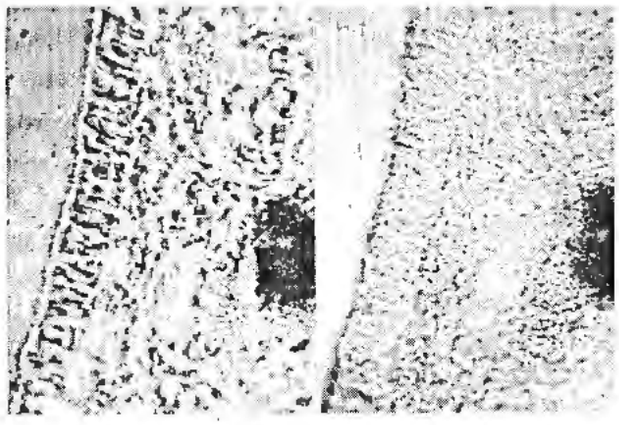

Figure 1. Exp. 125; prep. 524. Time: 3 hours after intraperitoneal injection of S^{35}. Trachea. Aspect of the epithelium and submucosa. Localization of S^{35} in the apical margin of the epithelial cells (ciliate margin). In the subepithelial corium, there is moderate and intense incorporation in the mucous acinus of a gland. Unstained preparation. Left: phase contrast. Right: without phase contrast

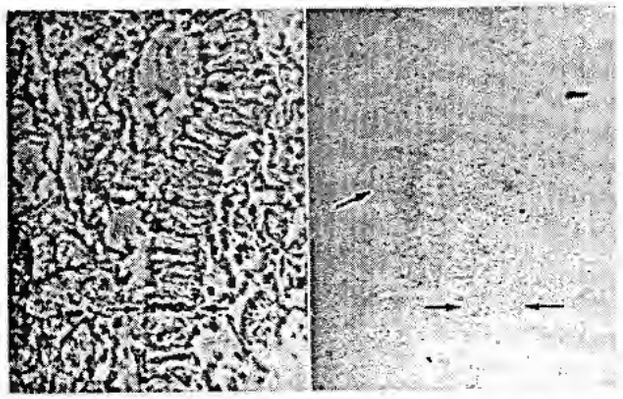

Figure 2. Exp. 124; prep. 246. Time: 57 hours after intraperitoneal injection of S^{35}. Trachea. Disappearance of the autoradiograph of the ciliate margin of the epithelial cells. Autoradiograph shows in the product of secretion, in the lumen of the trachea. Unstained preparation. Left: phase contrast. Right: without phase contrast

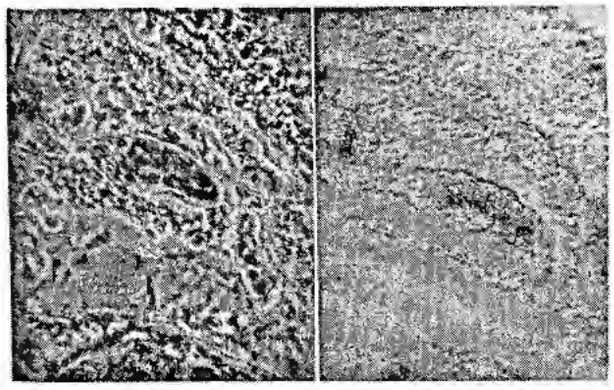

Figure 3. Exp. 125; prep. 524. Time: 3 hours after intraperitoneal injection of S^{35}. Trachea. Aspect of intense incorporation of S^{35} by the glandular mucous acini; discrete incorporation in the connective tissue of the vicinity. Unstained preparation. Left: phase contrast. Right: without phase contrast

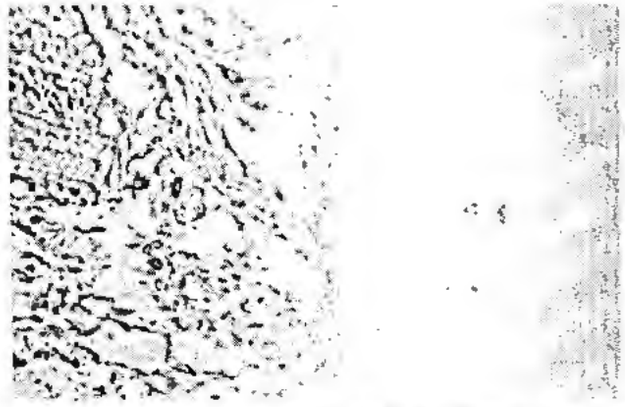

Figure 4. Exp. 124; prep. 246. Time: 3 hours after intraperitoneal injection of S^{35}. Trachea. Intense incorporation by the cytoplasm of mastocytes present in the connective tissue of the submucous corium. Unstained preparation. Left: phase contrast. Right: without phase contrast

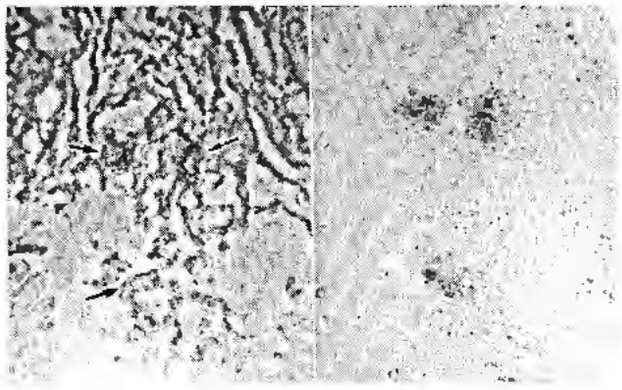

Figure 5. An area of Fig. 4, under greater magnification, in which the details of cytoplasmic incorporation of the radioelement by the mastocytes can be observed. Unstained preparation. Left: phase contrast. Right: without phase contrast

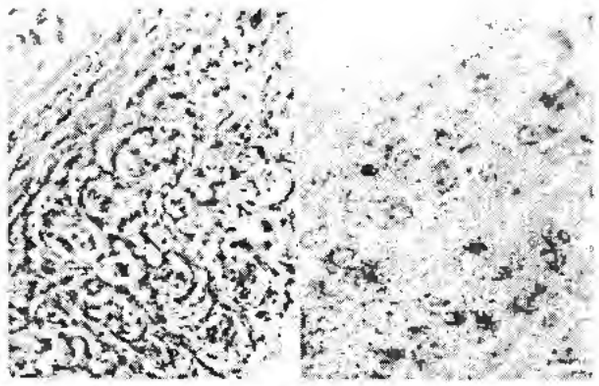

Figure 6. Exp. 124; prep. 524. Time: 3 hours after intraperitoneal injection of S^{35}. Trachea. Intense incorporation of S^{35} by the chondrocytes of the hyaline cartilage; moderate by the cellular capsule, slight by the intercellulary matrix. Unstained preparation. Left: phase contrast. Right: without phase contrast

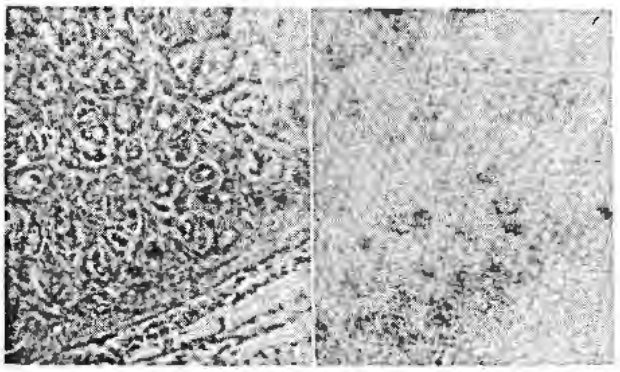

Figure 7. Exp. 124; prep. 317. Time: 12 hours after intraperitoneal injection of S^{35}. Trachea. Incorporation looks like that of Fig. 6, showing moderate autoradiograph in the cells of the deep layer of the perichondrium. Unstained preparation. Left: phase contrast. Right: without phase contrast

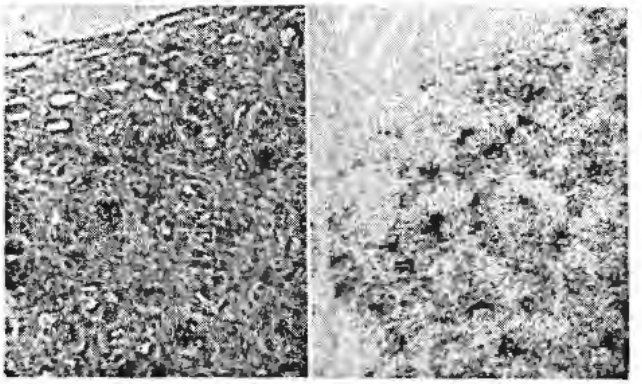

Figure 8. Exp. 124; prep. 246. Time: 57 hours after intraperitoneal injection of S^{35}. Trachea. Incorporation similar to that of Fig. 7, with intensification at the level of intracellular matrix. Unstained preparation. Left: phase contrast. Right: without phase contrast

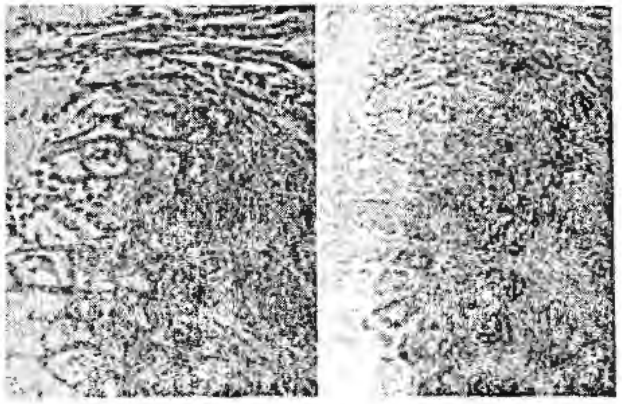

Figure 9. Exp. 124; prep. 237. Time: 12 hours after intraperitoneal injection of S^{35}. Sternum. Intense incorporation of S^{35} by the chondrocytes and the capsule, moderate incorporation by the intercellular matrix at the level of the seriate cartilage of the ossification zone. Unstained preparation. Left: phase contrast. Right: without phase contrast

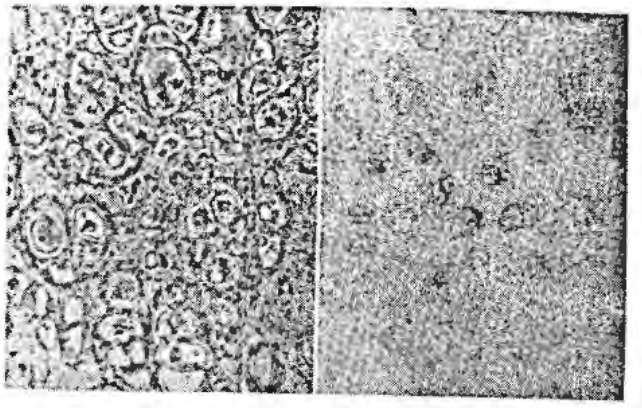

Figure 10. Exp. 124; prep. 237. Time: 12 hours after intraperitoneal injection of S^{35}. Sternum. Moderate incorporation by the cells and the capsule of the elastic cartilage of the body of the sternum; slight incorporation in the intercellular matrix. Unstained preparation. Left: phase contrast. Right: without phase contrast

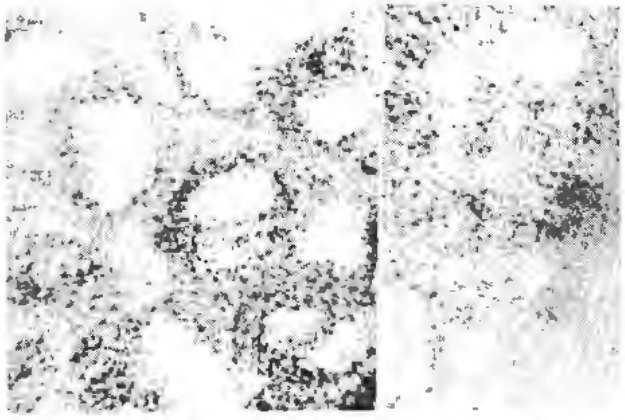

Figure 11. Exp. 124; prep. 245. Time: 57 hours after intraperitoneal injection of S^{35}. Sternum. Left: details of intense incorporation of S^{35} by the capsule; moderate by the cells. Right: another plane of the same preparation showing intense incorporation by the cellular matrix. Stain = Harris' hematoxylin. Homogeneous immersion

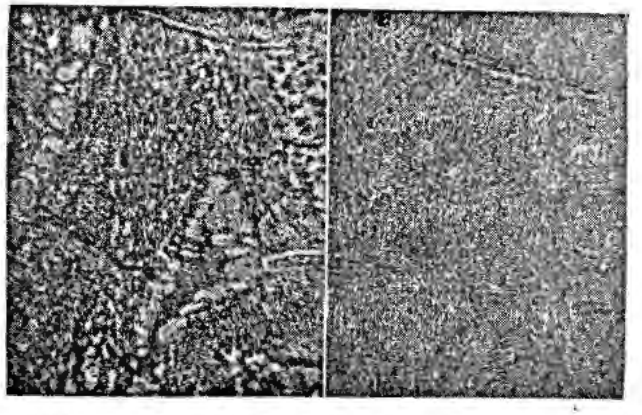

Figure 12. Exp. 124; prep. 176. Time: 6 hours after intraperitoneal injection of S^{35}. Aorta. Discrete incorporation of S^{35}, particularly in the tunica media (musculoelastic coat of the aortic wall). Unstained preparation. Left: phase contrast. Right: without phase contrast

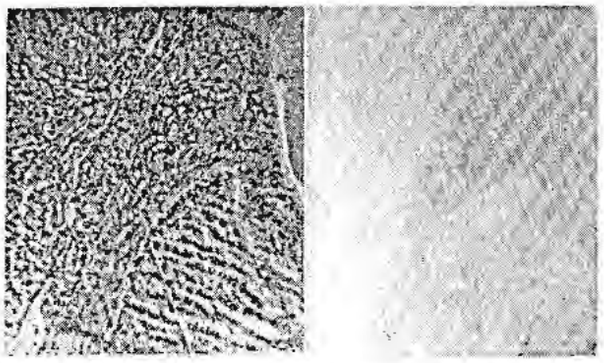

Figure 13. Exp. 124; prep. 180. Time: 12 hours after intraperitoneal injection of S^{35}. Aorta. Intense incorporation of S^{35} with same distribution of preparation as that shown in Fig. 12. Unstained preparation. Left: phase contrast. Right: without phase contrast

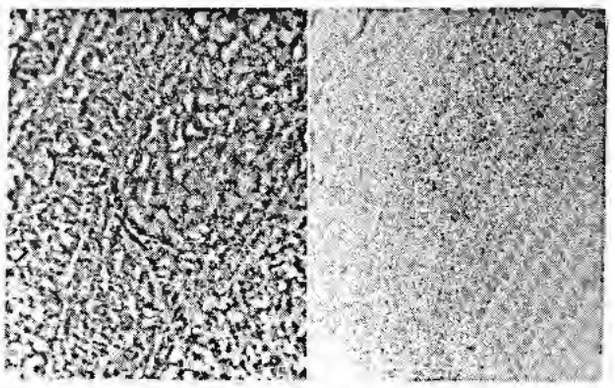

Figure 14. The same preparation as Fig. 13, seen under higher magnification, showing intensity of fixation by the media of the aorta. Unstained preparation. Left: phase contrast. Right: without phase contrast

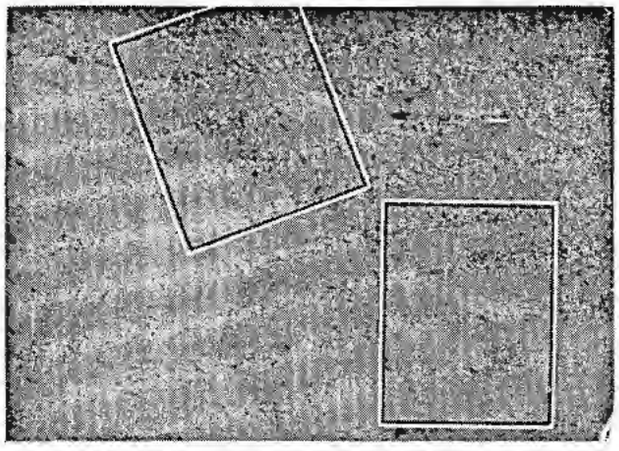

Figure 15. Exp. 125; prep. 507. Time: 24 hours after intraperitoneal injection of S^{35}. Atrioventricular valves. Intense incorporation by the stroma of the valves, throughout the thickness. We can also observe incorporation by the connective tissue annulus of one of the valves. Slight autoradiograph of the underlying myocardium. Unstained preparation. Without phase contrast

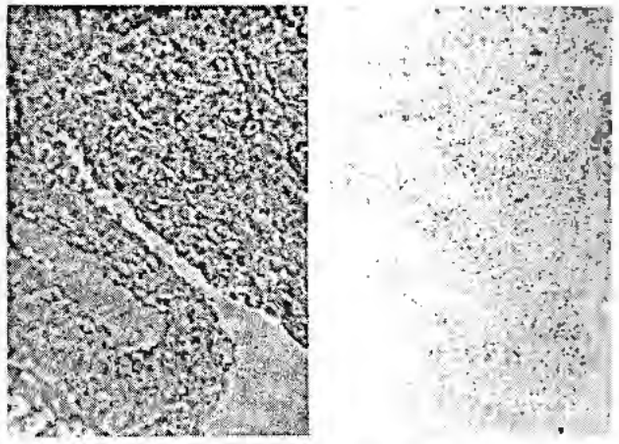

Figure 16. Exp. 125; prep. 496. Another section of the same zone of the preparation shown on Fig. 15, in which, under higher magnification, the details of incorporation by connective tissue of the atrioventricular valves can be shown. Unstained preparation. Left: phase contrast. Right: without phase contrast

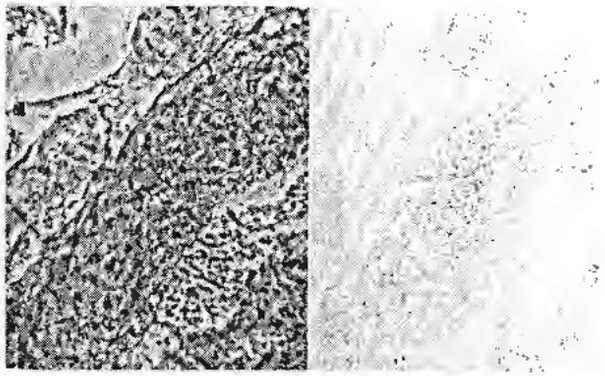

Figure 17. Exp. 125; prep. 507. Detail of both valves of Fig. 15, under higher magnification, showing uniform incorporation of S^{35} throughout the thickness of the stroma. Unstained preparation. Left: phase contrast. Right: without phase contrast

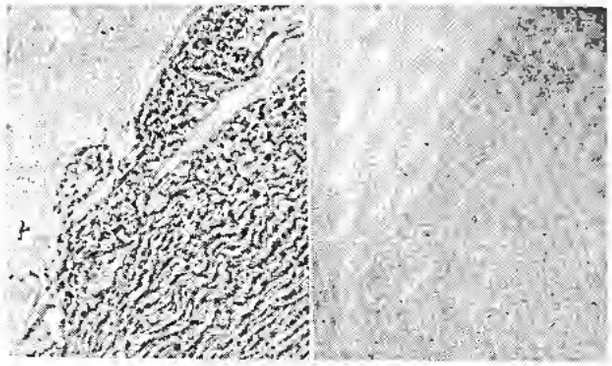

Figure 18. Exp. 125; prep. 507. Time: 24 hours after intraperitoneal injection of S^{35}. Auricle. Moderate incorporation of S^{35} by the connective tissue of the stroma of the auricle. Transition zone with the myocardium showing attenuated incorporation. Unstained preparation. Left: phase contrast. Right: without phase contrast

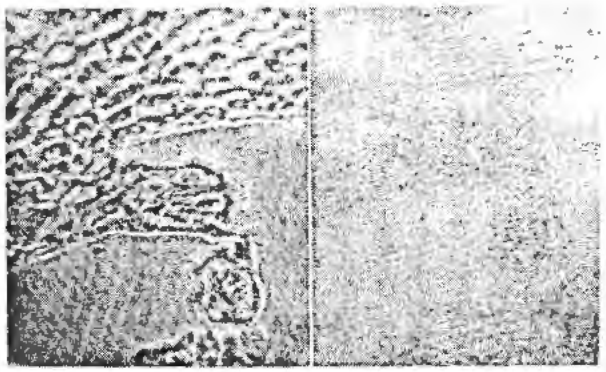

Figure 19. Exp. 125; prep. 507. Time: 24 hours after intraperitoneal injection of S^{35}. Auricle. Section of the wall of the other auricle, under higher magnification than Fig. 18, showing details of diffuse incorporation of S^{35} in the stroma of said structure. Unstained preparation. Left: phase contrast. Right: without phase contrast

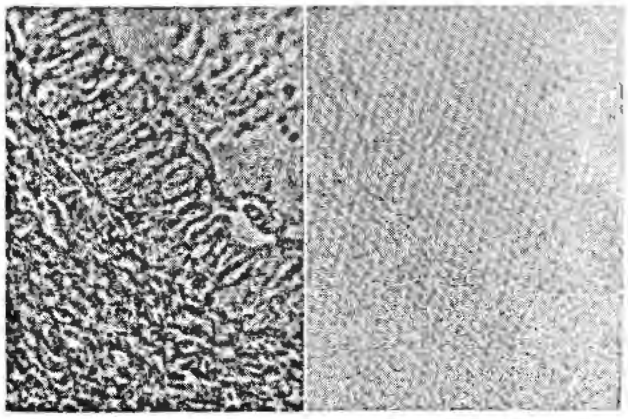

Figure 20. Exp. 124; prep. 290. Time: 12 hours after intraperitoneal injection of S^{35}. Kidney. Moderate incorporation of S^{35} by the connective tissue of the subepithelial corium of the renal papila. Unstained preparation. Left: phase contrast. Right: without phase contrast

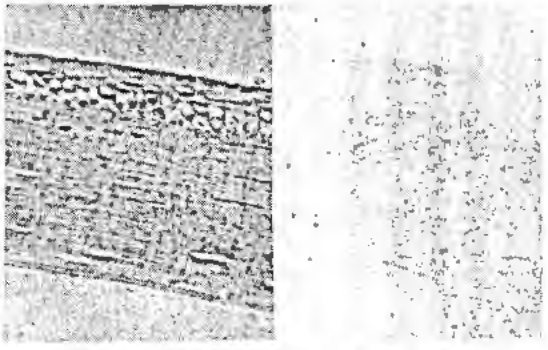

Figure 21. Exp. 124; prep. 431. Time: 24 hours after intraperitoneal injection of S^{35}. Cornea. Moderate incorporation by subepithelial connective tissue of corneal stroma. Negative autoradiograph at the level of the stratified epithelium. Unstained preparation. Left: phase contrast. Right: without phase contrast

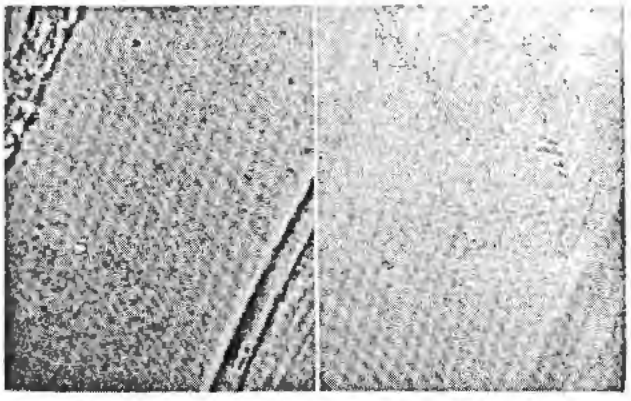

Figure 22. Exp. 125; prep. 431. Time: 24 hours after intraperitoneal injection of S^{35}. Eye. Moderate incorporation of S^{35} at the level of the vitreous humor. Left and below: lens capsule. Right and above: front layer of retina. Unstained preparation. Left: phase contrast. Right: without phase contrast

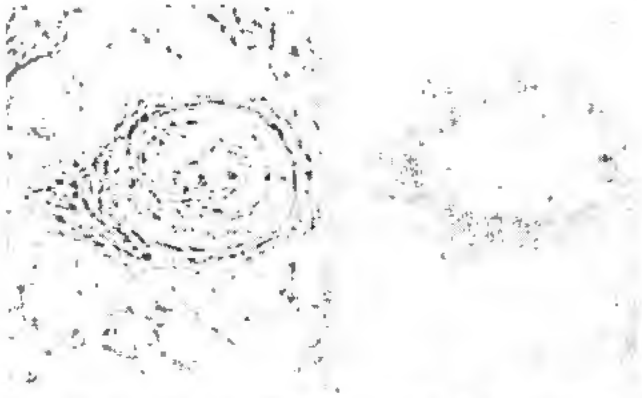

Figure 23. Exp. 124; prep. 337. Time: 6 hours after intraperitoneal injection of S^{35}. Skin. Moderate incorporation by the connective tissue of the outer root sheath of a hair follicle shown in transverse section. Negative autoradiograph at the level of inner epithelial sheath of same. Unstained preparation. Left: phase contrast. Right: without phase contrast

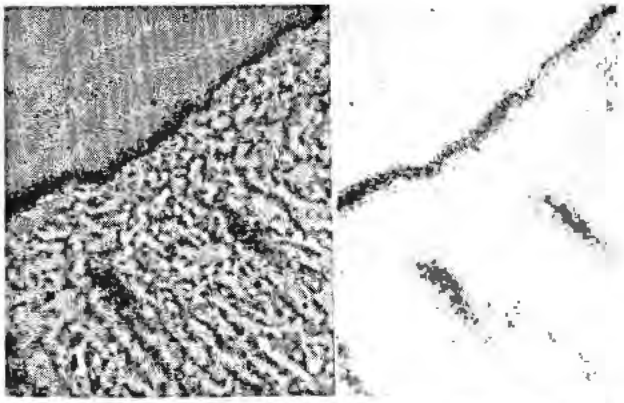

Figure 24. Exp. 124; prep. 207. Time: 3 hours after intraperifoneal injection of S^{35}. Stomach. Intense incorporation of S^{35} at the level of the neck of the superficial glands and superficial band of investing epithelium. Unstained preparation. Left: phase contrast. Right: without phase contrast

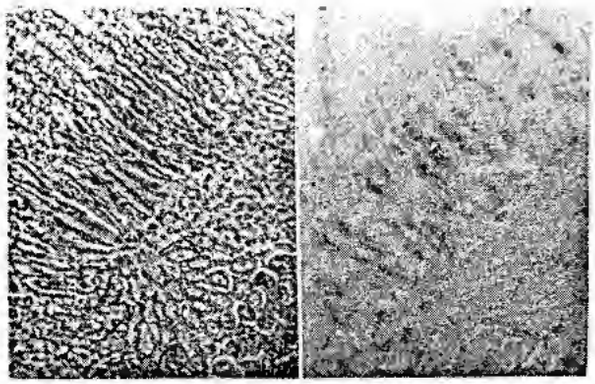

Figure 25. Exp. 124; prep. 10. Time: 12 hours after intraperitoneal injection of S^{35}. Stomach; pyloric section. Intense incorporation of S^{35} by epithelium of the superficial tubular glands. Unstained preparation. Left: phase contrast. Right: without phase contrast

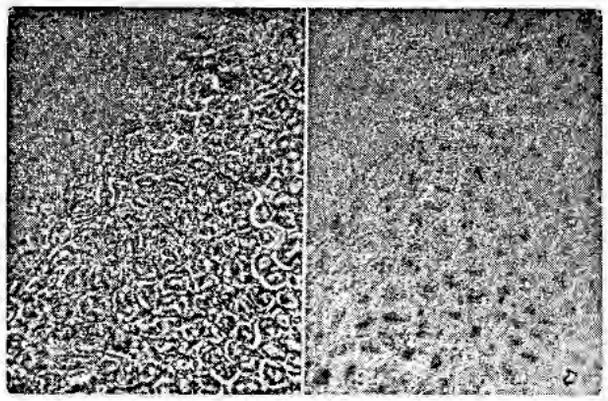

Figure 26. Exp. 124; prep. 10. Time: 12 hours after intraperitoneal injection of S^{35}. Stomach; pyloric section. Field of preparation of Fig. 25 showing intense incorporation of S^{35} at the level of epithelium of superficial tubular glands, in transverse section. Unstained preparation. Left: phase contrast. Right: without phase contrast

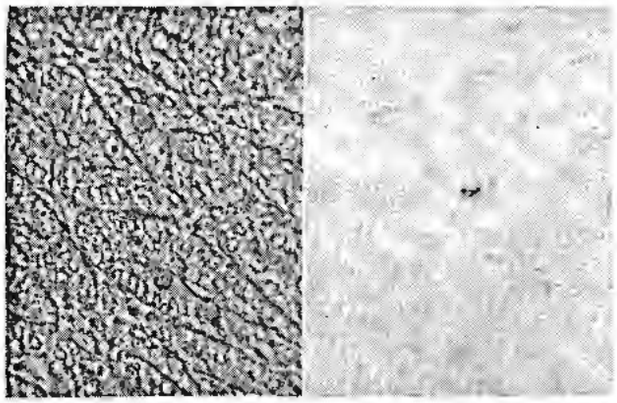

Figure 27. Exp. 124; prep. 85. Time: 3 hours after intraperitoneal injection of S^{35}. Small intestine. Moderate incorporation of S^{35} by goblet cells of epithelium of Lieberkuhn's glands. Slight incorporation by connective tissue of interglandular stroma. Observe mastocytes of the stroma giving intense autoradiograph. Unstained preparation. Left: phase contrast. Right: without phase contrast

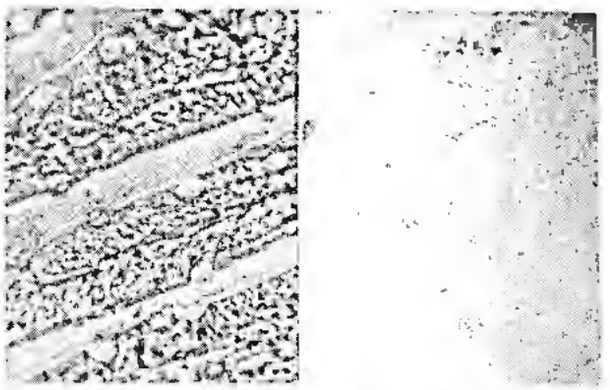

Figure 28. Exp. 124; prep. 87. Time: 12 hours after intraperitoneal injection of S^{35}. Small intestine. Moderate incorporation and secretion images of S^{35} by goblet cells of epithelium of Lieberkuhn's gland in longitudinal section. Unstained preparation. Left: phase contrast. Right: without phase contrast

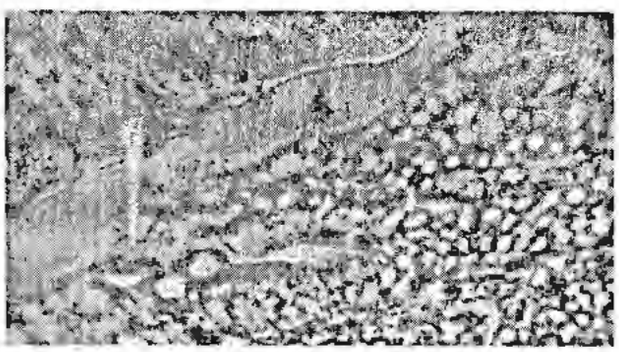

Figure 29. Exp. 124; prep. 87. Time: 12 hours after intraperitoneal injection of S^{35}. Small intestine. Incorporation of S^{35} in the product of secretion of the goblet cells of Lieberkuhn's glands. The picture shows the time at which this product reaches the lumen of the gland. Unstained preparation. With phase contrast

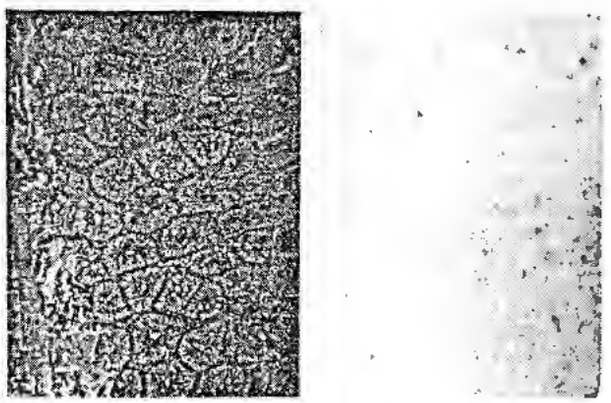

Figure 30. Exp. 125; prep. 507. Time: 24 hours after intraperitoneal injection of S^{35}. Small intestine. Transverse section of Lieberkuhn's glands. Intense incorporation of S^{35} by goblet cells of the glandular epithelium. Moderate incorporation in the interglandular stroma

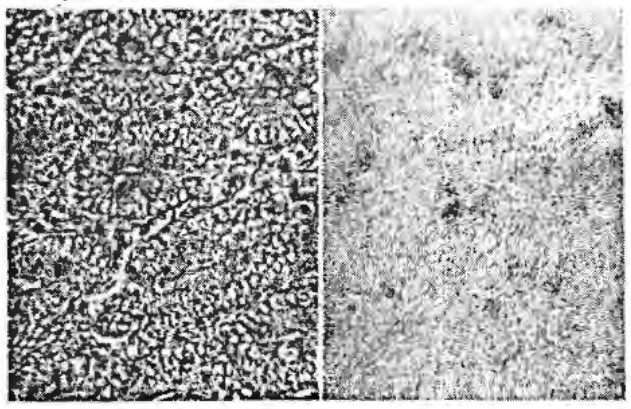

Figure 31. Exp. 125; prep. 507. Small intestine. One section of the preparation shown on Fig. 30, with higher magnification

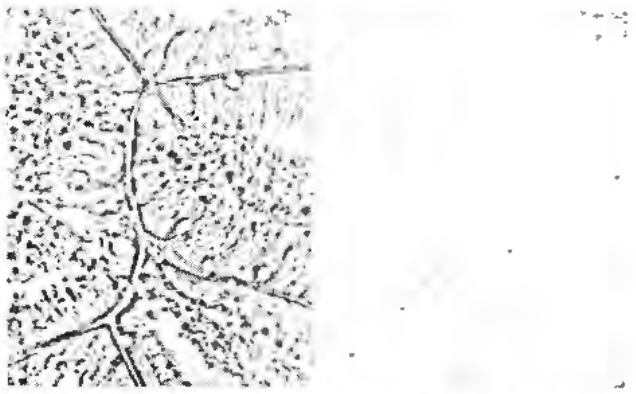

Figure 32. Exp. 124; prep. 101. Time: 24 hours following intraperitoneal injection of S^{35}. Small intestine. Transverse section of the intestinal villi, showing interglandular lumen, which contains the product of secretion charged with S^{35}. Unstained preparation. Left: phase contrast. Right: no phase contrast

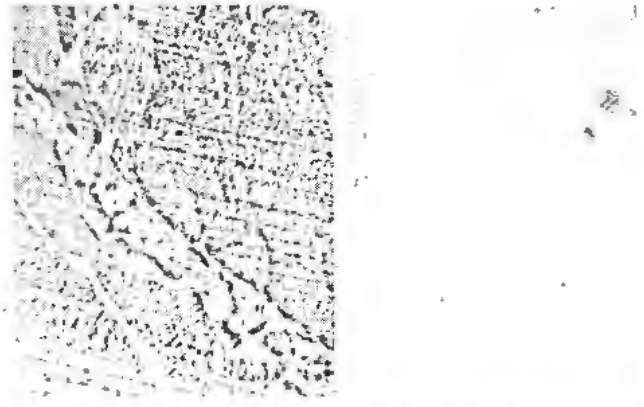

Figure 33. Exp. 125; prep. 408. Time: 24 hours after intraperitoneal injection of S^{35}. Tendon and striated muscle. Incorporation of S^{35} at the level of the tendon is moderate, and the autoradiograph is negative at the level of the muscle. Unstained preparation. Left: phase contrast. Right: without phase contrast

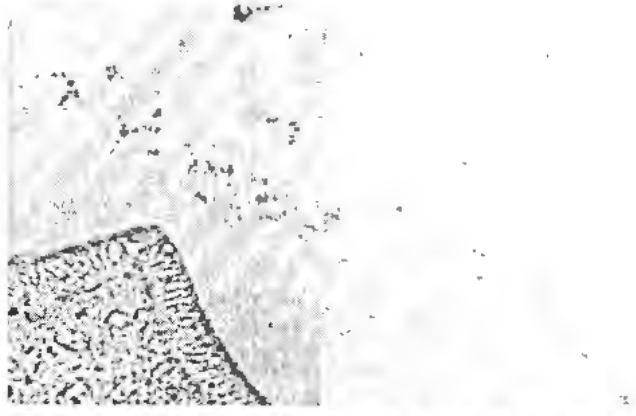

Figure 34. Exp. 125; prep. 421. Time: 3 hours after intraperitoneal injection of S^{35}. Uterus. Moderate incorporation at the level of the cells and ciliate margin of superficial epithelium. Slight incorporation by the fibroblasts of the subepithelial stroma. Unstained preparation. Left: phase contrast. Right: without phase contrast

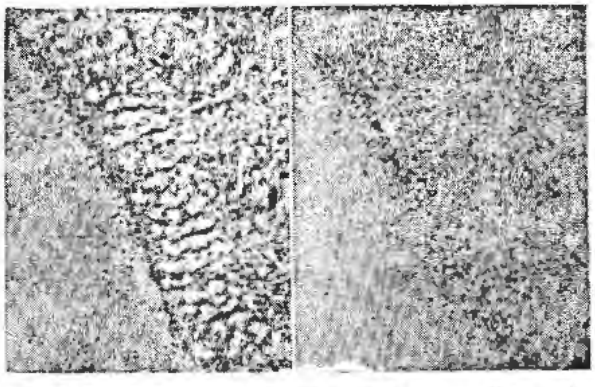

Figure 35. Exp. 125; prep. 421. One area of the preparation shown on Fig. 34, under larger magnification, showing predominant localization of S^{35} in the apical portion of the epithelium. Unstained preparation. Left: phase contrast. Right: without phase contrast

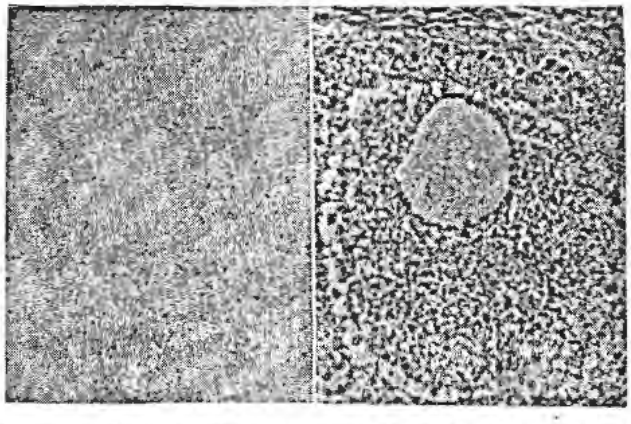

Figure 36. Exp. 125; prep. 432. Time: 24 hours after intraperitoneal injection of S^{35}. Ovary. Moderate incorporation by the cells of the epithelium of the granulosa of a follicle. Negative autoradiograph at the level of the ovum and pellucida. Unstained preparation. Left: phase contrast. Right: without phase contrast

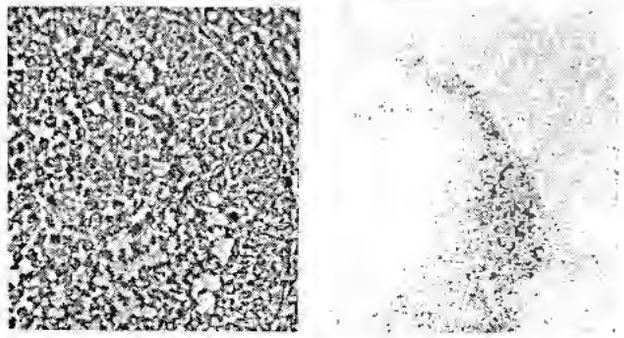

Figure 37. Exp. 125; prep. 432. Time: 24 hours after intraperitoneal injection of S^{35}. Ovary. Slight incorporation by the cells of the epithelium of the granulosa; intense localization in the intercellular spaces (Kall-Exner lacunae) of the growing follicle. Unstained preparation. Left: phase contrast. Right: no phase contrast

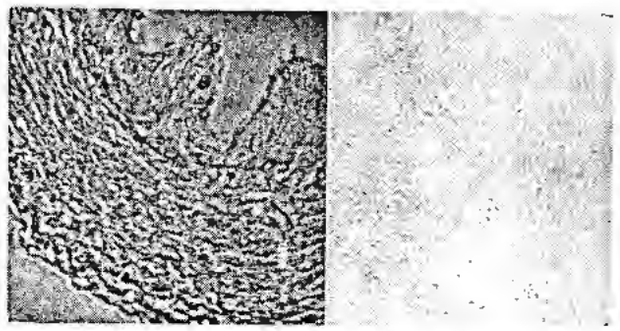

Figure 38. Exp. 125; prep. 421. Time: 24 hours after intraperitoneal injection of S^{35}. Fallopian tube. Moderate incorporation by the muscular and connective stroma of the wall. Negative autoradiograph at the level of the epithelium. Unstained preparation. Left: phase contrast. Right: without phase contrast

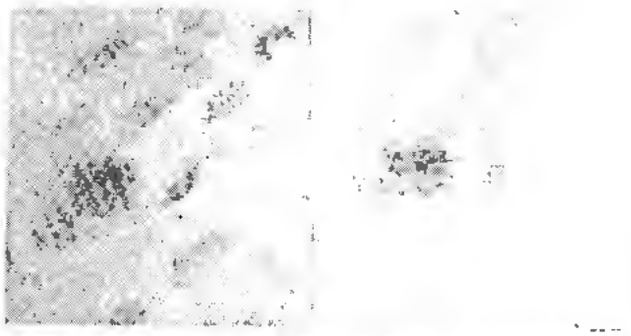

Figure 39. Exp. 124; prep. 182. Time: 24 hours after intraperitoneal injection of S^{35}. Cytological aspect of the wall of a marginal lymphatic sinus which contains a mastocyte between macrophages of this structure. Moderate incorporation in the cytoplasm of the mastocyte. Stained preparation, using Harris' Hematoxylin. Left: plane of stained preparation. Right: focusing on a higher plane in order to visualize the autoradiograph of said element

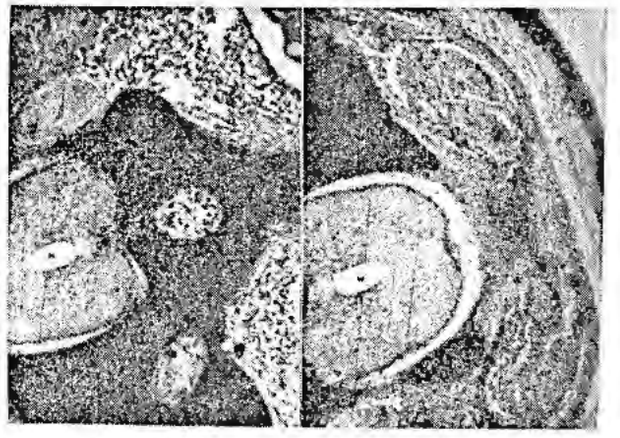

Figure 40. Exp. 132-D1. Time: 24 hours injection in the chorio-allantoid cavity of an 8-day chick embryo. Appearance of a part of the dorsal aspect of the embryo showing intense incorporation in the cartilage of the spine; moderate incorporation in the nervous tissue of the spinal medulla. Intensification at the level of the meninges. Moderate incorporation in the diffuse mesenchyma and retrovertebral myoblasts. The figure at right also shows intense incorporation at the level of a band of the dermic mesenchyma. Unstained preparation. Photomicrographs without phase contrast

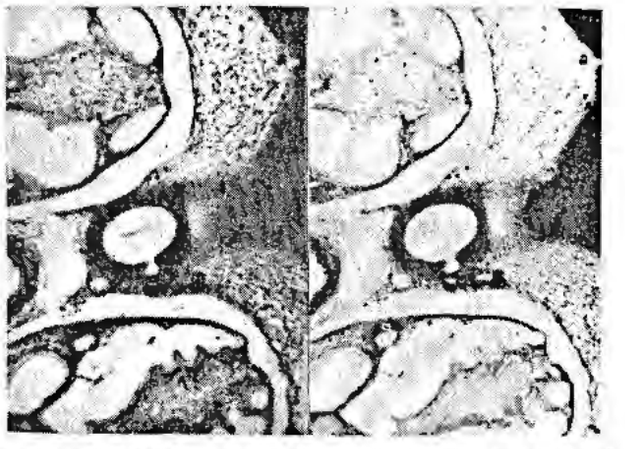

Figure 41. Exp. 132-D1. Time: 24 hours after injection into the chorio-allantoid cavity of an 8-day chick embryo. Appearance of a part of the middle aspect of the embryo. Intense incorporation at the level of the cartilage of the anterior part of the vertebral body. Intense incorporation in the musculo-connective part of the aorta, as well as in the wall of the esophagus (at upper limit of figure). Moderate incorporation in the cells of the intervisceral diffuse mesenchyme. Moderate incorporation by the epithelium of the pulmonary rudiments and through the subepithelial stroma of same. Unstained preparation. Left: phase contrast. Right: without phase contrast

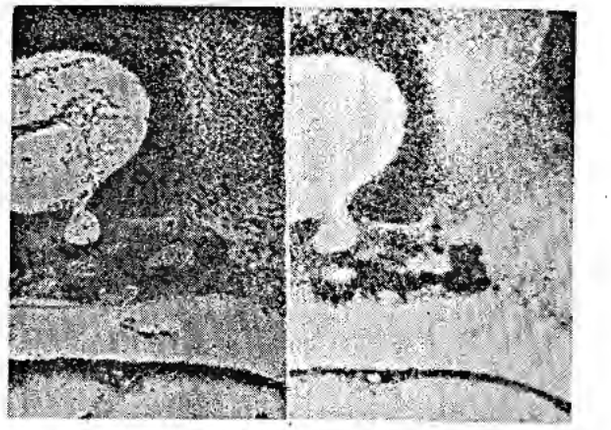

Figure 42. Exp. 132-D1. A part of the preparation which corresponds to Fig. 41, showing, under higher magnification, the rate of incorporation in the wall of the aorta and the mesenchyme interposed between the latter and the cartilage of the vertebral column. To the left of the aorta, a tangential section at the level of an arterial branch can be seen, showing intense incorporation of S^{35}. Unstained preparation. Left: phase contrast. Right: without phase contrast

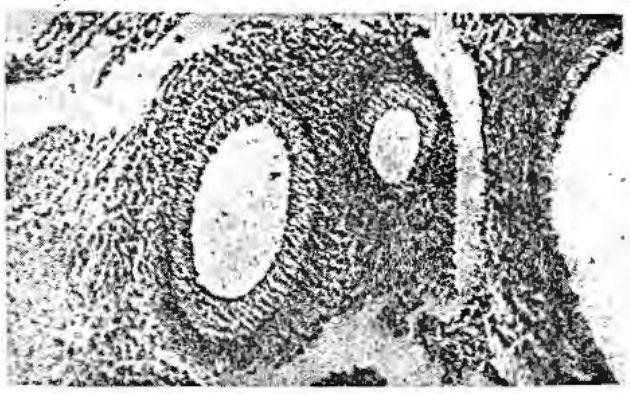

Figure 43. Exp. 132-D1. Time: 24 hours after injection in the chorio-allantoid cavity of an 8-day chick embryo. Appearance of the mesenchyme around the rudiments of the intestine. Intense incorporation by the histiocytes and moderate incorporation in the epithelium of an intestinal tube. Unstained preparation. Photomicrograph with phase contrast

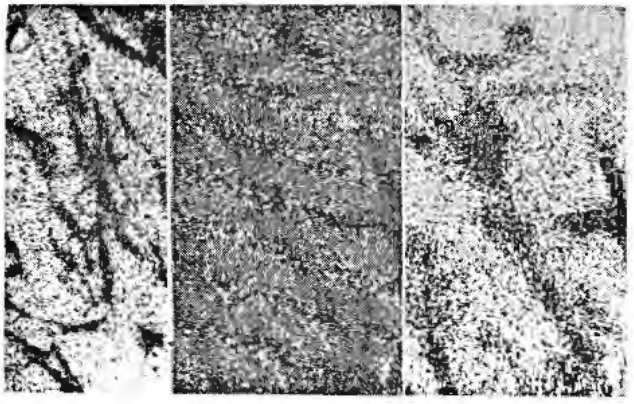

Figure 44. Exp. 131-2. Time: 48 hours following incorporation of S^{35} in a culture medium in a transplant of an 8-day chick embryo heart. There is intense incorporation in the cytoplasm of the fibroblasts, and moderate incorporation in the peri- and intercellular media. Unstained preparation

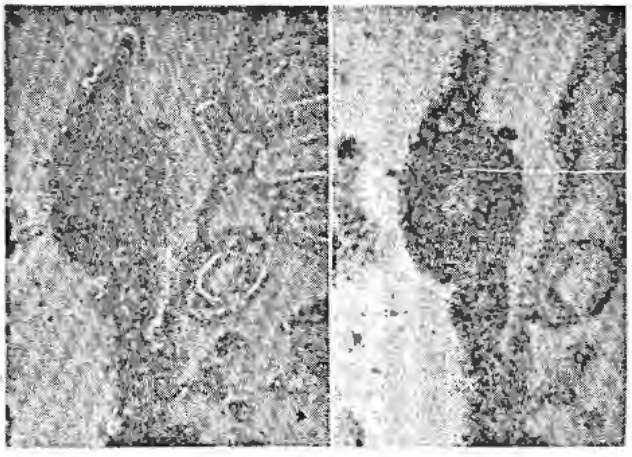

Figure 45. Exp. 132-D1. Time: 24 hours after injection of S^{35} in the chorio-allantoid cavity of an 8-day chick embryo. Appearance of a part of the middle zone of the embryo. Intense incorporation at the level of musculo-connective stroma of the wall of the ureter. Moderate incorporation in the capsule of the neighboring kidney. Slight incorporation in the cells of some renal tubules. Unstained preparation. Left: phase contrast. Right: without phase contrast

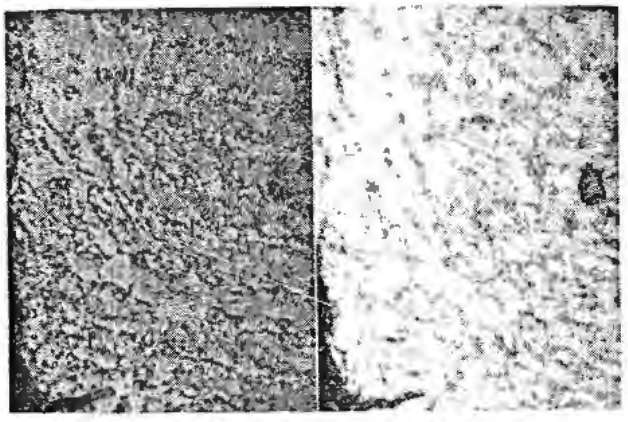

Figure 46. Exp. 132-D1. Time: 24 hours after injection of S^{35} in the chorio-allantoid cavity of an 8-day chick embryo. Appearance of a section at the level of the middle part of the body of the embryo. Observe moderate incorporation of S^{35} in the structure formed by cytoplasm of the histiocytes in the diffuse interviscerol mesenchyme. Unstained preparation. Left: phase contrast. Right: without phase contrast

muscular cells, pancreas, thymus, thyroid, lymphatic nodes, etc., the active substance of which is of unknown nature. It could be supposed that the sulfur-bearing amino acids already mentioned might intervene in this incorporation. This postulate is confirmed by the more marked collection of S^{35} in the embryo parenchymas in other words in parenchymas which are undergoing very rapid development, indicating intense protein synthesis. Some authors[15] have made similar findings in cultures of myeloid cells to which S^{35} had been added. A special mention of the finding of moderate fixation of S^{35} in the proximal and distal renal tubules is in order, particularly after about 24 hours. In this case, some amino acids or other sulfur-bearing proteins would seem to come into play, or else, as seems to be clear from the timing of the phenomena, the process would be one of sulfur elimination in the form of insoluble sulfo-conjugates. In this sense, we already know that a great part of the sulfur incorporated in its organic or inorganic form by an animal organism is eliminated by the principal organs of excretion[8]. In the last analysis, the exact nature of a substance which accounts for the incorporation of sulfur could only be determined by appropriately controlling chemical and enzymatic extraction processes, which is what we are now doing.

We should like to bring out the fact that the autoradiographic method used by us, in contrast with the methods applied by various other authors, has enabled us to make some observations of greater specificity and to achieve more accurate localization at a strictly cytological level, an objective not easily reached by other methods which give much poorer microscopic resolution.

SUMMARY

1. Adult rats were used for the experiments, along with chick embryos and chick embryo fibroblast cultures *in vitro*.

2. In the adult animals, S^{35} was administered intraperitoneally, in the embryos by the chorio-

allantoid route, and in the fibroblast cultures, it was incorporated directly to the medium.

3. Demonstration of the incorporation was made using the Pelc autoradiographic technique of the "stripping film."

4. The results observed were:

(*a*) In adult rats, the fixation rates, in general, were higher in some varieties of connective tissues and mucous epithelia, lower in the parenchymatous tissues.

(*b*) In chick embryo, the incorporation rate was maximal in the mesenchyme and tissues derived from the same. There also was some incorporation by various parenchymatous tissues, although in a smaller proportion.

(*c*) In *in vitro* cultures of fibroblasts incorporation was intense in the fibroblasts, much smaller in the intercellular medium.

CONCLUSIONS

The importance of connective tissue in the incorporation and metabolism of S^{35}, as compared with that of the parenchymata, is established. It is felt that, in the intercellular medium of connective tissue, substances are to be found, which account for this incorporation. Similarly, the fibroblasts appear to take an active part in the process.

Currently, we are carrying out experiments with small doses of S^{35} and enzymatic controls in an attempt at demonstrating the nature of the substance mentioned.

ACKNOWLEDGEMENT

The authors wish to thank Dr. E. Sacerdote de Lustig for preparing the tissue and embryo cultures.

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Comparative Study of Incorporation of S^{35} by Tissues of Adult Rats, Both as Sodium Sulfate and as DL-Methionine

By Constantino Nuñez and Roberto E. Mancini,* Argentina

The preceding paper[1] dealt with the incorporation of inorganic S^{35} by several tissues. Very active fixation by several varieties of connective and cartilaginous tissue was noted, as against light incorporation by parenchymas in general. This project was undertaken in order to draw a comparison between the results obtained by autoradiographic methods, using an S^{35} labeled essential amino acid, such as methionine, for the purpose of determining whether these tissues would metabolize the compound as inorganic S^{35}, or as the amino acid methionine. For this purpose, the first part of the present work will study the incorporation of S^{35} methionine in various organs and the second part will compare the results with those previously obtained with S^{35}.

MATERIALS AND METHODS

Four male adult rats of the C57B1 strain, weighing about 250 gm, were used. They all received 250 μc of S^{35}-tagged DL-methionine in 1 cm^3 normal saline. They were killed with illuminating gas after 24, 48 and 72 hours. The following organs were immediately dissected: trachea, heart and vascular pedicle, sternum, tendons and muscles, spleen, liver, small intestine, kidney, ureter, skin, esophagus, testicle, eye, brain and pancreas. These organs were fixed in Bouin's fluid, embedded in paraffin and cut in sections, 3-5 microns thick. The sections were then hydrated and mounted, and the Pelc[2] stripping-film autoradiographic method was used, developing after an exposure of 45 days. Observations were made using conventional and phase microscopy. Some samples were stained with Harris hematoxylin. The results were compared with those obtained from lots of animals treated with S^{35} tagged sodium sulfate and described in a parallel paper.[1]

RESULTS

The results obtained in each of the organs were as follows:

Trachea

Strong incorporation in the epithelial cells was observed after 24 hours. The corium gave a very slight autoradiograph, as did the cartilaginous tissue. The secretory acini of the corium mucosae indicated moderate fixation. The peri-and intercartilaginous bands of the muscle cells showed intense incorporation over their whole length. There was no major modification of these results during the 72-hour experimental period. There was a similarly moderate incorporation in the muscular layers of the medium-sized vessels.

Heart

There was diffuse and moderate incorporation throughout the cytoplasm of the muscle cells, beginning 24 hours after injection, which persisted, without alteration, until the end of the experiment. The autoradiograph was negative in the connective tissue of the stroma and serosae.

Aorta

Light incorporation was observed in the media of this vessel, corresponding to the muscle cells. No autoradiograph was given by the interstices of the media, the intima or the adventitia. This picture did not change appreciably up to the end of the experiment (72 hours).

Sternum

Except for very slight incorporation in the cartilaginous cells, the perichondrium, and neighboring connective tissue, there was no demonstrable autoradiograph during the whole course of the experiment.

Tendons and Muscles

There was moderate incorporation in the cytoplasm and muscular cells, while S^{35} fixation by the interfascicular connective and tendinous fasciculi was very slight (Fig. 9). This picture remained the same for the duration of the experiment.

Spleen

The autoradiographs showed slight incorporation in the cytoplasm of the cells belonging to the lymphoid line, as well as in some histiocytes. On the other hand, the autoradiographs of the capsular connective tissue and stroma were practically negative. As was indicated above, the picture showed no change in the 24 hour to 72 hour period.

Liver

Starting at the 24th hour, there was moderate incorporation, increasing progressively, although not

* Comisión Nacional de la Energía Atómica.
Original language: Spanish.

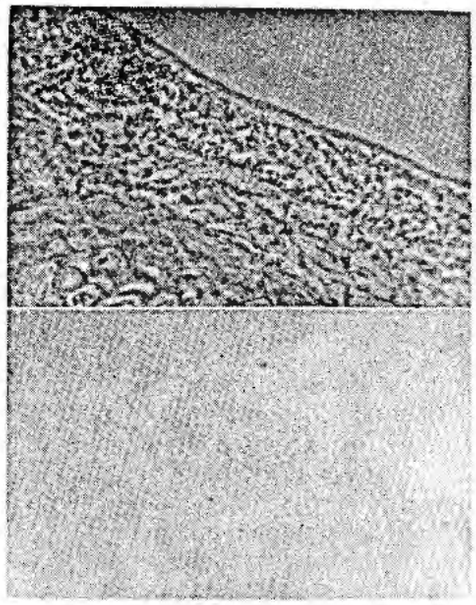

Figure 1. Exp. 123-1. Time: 24 hours after intraperitoneal injection of S^{35} labelled methionine. Trachea. Moderate incorporation at the level of the epithelial cells. Slight incorporation in the sub-epithelial connective tissue. Negative autoradiograph in the layer of underlying cartilage. Specimen not stained. Above: with phase contrast. Below: without phase contrast

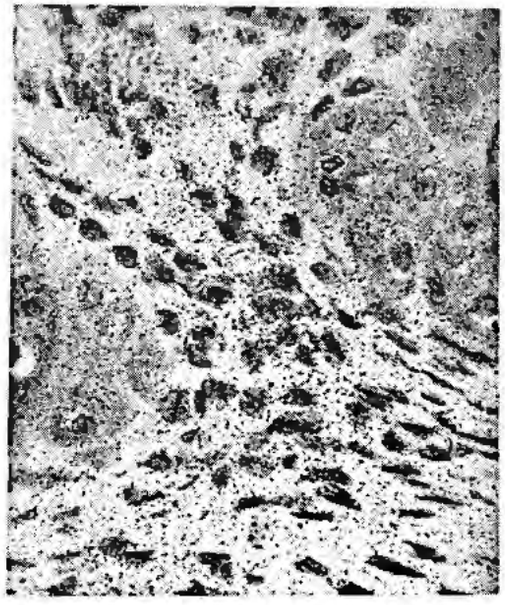

Figure 2. Exp. 23-1. Time: 24 hours after intraperitoneal injection of S^{35} labelled methionine. Trachea. Appearance of the intercartilaginous muscle cells. Moderate incorporation in the cytoplasm of these cells seen in cross and longitudinal cross sections. Slight incorporation in the cells of neighboring cartilage. Specimen stained with Harris hematoxylin

very markedly, up to the 72nd hour. This incorporation was uniform throughout the liver parenchyma. The connective tissue of the capsule, stroma walls of the bile tubes and tubules, showed a very slight autoradiograph.

Small Intestine

Incorporation was very moderate and predominant from the 24th to the 72nd hour in the cytoplasm of the epithelial cells. The stroma of the villi and of the rest of the wall showed slight incorporation, while the muscular cells of the external coat and of the axis of the villi absorbed S^{35} moderately, without any variation, for the duration uf the experiment. (Fig. 4).

Kidney

There was intense incorporation in the proximal convoluted tubules, a picture which persisted for the whole of the experiment. The distal tubules gave negative autoradiographs. The capsule and connective tissue of the intertubular stroma, as well as the epithelium of the renal papillae, showed slight incorporation during the whole of the experiment (Figs. 5 and 6).

Skin

The stratified epithelium and the internal epithelial sheaths incorporated S^{35} moderately. There was very slight incorporation throughout the corium. The hypodermis muscles showed moderate incorporation. All these pictures held unchanged between the 24th and 72nd hours.

Esophagus

Diffuse and moderate incorporation was observed, particularly in the stratified epithelium of the basal and intermediate layers, as well as in the muscular cells of the external coat. The mucosa and submucosa connective tissue incorporated S^{35} very slightly. None of these pictures was changed over the duration of the analyses.

Testicle

An evenly-distributed slight incorporation was observed in all the germinal epithelium layers (spermatogonium, spermatocytes, spermatids). Conversely, between the 48th and 72nd hours, incorporation became predominant in spermatids and spermatozoa close to the lumina of the seminiferous tubes. At the same time, there was a slight measure of incorporation in Leydig's cells. On the other hand, the autoradiographs were practically negative in the tube walls and the connective tissue of the intertubular spaces.

Eye

Intense incorporation was observed in the cornea, in the thickness of the epithelial layer. Conversely, incorporation was slight in the stroma and negative in the posterior basal membrane of Descemet. The lens gave signs of slight incorporation, which increased at the level of the equatorial layer cells. The capsule and the rest of the lens gave negative autoradiographs. The retina showed uniformly-distributed slight incorporation. The autoradiographs of the structures described did not suffer noticeable changes during the experiment (Figs. 3 and 8).

Cerebrum and Cerebellum

A diffuse autoradiograph of slight intensity was noted in all the samples. There was moderate incorporation in the cytoplasm of some cortical

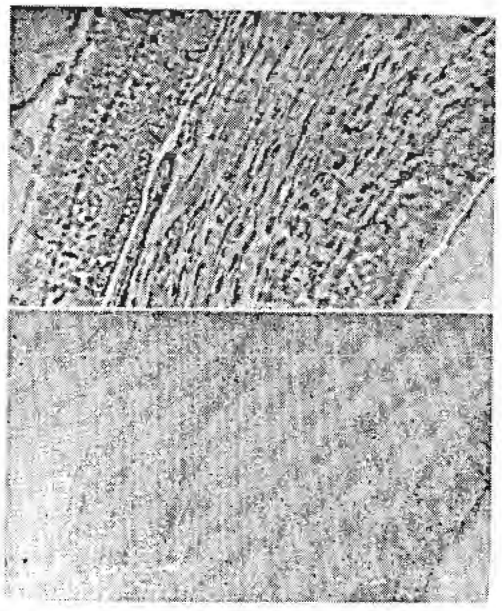

Figure 3. Exp. 123-1. Time: 24 hours after intraperitoneal injection of S^{35} labelled methionine. Cornea and retina. Strong incorporation in the corneal epithelium, slight fixation in the stroma. Slight incorporation may be seen in the retina which is shown artificially applied against the back of the cornea, throughout the thickness of the latter, with intensification of the autoradiograph through the thickness of the posterior layer, in contact with the margin of the cornea. Specimen not stained. Above: with phase contrast. Below: without phase contrast

neurons, particularly in the cerebellum, in the cytoplasm of the Purkinje cells. No autoradiograph of the meningeal connective tissue was in evidence.

Ureter

Moderate and diffuse incorporation throughout the epithelium, of a pseudo-stratified character, as well as in the muscle layers. The connective tissue of the mucosa gave a slight autoradiograph. This picture remained constant for the duration of the experiment.

DISCUSSION

Two facts are evident from this experiment. There is incorporation of methionine S^{35} by actively proliferating epithelia, such as in the testicle, stratified epithelium of the cornea, skin, esophagus and ureter, with a smaller measure of incorporation in those of lesser activity, such as the tracheal and intestinal epithelium. On the other hand, incorporation by the parenchymas was general, although of lesser intensity, as in the case of the glandular secretory acini, liver, lymphatic formations of the spleen and nodes, retina, etc. A similar finding was made in the muscle cells of the tunica media of the large and medium vessels such as the aorta, in the muscle layers of the intestinal and respiratory tract, and in the striated heart muscle. The intense incorporation noted in the proximal convoluted tubules of the kidney, compared with the very slight incorporation by the rest of the nephron is interesting. It should be pointed out that the incorporation described took place actively from the first hours after the intraperitoneal administration of the methionine S^{35} and that it persisted, without any major changes, for at least the 72 hours of the experiment.

Considering that methionine is an essential amino acid of importance in the synthesis of proteins in actively growing tissue, rapid and early incorporation in such formations during active proliferation or growth (epithelia) can readily be explained. In those tissues that are functionally and metabolically responsible for the main phases of amino acid metabolism, e.g., the liver, it was noted that the incorporation of this amino acid was of equal intensity and progressively increasing for at least 72 hours. In the case of the muscles, the moderate degree of incorporation may be accounted for by the possible participation of said amino acid in some of the phases related to its functional activity, possibly the synthesis of creatine and sarcosine. Presumably in the case of the cutaneous epithelium and proximal part of the esophagus of the rat, keratin may take some part in the phenomena, because it is composed of cysteine and other sulfur-containing amino acids, since, in this sense, the mutual metabolic relationships between methionine and cystine are well known. As regards incorporation by the cortical neurons and Purkinje cells, the most likely explanation would be found by assuming that it takes part in phospholipid metabolism.

In parenchymas of various kinds, such as those of the mucous or serous glands, such as the pancreas, or organs such as the lymphoid portion of the spleen and nodes, the slight incorporation of methionine could be similarly explained by its being an essential and irreplaceable amino acid for the several metabolic activities. In the special case of the kidney, the active and strong incorporation by the proximal convoluted

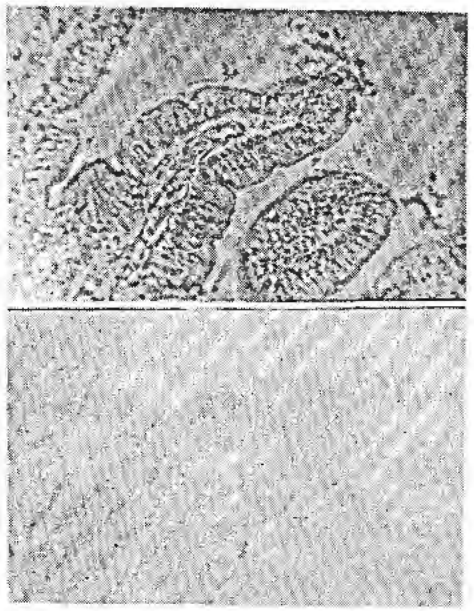

Figure 4. Exp. 123-1. Time: 24 hours after intraperitoneal injection of S^{35} tagged methionine. Small intestine. Moderate and diffuse incorporation in all the epithelial cells of the villi. Slight incorporation in the stroma of the musculo-connective axis of said villi. Specimen not stained. Above: phase contrast. Below: without phase contrast

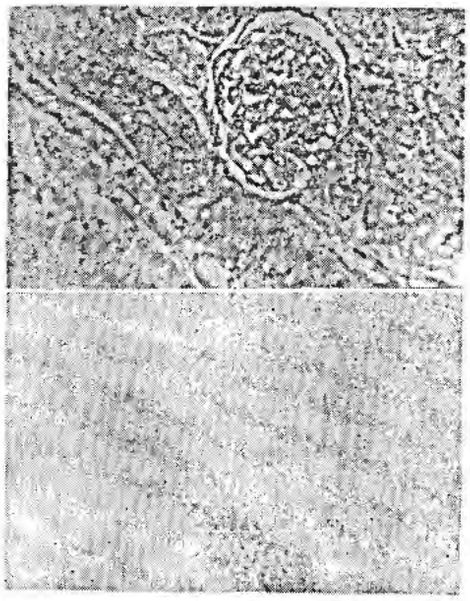

Figure 5. Exp. 123-1. Time: 24 hours after intraperitoneal injection of S^{35} tagged methionine. Kidney. Strong incorporation in the proximal convoluted tubules. Negative autoradiograph of neighbouring glomerule. Specimen not stained. Above: with phase contrast. Below: without phase contrast

tubules may possibly be explained, not only by its function as an essential element in cells of such high activity as are those of the portion of the nephron just mentioned, but also by the known fact that a certain part of the amino acids is excreted through the renal tubules. In this case, the autoradiographic method would make a contribution to the localization of the exact point of elimination of the substance in the nephron. This assumption would receive support from the fact that, during the metabolism of sulfur-containing amino acids (oxidation and sulfur elimination) brought into the organism, the sulfur released is eliminated mostly by the renal tract.

As regards the incorporation by the connective tissue of the capsules and stroma of various organs, by vascular connective tissue, the respiratory and intestinal tracts, the submucosa and corium connective tissue, the connective tissue of the serosae, the tendinous fasciculi and cartilaginous tissue, there is obvious incorporation of light intensity, diffusely distributed throughout the structures just mentioned, without any particular localization in the cellular portions. In the interpretation of fixation by connective tissue, one cannot rule out the hypothesis that, independent of the direct incorporation of said amino acids, and as a result of oxidation and deamination processes, the S^{35} is liberated and, if not eliminated, free to circulate through the organism and be incorporated as a free inorganic element by all the types of connective tissue. The lightness of the autoradiographs obtained in these structures would be explained by the fact that the amount in circulation is very minute.

Using the results of our earlier work[1] on the incorporation of S^{35} as sodium sulfate by the different tissues as a base, and making a study of it all by the same autoradiographic technique, interesting conclusions may be drawn by comparison with the above-described results of the use of S^{35} tagged DL-methionine. Thus it is that, in contradistinction with what happens in the parenchymas, S^{35} was actively incorporated by all types of connective tissue which, containing sulfur-bearing mucopolysaccharides, made up the capsules and stroma of the different organs. Incorporation by the hyaline and elastic varieties of cartilaginous tissue was very marked. However, it should be stated that S^{35} also was incorporated (although very slightly) in certain parenchymas such as the liver, spleen, glands, etc. Working on bone-marrow cultures, with methionine S^{35}, Lajtha[2] showed, on the other hand, the existence of its uniform distribution throughout all the cellular lines of the bone marrow, a point he was unable to show with S^{35} as a sulfate, the incorporation of which was limited to the myeloid series.

The interpretation of the different distribution of S^{35}, when administered in the inorganic form or incorporated in the molecule of an amino acid, such as methionine, would be based on the fact that, in the first case, metabolism would follow the elective affinity of the tissues which contain substances, such as the mucopolysaccharides, which are capable of incorporating S^{35} in the inorganic form. On the other hand, the S^{35} present in the methionine molecule must be used in the metabolism of said amino acid and be incorporated by the tissues in active development or by other parenchymas, requiring it for some phases of their metabolism.

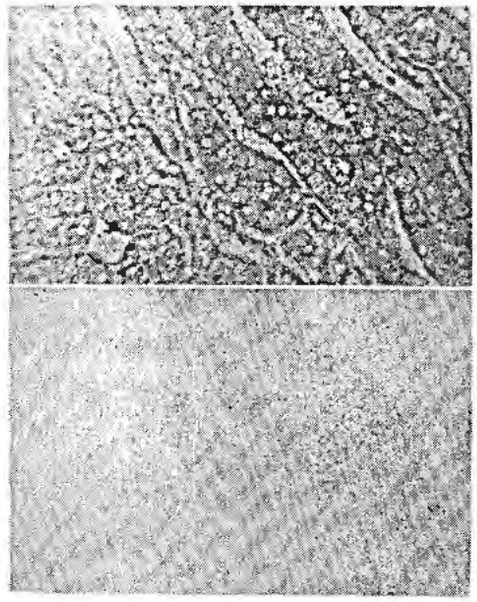

Figure 6. Exp. 123-1. Time: 24 hours after intraperitoneal injection of S^{35} tagged methionine. View of a zone showing proximal convoluted tubules in longitudinal section, distal tubules in transverse cross section. Intense incorporation in the interstitial stroma of the proximal convoluted tubules. Specimen not stained. Above: with phase contrast. Below: without phase contrast

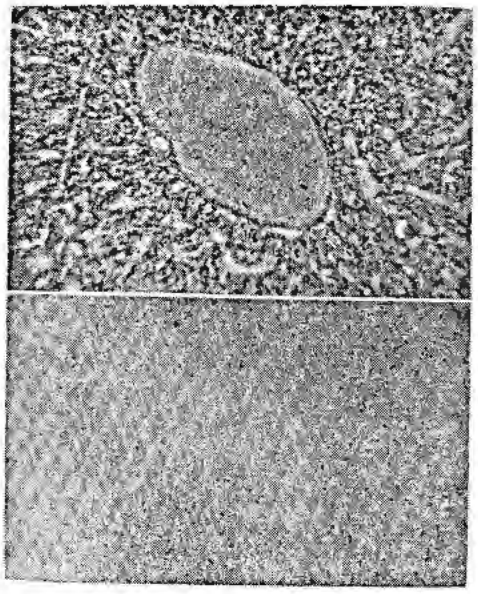

Figure 7. Exp. 123-1. Time: 24 hours after intraperitoneal injection of S^{35} tagged methionine. Liver. Moderate and diffuse incorporation in the cells of the trabeculae of a lobule. Negative autoradiograph of walls of the central lobular vessel. Specimen not stained. Above: with phase contrast. Below: without phase contrast

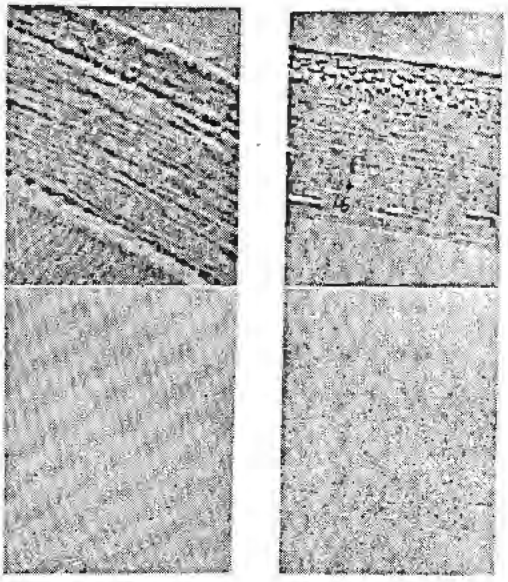

Figure 8. Comparison between incorporation of S^{35} and S^{35} tagged methionine in the cornea. Incorporation of S^{35} tagged methionine may be seen at the level of the corneal epithelium, as well as a very slight autoradiograph of the stroma, in contrast to the moderate incorporation of S^{35} in the stroma, and very slight incorporation by the corneal epithelium. Specimens not stained. Above: with phase contrast. Below: without phase contrast

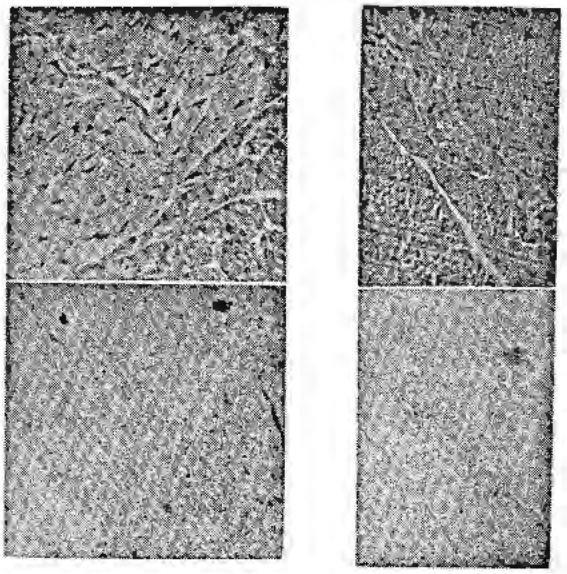

Figure 9. Comparison of the incorporation in two muscle tendon specimens of S^{35} tagged methionine and inorganic S^{35}. Moderate incorporation of S^{35} tagged methionine in the muscle cells, very slight in the tendinous fascicle. There is, instead, moderate incorporation of S^{35} in the tendon, very slight in the adjacent muscle. Specimens not stained. Above: with phase contrast. Below: without phase contrast

SUMMARY

1. Adult rats were used in the experiment. The detection technique used was autoradiography with stripping-film (Pelc's method).

2. Both sodium sulfate S^{35} and methionine S^{35} were administered by the intraperitoneal route.

3. The results achieved show definite incorporation, metabolism and distribution in the tissues of a fraction of both compounds. As regards S^{35} sodium sulfate, it has been observed that incorporation was predominant in some varieties of connective tissue; on the other hand there was less incorporation in the parenchymas. The S^{35} belonging to the tagged amino acid (methionine S^{35}) was captured mostly by the parenchymas, more so in those which showed proliferative activity. On the contrary, incorporation by connective tissue in general was much less marked.

CONCLUSIONS

The incorporation of sulfur is totally different when administered in the inorganic form, than when it is contained in an organic molecule. The apparent reason would be that the mechanism of incorporation in the organic form depends essentially on the features of metabolism of the organic compound and not on the sulfur incorporated in it. In support of this hypothesis, let us mention the fact that the incorporation of this type of compound mainly takes place in tissues which are functionally very active and in which protein synthesis is very intense.

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Nature of the I^{131} Compounds Appearing in the Thyroid Vein After Injection of Iodide I^{131}

By I. L. Chaikoff, A. Taurog and J. D. Wheat,* USA

The concentration of organic iodine in the plasma of euthyroid humans is approximately 5 μg per 100 cm^3. This is an exceedingly small concentration from the point of view of chemical identification; nevertheless, it has been possible to learn a great deal about the nature of the circulating thyroid hormone with the aid of several powerful tools developed during the last 15 years. These include (*a*) the 8-day radioactive isotope of iodine, I^{131}; (*b*) chromatographic and electrophoresis techniques, especially those involving filter paper, and (*c*) sensitive methods for the determination of chemical iodine.

There can be little doubt that the so-called protein-bound iodine of plasma differs from the iodine-containing protein of the thyroid gland, thyroglobulin. The latter contains various iodinated amino acids as part of a peptide chain, but the organic iodine in plasma is only loosely bound to protein. Recent evidence indicates that the circulating thyroid hormone consists primarily of thyroxine bound to a plasma protein having an electrophoretic mobility intermediate between that of α-1 and α-2 globulin.[1]

The recent discovery of triiodothyronine[2,3] has raised the question to what extent this highly active substance is normally present in the thyroid gland and in the circulation. Since injected triiodothyronine disappears from the peripheral circulation much more rapidly than does injected thyroxine,[4] the relative concentrations of these compounds in the general circulation at any time may not be indicative of their relative rates of secretion by the thyroid gland. In this connection, therefore, it seemed of interest to investigate the relative concentrations of the various I^{131}-compounds appearing in the blood of the thyroid veins of animals injected with iodide-I^{131}.

By sampling the venous blood draining the thyroid gland before it is diluted with the entire circulatory volume, we thought that it should be possible to determine which of the iodinated compounds in the thyroid gland are actually released into the circulation.

Withdrawal of blood from the thyroid vein was facilitated by the use of large animals. Most of the work described here was carried out on sheep. One horse and one dairy calf were also used as experimental animals.

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MATERIALS AND METHODS

Two different groups of sheep were employed in these experiments: (1) Suffolks, bred and raised on the Armstrong tract of the University of California College of Agriculture and (2) Western White Face, purchased from a near-by rancher. The results were not the same with these two groups, presumably because of widely differing rates of thyroid hormone formation (see below). The horse and the dairy calf were stock University tract animals. All animals were on pasture before they were injected with I^{131} except the calf, which was still suckling.

Injection of I^{131}

Large doses of I^{131} were required in these experiments to permit detection of weak components on the filter paper chromatograms of plasma. Oak Ridge I^{131} was used without further purification. All injections were made via the external jugular with the aid of a shielded syringe operated by a crank. The sheep, weighing 37–74 lb, were injected with 25 mc of I^{131}. The horse, weighing 1120 lb, received 175 mc. The calf, weighiog 180 lb, received 45 mc.

Treatment with Thyrotropic Hormone

Two sheep were treated with thyrotropic hormone. (We are indebted to Parke, Davis and Company for a supply of Thyrotropic Hormone Preparation Rx X5094, which contained 2 USP units per mg.) One animal (No. 3, Table I) received, intravenously, 50 mg of thyrotropin daily for 3 days before it was injected with I^{131}. The last dose of thyrotropin was given 2 hours before the I^{131} was injected. The other animal (No. 10, Table I) received two injections

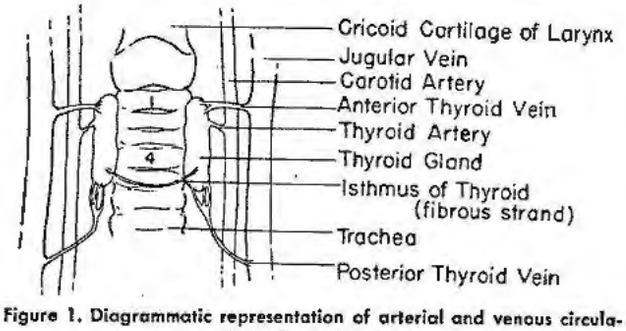

Figure 1. Diagrammatic representation of arterial and venous circulation of the sheep thyroid gland

of 50 mg of thyrotropin, separated by an interval of 8 hours. The I^{131} was administered immediately after the first TSH injection.

Surgical Techniques

All surgical procedures were performed in the School of Veterinary Medicine of the University of California College of Agriculture at Davis, California. At the desired time after I^{131} injection, the animals were anesthetized by intravenous injections of Nembutal (sheep and calf) or chloral hydrate (horse). The thyroid gland, thyroid veins, and carotid artery on one side were exposed. The anatomical details varied somewhat as follows:

Sheep

In the sheep, one large thyroid vein was observed emerging from the anterior pole of the gland and entering the jugular (Fig. 1). Several smaller veins emerged from the posterior pole of the gland. A mass ligature was first placed around the posterior veins close to their origin within the gland. The large anterior vein was ligated distally from the gland, close to its junction with the jugular, and blood was drawn from this vein directly into a heparinized syringe. It was usually possible to obtain 5 cm^3 of thyroid venous blood in this manner. Immediately after the venous sample had been taken, blood was removed from the carotid artery, and both lobes of the thyroid were removed.

Horse

The major thyroid vein in the horse was a fairly large vessel, approximately $^1/_8$ inch in diameter, emerging from the posterior pole of the gland. Also visible were a smaller anterior vein and several much smaller posterior veins. All but the large posterior vein were ligated near their origin within the gland. The major thyroid vein was ligated near its junction with the jugular, and blood was drawn from it directly into a syringe.

Calf

The arrangement of the thyroid veins in the calf was similar to that in the sheep, but the vessels were considerably smaller in diameter than those of the sheep, even though the body weight of the animal was much greater. The procedure for obtaining thyroid venous blood was similar to that described for the sheep, but considerable difficulty was encountered because of the small size of the vein.

Measurement of Uptake of I^{131} by the Thyroid Gland

Because of the large amount of radioactivity contained in the thyroid glands, it was not desirable to handle the entire gland for measurement of uptake. A weighed aliquot of the entire gland was homogenized with a measured volume of bicarbonate-Ringer's solution to yield an homogenate containing approximately 50 mg of tissue per cm^3. An aliquot of this homogenate was used for I^{131} assay, and another aliquot (0.5 cm^3) was digested with 10 mg of pancreatin for 15–18 hr for chromatographic analysis.[5] Because of the limitation imposed by the high radioactive content, the thyroid glands could not be carefully trimmed of extraneous tissue before they were weighed. The uptakes calculated in the above manner, therefore, were probably too high by several per cent.

Precipitation of Protein-Bound I^{131} of Plasma with Trichloroacetic Acid

0.5 cm^3 of plasma was added to 2 cm^3 of 20 per cent trichloroacetic acid; the mixture was stirred well with a glass rod, and 3 cm^3 of 10 per cent trichloroacetic acid were added with stirring. After centrifugation, the supernatant was transferred to a volumetric flask and the precipitate was washed once with 5 cm^3 of 10 per cent trichloroacetic acid. The supernatants were combined and an aliquot was taken for I^{131} assay in a well-type scintillation counter. The precipitate was dissolved in 2*N* NaOH, with warming, the solution was made up to volume, and an aliquot was taken for counting. An appreciable percentage of inorganic I^{131} (2–5 per cent) may remain with the trichloroacetic acid precipitate in the above procedure. The chromatographic procedure described below is much more satisfactory for the detection of small percentages of protein-bound I^{131} in plasma.

Chromatographic Procedures

The techniques used for filter paper chromatography of thyroid hydrolysates and plasma have been described in previous communications.[5,6] The following two additions to the general procedures were made in the present investigation: (1) The use of a solvent consisting of butanol-ethanol-2*N* NH_4OH, 5: 1: 2. This solvent gives results similar to the butanol-dioxane-NH_4OH described by Gross and Leblond.[7] It is useful for detecting triiodothyronine, which it separates very well from thyroxine. However, it does not separate mono- and diiodotyrosine, and gives only a poor separation of thyroxine from inorganic iodide. (2) The use of concentrated butanol extracts of plasma for filter paper chromatography. In previous work we have generally employed whole plasma for chromatographic analysis rather than concentrated butanol extracts, to avoid as much as possible introducing I^{131} artifacts into the results. We have found that much of the difficulty previously encountered in this regard can be eliminated by the addition of a small amount of thiouracil to butanol extracts of plasma just before they are concentrated. The details of the procedure are as follows: 1.5 cm^3 of plasma were extracted in a 15-cm^3, conical centrifuge tube, first with 3 cm^3, then 3 more times with 2 cm^3 of butyl alcohol. The butanol extracts were separated by centrifugation and drawn off into a second tube with the aid of a capillary and suction. The combined butanol extracts were transferred to a 10-cm^3 beaker, and a small amount of thiouracil (0.1–0.3 mg) was mixed in with a stirring rod. The solvent was removed at 40–50° in a stream of nitrogen under reduced pressure. As soon as the

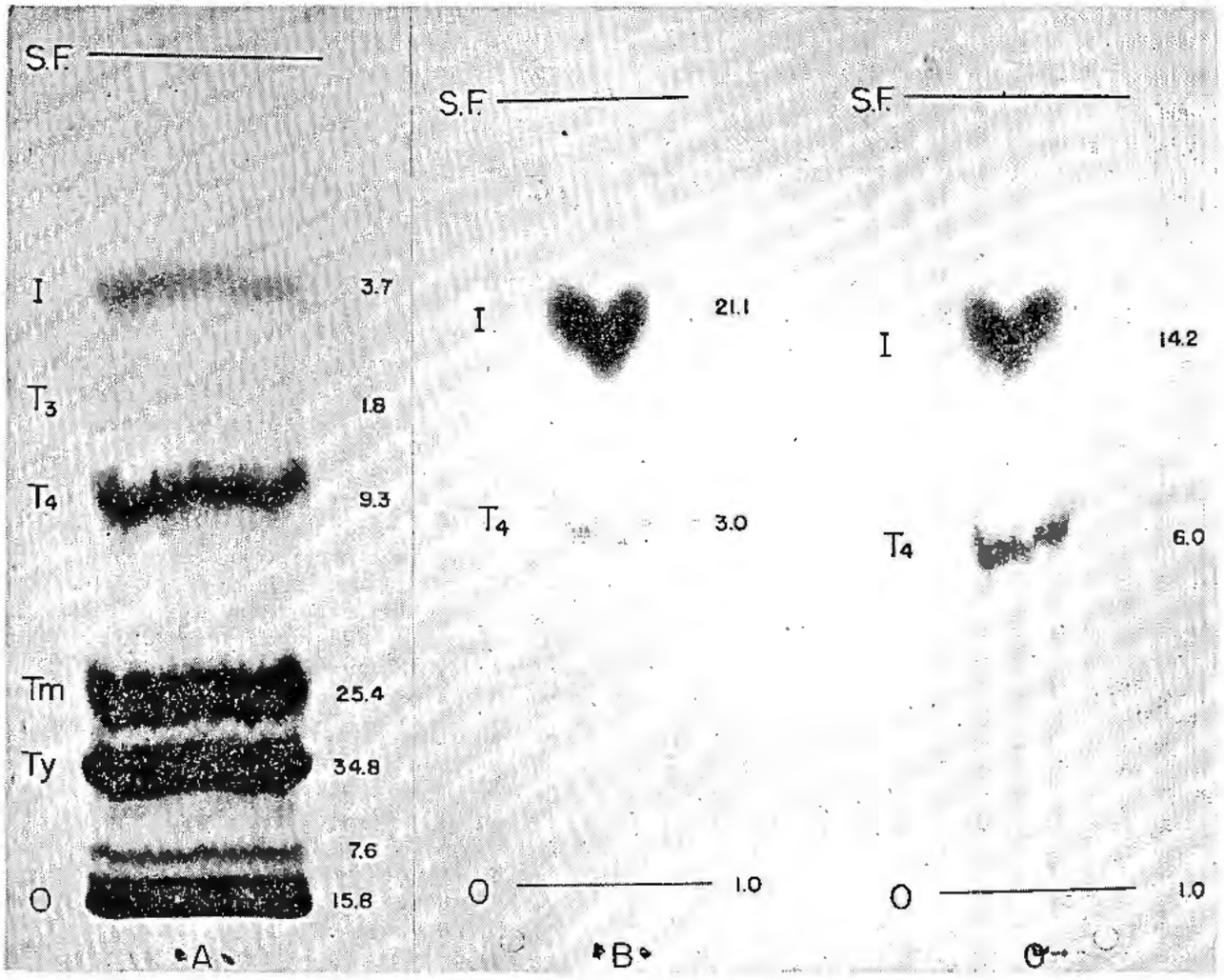

Figure 2. Radioautographs of chromatograms showing the nature of the I^{131}-compounds in thyroid gland, carotid arterial plasma and thyroid venous plasma of Sheep No. 1, Table I, 24 hours after intravenous injection of 29 mc of carrier-free I^{131}. A, 20 μl of a pancreatin digest of the thyroid gland; B, 40 μl of whole plasma obtained from the carotid artery; C, 40 μl of whole plasma obtained from the thyroid vein. Solvent: collidine-water-ammonia. Symbols: O, origin; Ty, diiodotyrosine; Tm, monoiodotyrosine; T_4, thyroxine; T_3, triiodothyronine; I, inorganic iodide; S F, solvent front. The numbers alongside the bands refer in A, to the percentage of the total I^{131} in the various fractions; in B and C to the actual counts per second in the various fractions. The radioautographs of the plasma chromatograms were exposed for 13 days

residue was dry, it was rubbed up thoroughly with 200 μl of 1 : 1 ethanol-2*N* NH_4OH. The resulting suspension was used directly for filter paper chromatography. Two or three deliveries of 20 μl each were made for this purpose.

Extraction of plasma I^{131} with butanol was usually only 75–80 per cent complete. Further loss of I^{131} occurred in the concentration procedure. It must be emphasized, therefore, that the relative amounts of I^{131} in the chromatograms prepared from concentrated butanol extracts were not necessarily the same as they were in the original plasma. However, even though the concentration procedure may have altered the relative amounts of I^{131} compounds, it was still useful for detecting the presence of weakly labeled components.

For quantitative estimation of the I^{131} in the various compounds on the chromatograms, the sections of the chromatograms corresponding to the bands observed on the radioautographs were cut out, folded, and placed in vials for counting in a well-type scintillation counter. The counter had a sensitivity of 10^4 counts per second per μc of I^{131}, and a background of 1.5 counts per second.

Determination of Protein-Bound Iodine Levels of Plasma

In a few of the animals, the level of protein-bound iodine in the peripheral circulation was determined by a method previously described.[8] In the group of Western White Face sheep, the level of protein-bound iodine was also determined in pooled samples of thyroid venous plasma.

RESULTS

Table I summarizes the nonchromatographic data obtained in the present investigation. Body weights, thyroid gland weights, and thyroid uptakes of I^{131} are shown for all the animals used. Also shown in Table I are results of protein-bound I^{131} studies on

Table I. Relative Concentration of Protein-bound I^{131} in Carotid Artery and Thyroid Vein of Various Animals Injected with I^{131}

Animal	Wt of animal, lb	Wt of thyroid gland, gm	Interval after I^{131}, hr	Uptake of I^{131} by thyroid, % ID*	Protein-bound I^{131} Total I^{131} Carotid artery, %	Protein-bound I^{131} Total I^{131} Thyroid vein, %	Protein-bound I^{127} of arterial plasma, $\mu g/100\ cm^3$	Protein-bound I^{127} of thyroid venous plasma, $\mu g/100\ cm^3$
1. Suffolk sheep ♀	47	1.4	26	14	22	37		
2. Suffolk sheep ♀	54	1.75	6	10	4.8	7.4		
3. Suffolk sheep ♂ †	46	4.0	25	21	45	56		
4. Suffolk sheep ♀	74	2.3	22	22	20	51	5.4	
5. Western White Face sheep ♀	37	1.9	3	2.5	3.9	3.7		
6. Western White Face sheep ♀	40	2.4	6	5.7	2.3	2.3		
7. Western White Face sheep ♂	52	2.7	12	3.6	3.1	3.5	2.5‡	2.4‡
8. Western White Face sheep ♂	44	3.4	24	16	3.8	5.7		
9. Western White Face sheep ♂	52	7.3	48	32	2.8	3.9		
10. Western White Face sheep ♂‡	66	4.1	22	22	2.7	3.8		
11. Horse	1120	43	24	8	8	43		
12. Dairy calf	180	4.8	20	5.6	6.3	73	4.4	

* Per cent of injected dose.
† Treated with thyrotropic hormone (see "Methods").
‡ Values obtained using pooled samples from sheep 5-9.

carotid arterial and thyroid venous plasma, as determined by trichloroacetic acid precipitation. A few determinations of protein-bound iodine levels are also reported.

Most of the effort in the present investigation was devoted to filter paper chromatographic analyses of the arterial and venous plasma of the thyroid, and it is the results of those analyses (shown in Figs. 2–6) that will be described in detail. Each section below deals with the chromatographic results obtained with a particular group or species of animals.

Suffolk Sheep

Figure 2 shows radioautographs of filter paper chromatograms illustrating the nature of the I^{131} compounds in samples of carotid arterial plasma, thyroid venous plasma, and thyroid gland, all simultaneously removed from a 47-lb female sheep 26 hr after injection of 29 mc of I^{131}. The total uptake of I^{131} by the thyroid at that time was 14 per cent of the injected dose (Sheep No. 1, Table I).

Thyroid Gland

Figure 2A shows the nature of the I^{131} compounds in a pancreatin digest of the thyroid gland. The numbers beside the various bands indicate the percentages of the total I^{131} found in the various I^{131} fractions as determined by cutting out the corresponding sections of the filter paper chromatograms and determining their activity in a well-type scintillation counter. Mono- and diiodotyrosine comprised by far the largest part of the I^{131} in the thyroid gland. Thyroxine-I^{131} accounted for only 9.3 per cent of the total. The presence of 16 per cent of the I^{131} at the origin most likely reflects the incompleteness of the hydrolysis. The band between thyroxine and inorganic iodide is the region to which triiodothyronine moves in the collidine solvent which was used. This area contained 1.8 per cent of the total I^{131}. For more accurate estimation of triiodothyronine-I^{131}, however, we prefer to use the results of chromatography in butanol-ethanol-NH_4OH since, under these conditions, the triiodothyronine band is more discrete and is better separated from adjacent bands. With this solvent, triiodothyronine-I^{131} comprised 1.3 per cent of the total I^{131}, and the ratio (triiodothyronine-I^{131})/(thyroxine-I^{131}) (using the value for thyroxine-I^{131} obtained with the collidine solvent) was 0.14.

Plasma from Carotid Artery and Thyroid Vein

Figures 2B and 2C show radioautographs of filter paper chromatograms illustrating the nature of the I^{131} compounds in the carotid artery and the thyroid vein, respectively. These chromatograms were prepared with whole plasma to avoid losses of I^{131} which occur during extraction procedures. The numbers appearing on the autographs refer to the counts per second in the various bands, as determined by cutting out the corresponding sections of the filter paper chromatograms and counting them in a well-type scintillation counter. These numbers also give the relative concentrations of I^{131} in artery and vein since the volume delivered for chromatography was the same for both (40 μl).

Comparison of Figs. 2B and 2C indicates that the thyroxine-I^{131} concentration was twice as great in the thyroid vein as in the carotid artery. The concentration of iodide-I^{131}, on the other hand, was less in the vein than in the artery, indicating active removal of iodide by the gland. The A-V difference might have been even greater were it not for deiodination of iodotyrosines, which probably occurs in the thyroid gland and which may release extra iodide-I^{131} into the thyroid vein.

Perhaps the most revealing feature of the radioautograph prepared from thyroid venous plasma (Fig. 2C) is the absence of the I^{131}-iodotyrosines which are so abundant in the thyroid gland itself. In all the animals examined in the present investiga-

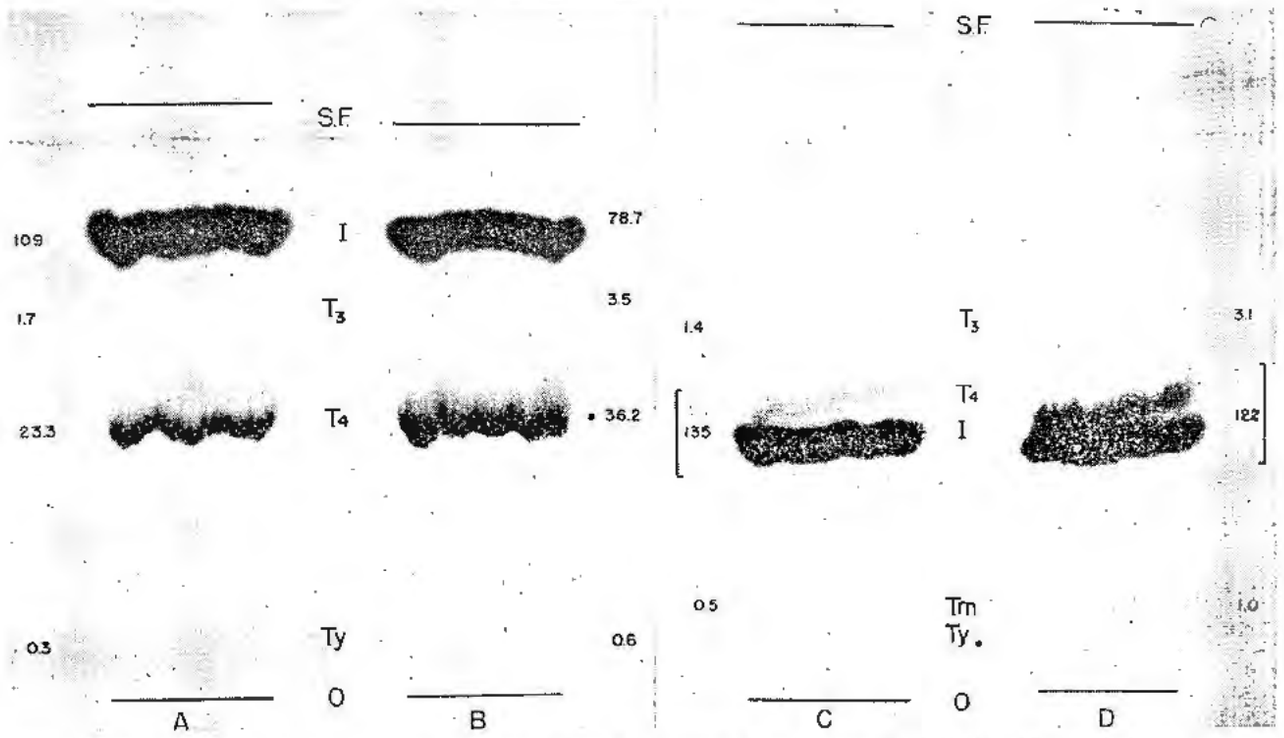

Figure 3. Radioautographs of chromatograms of concentrated butanol extracts of the same plasma samples used for the preparation of Fig. 2. See text for procedure. A, 40 μl of concentrated extract of carotid arterial plasma; solvent, collidine-water-ammonia. B, 40 μl of concentrated extract of thyroid venous plasma; solvent, collidine-water-ammonia. C, 40 μl of concentrated extract of carotid arterial plasma; solvent, butanol-ethanol-2N NH_4OH 5:1:2. D, 40 μl of concentrated extract of thyroid venous plasma; solvent, butanol-ethanol-2N NH_4OH. All radioautographs exposed 11 days. Numbers alongside the bands refer to counts per second in the various fractions. See Fig. 2 for meaning of symbols

tion (except in one treated with thyrotropic hormone) there was never more than a trace of I^{131}-iodotyrosine in the thyroid vein. Either the iodotyrosines are quantitatively converted into thyroxine in the thyroid gland or else, as suggested by Roche and Michel,[9] deiodination by thyroid enzyme(s) is an important alternate fate for these compounds. The latter explanation is probably correct.

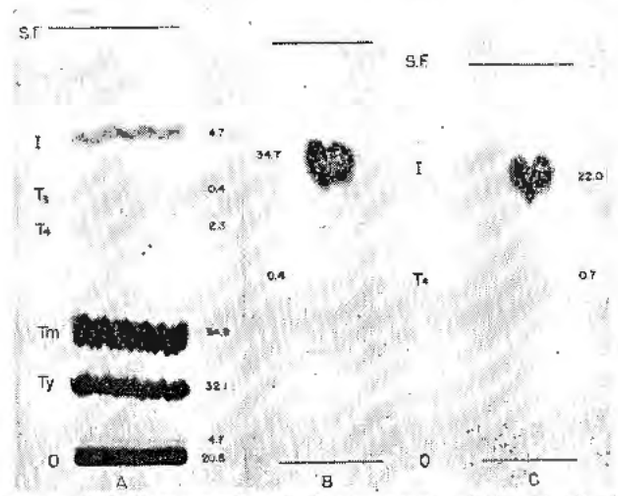

Figure 4. Radioautographs of chromatograms of thyroid gland, carotid arterial plasma and thyroid venous plasma of Sheep No. 9, Table I, 48 hours after intravenous injection of 30 mc of carrier-free I^{131}. A, 20 μl of a pancreatin digest of the thyroid gland. B, 40 μl of whole plasma obtained from the carotid artery. C, 40 μl of whole plasma obtained from the thyroid vein. Solvent: Collidine-water-ammonia. Plasma radioautographs exposed 11 days. See Fig. 2 for meaning of symbols and numbers

Chromatography of Concentrated Butanol Extracts of Plasma

To permit the detection of weak I^{131} components in plasma, such as triiodothyronine, it was necessary to concentrate the I^{131} before chromatography. This was accomplished by preparing butanol extracts of plasma and then concentrating them as described above under "Methods". Figure 3 shows radioautographs of chromatograms prepared from concentrated butanol extracts of the same sheep plasma samples used for the preparation of Fig. 2. Results with two different chromatographic solvents were obtained.

The results with *collidine-water-ammonia* are shown in Figs. 3A and 3B, and agree in general with those obtained with whole plasma (Fig. 2). Quantitative discrepancies can be explained (as described under "Methods") by losses of I^{131} involved in the extraction procedure. Triiodothyronine-I^{131} appeared as a definite band in the concentrated extract of thyroid venous blood, whereas it was too weak to be detected in whole plasma. A very faint band also appeared in the region of diiodotyrosine. The latter comprised less than 0.5 per cent of the total I^{131}, and might possibly have arisen as an artifact during the concentration procedure.

The results with *butanol-ethanol-2N* NH_4OH are shown in Figs. 3C and 3D. The separation of thyroxine from inorganic iodide was rather poor, but triiodothyronine appeared as a discrete band, well separated from neighboring components. In this solvent also, a faint band (<1 per cent) appeared in the iodotyrosine region, but it is difficult to be certain of its physiological significance.

The ratio (triiodothyronine-I^{131})/(thyroxine-I^{131}) in thyroid venous plasma was estimated from these chromatograms to be approximately 0.10, somewhat less than the same ratio for the thyroid gland.

Treatment with Thyrotropin

One of the sheep (No. 3, Table I) was treated with thyrotropin to determine whether this would have any effect on the nature or on the distribution of I^{131} in the thyroid vein or in the thyroid gland. The uptake of I^{131} by the thyroid of this animal was in the normal range at 25 hours, but the peak uptake may have occurred at an earlier interval. The chromatograms of the thyroid venous plasma of the TSH-treated sheep showed two significant differences from the untreated animal described above: (*a*) weak, but possibly significant, bands were present in the area of the iodotyrosines and (*b*) a large percentage (approximately 40 per cent) of the I^{131} remained at the origin of the chromatograms. At first it was thought that this indicated release of I^{131}-thyroglobulin into the circulation, but subsequent experiments on other animals not treated with TSH raised the possibility that some other I^{131}-containing protein may also have been released into the circulation.

Western White Face Sheep

A group of these animals was injected with I^{131}. At intervals ranging from 3 to 48 hours following injection, carotid blood, thyroid venous blood, and thyroid glands were removed and examined as described above. Although the total uptake of I^{131} by the thyroids of these sheep was as high as that obtained for the Suffolk sheep, the startling observation was made that very little thyroxine-I^{131} was released into the circulation. Only after 24 hours was there any detectable thyroxine-I^{131} in the thyroid vein. Analysis of the thyroid gland itself at that interval revealed the presence of relatively low, but definite amounts of thyroxine-I^{131}. In fact, thyroxine-I^{131} was readily detectable in the gland at the 3-hour interval.

Figure 4 shows radioautographs of chromatograms of the 48-hour samples in this group of sheep.

Thyroid Gland (Fig. 4A)

The total uptake of I^{131} in the thyroid gland at this interval was 32 per cent and over 95 per cent of this was organically bound. The ratio (thyroxine-I^{131})/(iodotyrosine-I^{131}) was considerably less in this sheep than in the Suffolk sheep, most likely indicating a lower rate of thyroxine formation. Perhaps, in these animals, deiodination of diiodotyrosine by the

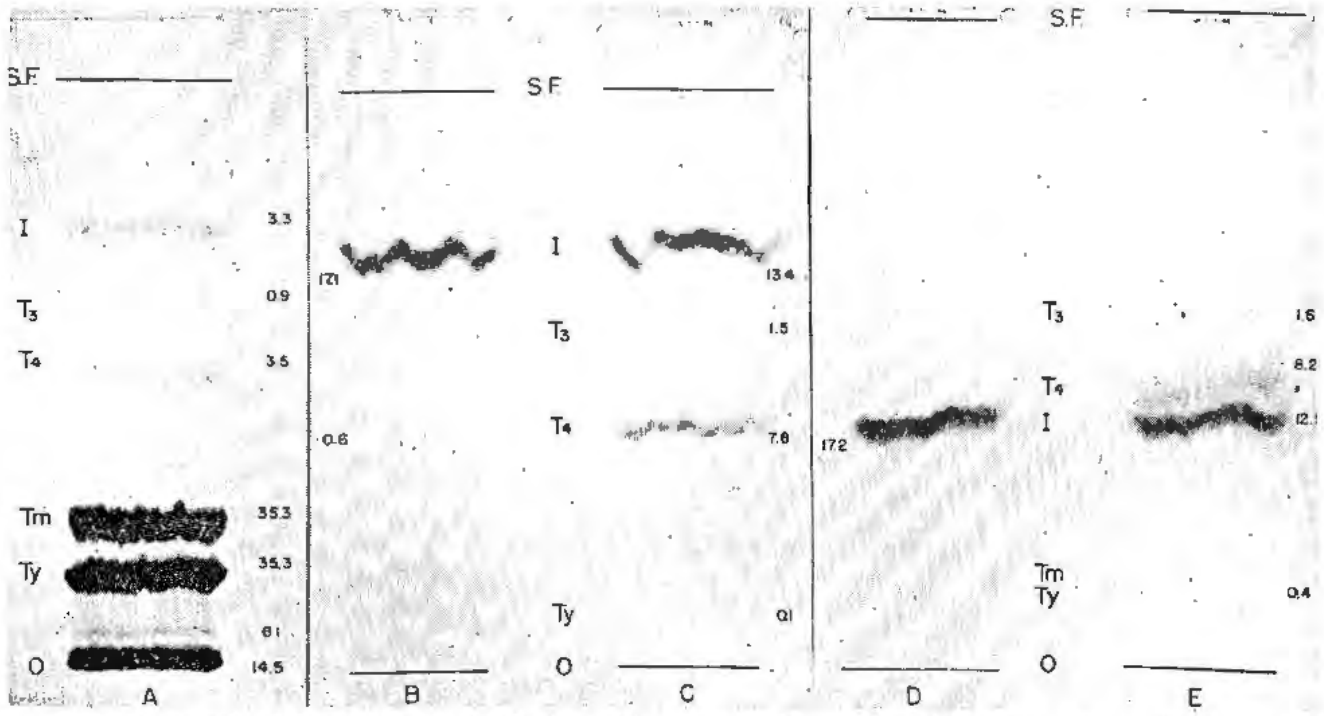

Figure 5. Radioautographs of chromatograms of thyroid gland and of concentrated butanol extracts of carotid arterial and thyroid venous plasma of a horse 24 hours after intravenous injection of 175 mc of I^{131}. A, 20 μl of a pancreatin digest of the thyroid gland; solvent, collidine-water-ammonia. B, 40 μl of a concentrated butanol extract of carotid arterial plasma; solvent, collidine-water-ammonia. C, 40 μl of a concentrated butanol extract of thyroid venous plasma; solvent, collidine-water-ammonia. D, 40 μl of a concentrated butanol extract of carotid arterial plasma; solvent, butanol-ethanol-2N NH_4OH 5:1:2. E, 40 μl of a concentrated butanol extract of thyroid venous plasma; solvent, butanol-ethanol-2N NH_4OH. All plasma radioautographs exposed 11 days. Numbers alongside the various bands refer to counts per second except in radioautograph A, in which they refer to per cent of the total I^{131} in the various fractions. See Fig. 2 for meaning of symbols

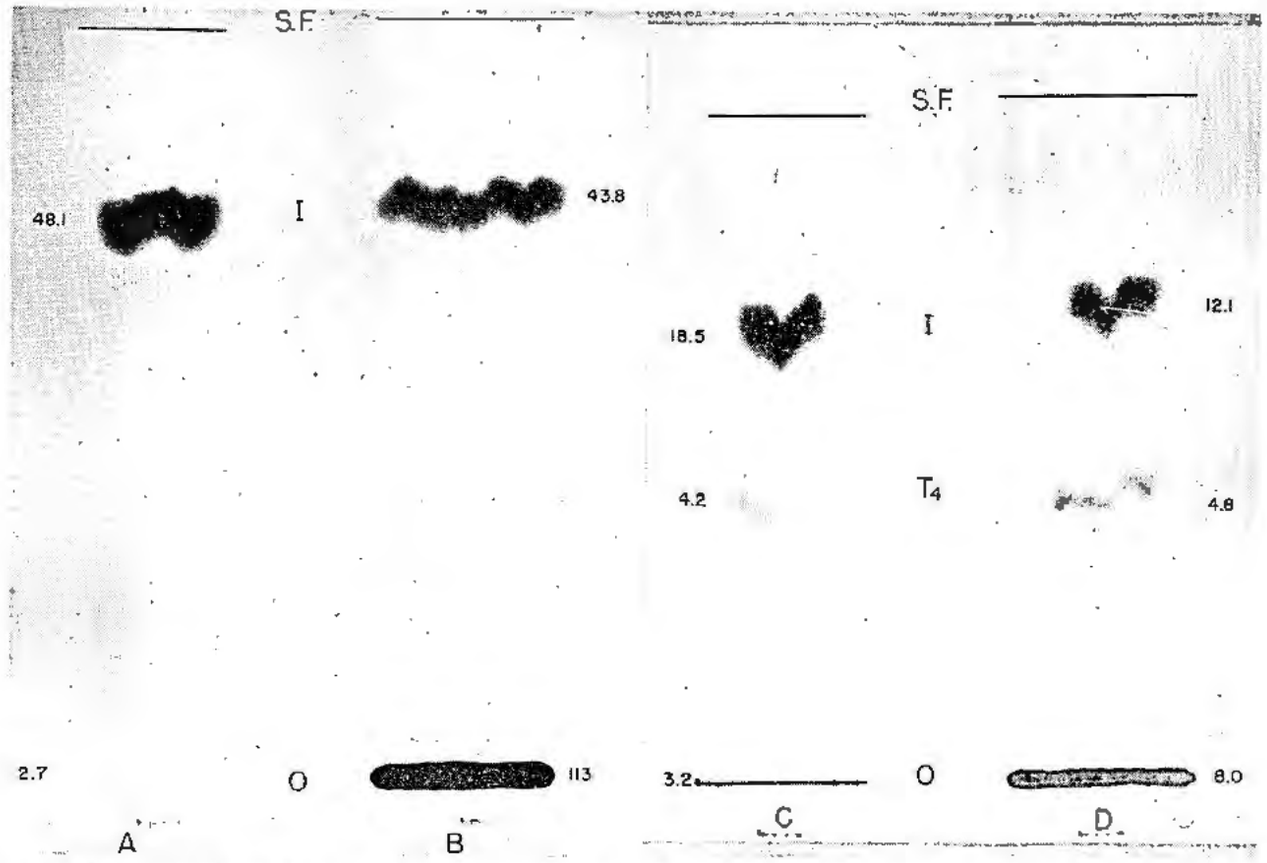

Figure 6. Radioautographs of chromatograms of plasma obtained from calf and sheep. A, 40 μl of whole plasma from carotid artery of a calf 20 hours after intravenous injection of 45 mc of carrier-free I^{131}. B, 40 μl of whole plasma from thyroid vein of same animal. C, 40 μl of whole plasma from carotid artery of Sheep No. 4, Table I, 22 hours after intravenous injection of 26 mc of carrier-free I^{131}. D, 40 μl of whole plasma from thyroid vein of same animal. Solvent: collidine-water-ammonia. Radioautographs A and B were exposed for 11 days, C and D for 14 days. Numbers alongside the various bands refer to counts per second in the various fractions. See Fig. 2 for meaning of symbols

thyroid gland proceeded at a more rapid rate than did the conversion of diiodotyrosine to thyroxine, so that in spite of the high I^{131} uptake, only little thyroxine-I^{131} was formed.

Carotid Artery and Thyroid Vein (Figs. 4B, 4C)

The thyroid vein contained only 3 per cent of its I^{131} in the form of thyroxine, indicating that very little hormone was being released into the circulation. This view was confirmed by the finding that the protein-bound iodine level of the pooled plasma from this group of sheep was considerably lower than that observed in the Suffolk sheep (Table I).

The concentration of I^{131}-thyroxine in the thyroid vein was only slightly greater than that in the carotid artery. A very significant drop in iodide-I^{131} concentration was observed in the vein, indicating that the thyroid was actively taking up iodide.

Effect of Thyrotropin Injection

After it had been found that the thyroids of this group of sheep were quite inactive, one of the previously unused animals in the group (No. 10, Table I) was injected with thyrotropin, both immediately before and 8 hours after receiving I^{131}, to test whether this would increase the rate of I^{131}-thyroxine secretion into the thyroid vein. As shown in Table I, no effect was observed, as measured by the trichloroacetic acid precipitation procedure. However, application of the filter paper chromatographic procedures did reveal a very small, but definite, increase over the control animal. It is not possible, from this single experiment, to decide whether this sheep was particularly insensitive to TSH injection or whether the dosage or timing of the TSH injections was responsible for the low response.

Horse

Figure 5 shows radioautographs of chromatograms of plasma and thyroid removed from a horse 24 hours after injection of 175 mc of I^{131}. The total uptake of I^{131} in the thyroid glands at that time was 8 per cent of the injected dose.

Thyroid

The distribution of I^{131} in the various iodine compounds of the thyroid gland found after enzymatic hydrolysis is shown in Fig. 5A. The results are qualitatively similar to those obtained with the sheep. Quantitatively, the ratio of thyroxine-I^{131} to iodotyrosine-I^{131} is quite low, resembling that of the Western White Face sheep. The ratio (triiodothyronine-I^{131})/(thyroxine-I^{131}) calculated as described for the Suffolk sheep, was 0.22 for the horse

thyroid. This was the highest value for this ratio observed for any animal in the present investigation.

Carotid Artery and Thyroid Venous Plasma

As illustrated in the radioautographs shown in Figures 5B-5D, the thyroxine-I^{131} concentration in the thyroid vein of the horse was much greater than its concentration in the carotid artery. The chromatograms in this case were prepared from concentrated butanol extracts of plasma and were developed with collidine-water-ammonia (Figs. 5B–C) or with butanol-ethanol-2*N* NH_4OH (Figs. 5D–E). With both solvents, scarcely any thyroxine-I^{131} was detectable in the arterial plasma, but in the venous plasma the band corresponding to thyroxine-I^{131} was very prominent. The ratio (triiodothyronine-I^{131})/(thyroxine-I^{131}) in the thyroid venous plasma, calculated as for the Suffolk sheep, was 0.19, roughly the same as that obtained for the gland.

In the horse, as in the other animals examined, there was only a faint band present in the iodotyrosine area of the chromatogram of the thyroid venous plasma. It is questionable whether this small activity in a concentrated butanol extract of plasma has any physiological significance.

Calf

Chromatographic analysis of the thyroid arterial and venous plasma obtained from a young dairy calf 20 hours after injection of iodide I^{131} is shown in Figs. 6A and 6B, respectively. The results are much different from those described above for the sheep or horse. The thyroid venous plasma of the calf contained as its major I^{131} component a thyroglobulin-like band which remained at the origin of the chromatogram and which was not extractable with butanol. A similar, but much weaker, band was observed in the arterial plasma. No detectable thyroxine-I^{131} was present in either artery or vein. The distribution of I^{131} in the thyroid gland resembled that of the sheep shown in Fig. 4A.

The presence of a thyroglobulin-like I^{131} component in the plasma of rats treated with destructive doses of I^{131} was reported in a previous communication from this laboratory.[10] However, this component was not observed in the circulation at an interval as early as 24 hours after the injection of I^{131}. Similarly, Robbins *et al.*[11] examined the serum of I^{131}-treated hyperthyroid patients four or more *days* after administration of I^{131} in order to identify thyroglobulin-I^{131} in the circulation. It is questionable, therefore, whether sufficient tissue damage had occurred in the thyroid of the calf after only 20 hours (uptake only 5.6 per cent) to release relatively large amounts of thyroglobulin into the circulation. Unfortunately, the small amount of blood obtained from the thyroid vein of the calf precluded further study of the nature of the protein-like I^{131} component at the origin of the chromatogram. In connection with these results, it is of interest to point out that Larson *et al.*[12] described, in the serum of hyperthyroid patients receiving therapeutic doses of I^{131}, a non-butanol-extractable I^{131} component which migrated with albumin in electrophoresis studies. A similar I^{131} component in the plasma of I^{131}-treated patients has also been observed by Robbins (personal communication) and by Dingledine *et al.*[11]

The formation of an I^{131}-containing component which remained at the origin of the chromatogram was also encountered in one of the Suffolk sheep (No. 4, Table I; Figs. 6C and 6D). In the thyroid vein of this animal, over 30 per cent of the total I^{131} behaved like I^{131}-thyroglobulin. The corresponding figure for the peripheral circulation was 12 per cent. In contrast to the calf, however, thyroxine-I^{131} was definitely detectable in both the thyroid vein and the thyroid artery of this animal. The concentration of the hormonal I^{131} was only slightly greater in the vein than in the artery.

DISCUSSION

In previous studies in this laboratory dealing with the nature of the I^{131} compounds in the plasma of rats injected with large doses of iodide I^{131}, we did not encounter (on our filter paper chromatograms) significant amounts of I^{131} compounds other than thyroxine and inorganic iodide. The reports by Gross and Pitt-Rivers and by Roche and his coworkers that appreciable amounts of I^{131}-triiodothyronine may also be present in plasma induced us to reinvestigate our earlier findings and to carry out further experiments on the nature of the I^{131} compounds released into the circulation by the thyroid gland. It occurred to us that the best place to look for I^{131}-triiodothyronine would be in the thyroid vein since the rapid rate of removal of this compound from the circulation might explain our failure to detect it in past experiments. Furthermore, in the present investigation we have made use of the following additions to our filter paper chromatographic technique for separating the I^{131} components of plasma: (1) a chromatographic solvent consisting of butanol-ethanol-2*N* NH_4OH 5 : 1 : 2, patterned after the butanol-dioxane-2*N* NH_4OH solvent of Gross and Leblond.[7] This solvent is especially valuable for separating triiodothyronine from thyroxine. (2) A procedure for concentrating the I^{131} in plasma involving extraction of the plasma with butanol, concentration of the extract to dryness in the presence of a small concentration of thiouracil, and solution of the residue in ammoniacal ethanol.

With the aid of the above procedures, it was possible to detect the presence of I^{131}-triiodothyronine in the thyroid veins of several sheep and one horse after injection of large doses of iodide-I^{131}. The same procedures applied to the peripheral circulation were not sensitive enough to detect I^{131}-triiodothyronine with certainty.

The triiodothyronine-I^{131} in the thyroids of the animals examined here amounted to 10–20 per cent of the thyroxine-I^{131}. These values do not necessarily indicate the ratio of stable triiodothyronine to thyroxine. Only chemical analysis can yield this in-

formation, since it is impossible in the experiments described here to be certain that the I^{131} had the same specific activity in both of these compounds.

The failure of animals such as the Western White Face sheep and the dairy calf to show any appreciable amount of I^{131}-thyroxine in the thyroid vein even 24 hours after I^{131} administration was most surprising. In the case of the sheep, this slow release of I^{131}-thyroxine into the circulation was coupled with a fairly rapid uptake of I^{131} by the thyroid gland. Chromatographic analysis of the gland at the 24-hour interval revealed that practically all the I^{131} was organically bound, and that an appreciable, but low, fraction (2–3 per cent) of the total I^{131} was in the form of thyroxine. This interesting observation raises the question whether I^{131} uptake by the thyroid gland is truly an index of thyroid function, since mere uptake of I^{131} is not necessarily a reflection of the rate of thyroxine secretion into the circulation.

The most consistent finding in all the experiments reported here was the absence of I^{131}-iodotyrosines from the thyroid vein of all animals (except one thyrotropin-treated sheep). Even at a time when the thyroid gland contained 75 per cent or more of its I^{131} in the form of iodotyrosines, no more than a trace of these compounds normally appeared in the thyroid vein. It may be concluded, therefore, that iodotyrosines do not normally leave the thyroid gland as such. Deiodination probably plays an important role in the fate of these compounds in the thyroid gland, as previously suggested by Roche and Michel.

SUMMARY

1. An investigation was made of the nature of the I^{131} compounds appearing in the thyroid vein of 10 sheep, one horse, and one dairy calf that had been injected with large doses of I^{131}. Thyroid venous plasma, carotid arterial plasma, and thyroid tissue, obtained simultaneously, were examined by filter paper chromatography. To detect weak I^{131}-containing components in plasma such as triiodothyronine and iodotyrosines, concentrated butanol extracts of plasma were first prepared before chromatography. Whole plasma was always chromatographed for comparison.

2. I^{131}-thyroxine was generally present in higher concentration in the thyroid vein than in the carotid artery. Iodide-I^{131}, on the other hand, was almost always more concentrated in the artery than in the vein, indicating active uptake of I^{131} by the thyroid.

3. I^{131}-triiodothyronine was not detectable in the peripheral circulation even when concentrated butanol extracts of plasma were chromatographed. In the vein, on the other hand, I^{131}-triiodothyronine appeared to be present to the extent of 10 to 20 per cent of the I^{131}-thyroxine. Similar values were obtained for the ratio of the two compounds in the thyroid gland.

4. I^{131}-thyroxine was not detected in the thyroid vein of all the animals examined. One entire group of sheep failed to show any appreciable I^{131}-thyroxine in the thyroid vein until 48 hours after I^{131} injection, despite the fact that I^{131}-thyroxine was present in significant amounts in the thyroid gland as early as 3 hours after the injection, and notwithstanding the finding that the uptake of I^{131} and its conversion to iodotyrosines were well within the normal range.

5. No I^{131}-thyroxine was detected in the thyroid vein of the calf 20 hours after I^{131} injection. Over 70 per cent of the I^{131} in this case was in the form of a component which remained at the origin of the chromatogram, characteristic of I^{131}-thyroglobulin or other I^{131}-proteins. A component with similar mobility was found in the thyroid vein of one normal sheep and one sheep treated with thyrotropin.

6. A consistent finding throughout this investigation was the almost complete absence of I^{131}-iodotyrosines from the thyroid vein, even at times when the thyroid gland itself contained 75 per cent or more of its I^{131} in this form. Mono- and diiodotyrosine do not leave the thyroid gland as such, suggesting that deiodination is an important metabolic fate for these compounds.

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A Simplified Method of Obtaining Orientation Autoradiographs of High Resolving Power with Nuclear Emulsions

By Constantino Nuñez,* Argentina

Pelc's stripping-film process[1] is a great advance in the autoradiographic technique. Its resolving power is very high. The theoretical requirements of emulsion thickness, emulsion-sample distance, and grain size are satisfactorily met by it. Nevertheless, the use of the Kodak autoradiographic plate (stripping film) entails certain difficulties despite its simplicity, the principal one being the fact that the emulsion is slow. The melted-emnlsion plating techniques[2] have been used for several years. Zagdela's process[3], involving the use of a nuclear emulsion, is an improvement, as he obtained very good preparations, despite the fact that the use of separation elements, such as celloidin, between sample and emulsion, tends to reduce the resolving power of the method.

Even though Pelc's stripping-film method is suitable for high resolving power autoradiographs, an easier method is needed for quicker information on the distribution of a given radioisotope or labeled substance of a given compound, since the use of Kodak stripping film requires weeks of exposure for certain autoradiographs. Pelc himself uses, in certain cases, an X-ray type emulsion prepared by Kodak in the same way as the stripping plate (Kodak Experimental Plates). Pelc follows the same plating technique with this emulsion. Due to the higher speed of an X-ray emulsion, it is possible to obtain an autoradiograph in a few days, which will always be adequate to give a general idea. However, the shorter time is obtained at the expense of a considerable loss in resolution, due to grain coarseness.

This situation has led us to look for an easier screening process, having a higher resolving power than Pelc's, making use of the main advantages of the nuclear emulsion, i.e., its speed and fine grain, and free from the disadvantages of a cumbersome technique, or weakening of the resolving power of the autoradiograph.

The following method, evolved by the Laboratories of the Radiobiological Research Division of the National Atomic Energy Commission, is very easy to use, and has yielded very good results.

(*a*) The preparations were made in the usual way. Once dewaxed, they were placed in 70% alcohol, and later in distilled water (one hour). A small amount of Ilford G5 nuclear emulsion is melted in a double boiler (at 40°C) in the dark room. A very fine brush (we discarded the camel hair brush and use Windsor and Newton marten bristle hrush for watercolors) is dipped in the emulsion, and passed just *once* over the preparation, painting a white stripe wide enough to cover it (Fig. 1). This yields a very close contact between the very fine nuclear-emulsion film and the compound. After drying (2–5 minutes), the slides prepared in tbis fashion are placed in a slide-box which later is wrapped in dark paper and placed in the refrigerator. Exposure time is very short. Generally, 3 to 72 hours suffice. It would be convenient to use the trial and error test, i.e., to develop a sample in tbe estimated minimum exposure time and, later, develop the rest according to these results.

(*b*) After exposure, the compounds are taken back to the dark room and developed with high-contrast type D19b Kodak developer for 6 minutes at 18°C. D19b: elon, 2.2 gm; anhydrous sodium sulfide, 72 gm; hydroquinone, 8.8 gm; anhydrous sodium carbonate, 48 gm; potassium bromide, 4 gm, and water, q.s. 1000 cm^3.

Wash in distilled water; fix for 10 minutes in a standard fixer.

(*c*) For microscopic observation it is convenient, as done by Pelc, to put a large water drop on the compound, place it under a slide cover, drying the excess water with gauze or filter paper. Complete evaporation of the water must be avoided. Observation may be made without previous staining by using a phase-contrast microscope. The compounds can be stained through the emulsion in the usual manner with Delafield or Harris hematoxylin for about 5 minutes. The Lajtha method may be used for the preparation of blood cells. The artifacts that may eventually be produced are easily identifiable as such, particularly using control samples prepared in a similar manner.

Tbe only original aspect of the process resides in the extreme simplification of the technique, which makes it possible to obtain samples rapidly, with a minimum of handing. It has the following additional advantages:

Original language: Spanish.

* Comisión Nacional de la Energía Atómica, Argentina.

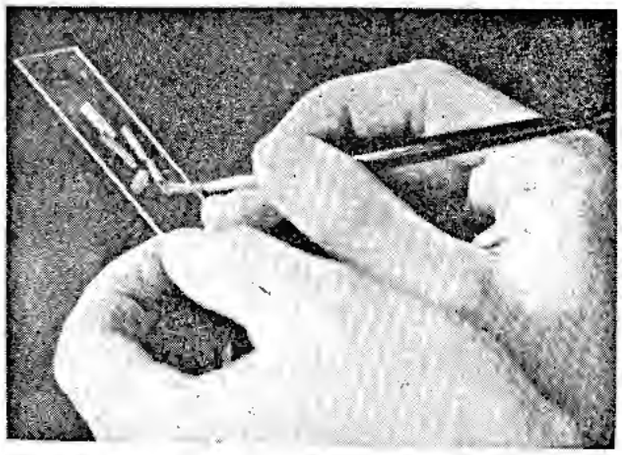

Figure 1. Marten bristle brush (for watercolors) used to spread with a single stroke the liquid nuclear emulsion over each one of the smears. The thickness of the emulsion depends on the time of application

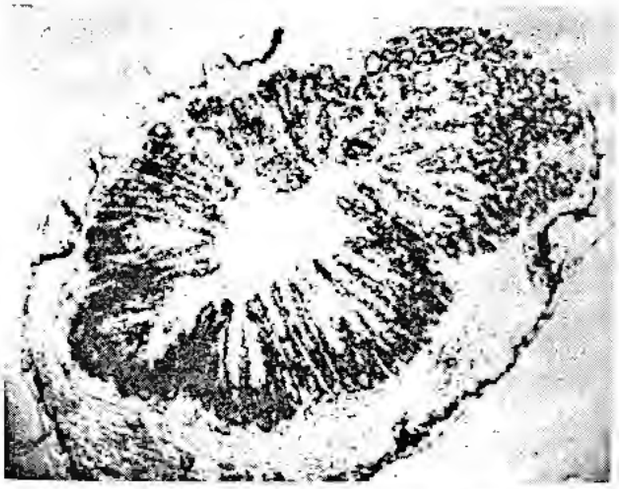

Figure 2. Small intestine of rat injected intraperitoneally with C^{14} adenine and killed 24 hours later

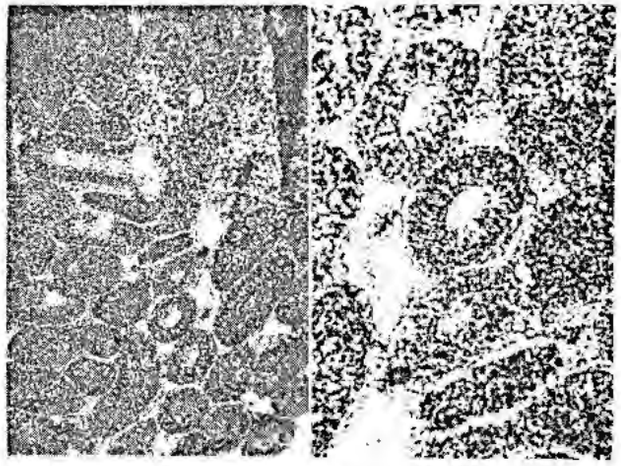

Figure 3. Testicle of rat killed 24 hours after an intraperitoneal injection with C^{14} adenine

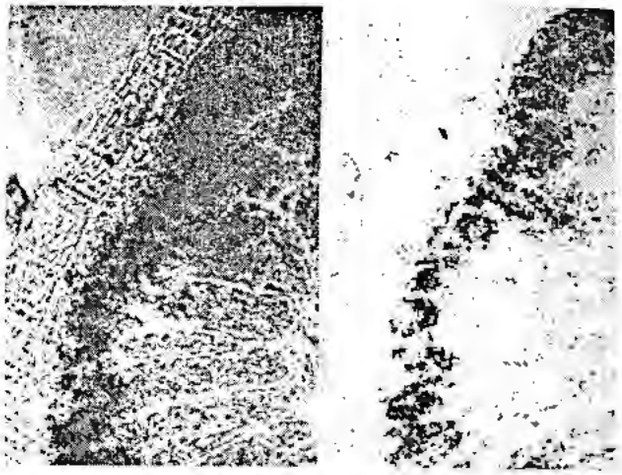

Figure 4. Small intestine of rat killed 24 hours after an intraperitoneal injection with C^{14} adenine. Preparation not stained. Left: phase contrast. Right: without phase contrast

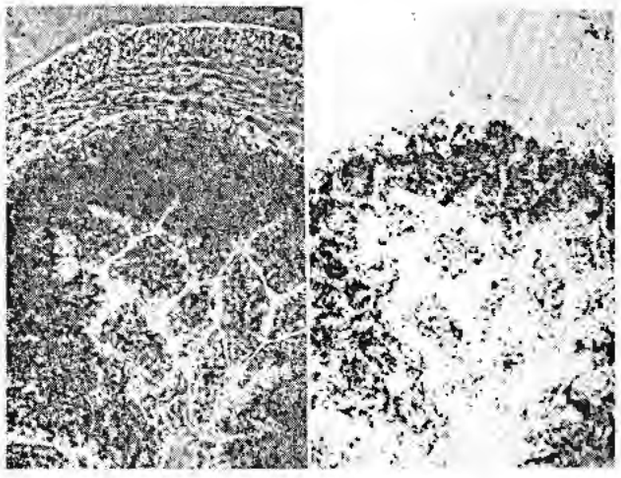

Figure 5. Small intestine of rat killed 24 hours after an intraperitoneal injection with C^{14} adenine. Compound not stained. Left: phase contrast. Right: without phase contrast

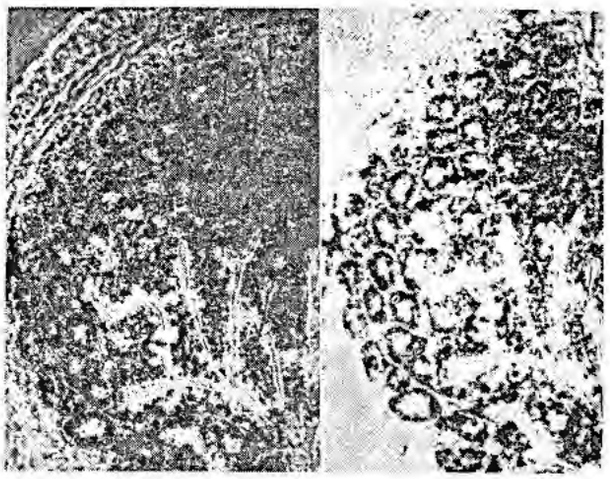

Figure 6. Small intestine of rat killed 24 hours after an intraperitoneal injection with C^{14} adenine. Compound not stained. Left: phase contrast. Right: without phase contrast

(*a*) Preparatory operations are simple.

(*b*) The Ilford G5 emulsion requires only brief exposure, thus making a rapid evaluation of the results possible.

(*c*) It is an ideal method for obtaining preliminary screening data, when final operations are more elaborate and require much longer exposure.

(*d*) A dark orange filter affords good visibility without interfering with the emulsion, and facilitates operations in the dark room.

The process has the following disadvantages:

(*a*) An accurate quantitative evaluation is made difficult by the unevenness of the spreaded emulsion.

(*b*) Artifacts may be produced.

(*c*) A lower resolving power is obtained than with the Pelc stripping film, due to the size of grain of the emulsion.

The listed advantages and disadvantages make this process suitable for obtaining screening or preliminary data before engaging in processes such as Pelc's, which are basic in autoradiography.

ACKNOWLEDGEMENT

I wish to thank for her help Miss Emma V. Pérez Ferreyra, of the Nuclear Plates Laboratory, National Commission of Atomic Energy.

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The Utilization of the Sulfites by the Higher Animals

By P. Fromageot and F. Chapeville,* France

Many papers have been published on the utilization of sulfur by the higher animals, in its oxidized and mineral form for the synthesis of cystine. Neither Tarver and Schmidt,[1] nor Bostrom and Aqvist,[2] could find clear proof of incorporation, in liver cystine, of the radioactivity of the sulfate used. On the contrary, Dziewiatkowski[3] observed, in young rats, a marked degree of incorporation of the radioactive sulfur from the tagged sulfate, without using any carrier. After injection to chickens, Machlin[4] reached the same conclusion. The results of the work of Dziewiatkowski[3] and those of the authors just mentioned are not contradictory, since the experimental conditions chosen by Dziewiatkowski were much more favorable for the visualization of a phenomenon which remains discrete. One may wonder, on the one hand, whether the bacterial flora of the digestive tract may not be made responsible for this transformation of the sulfate into a reduced organic compound, as is manifest from the work of Block[5] on the cudchewing animals; on the other hand, one may also wonder whether it is not the sulfur as a sulfite, rather than as a sulfate, which may be incorporated. We showed, *in vitro*, that the acetonic powder of rabbit kidney could utilize radioactive sulfur as a sulfite to form cysteine sulfinic acid.[6] The latter may be the product, either of the reverse reaction to that which leads to pyruvic acid and SO_2, or of a very different set of reactions, ending up in the production of cystine, from which it is known that cysteine sulfinic acid can readily be produced,[7] or, finally, from an exchange between the sulfite molecule and the sulfinyl group of cysteine sulfinic acid.[8]

In the present work we compared, in the rabbit, the incorporation of radioactive sulfate and sulfite in cysteine sulfinic acid, taurine and cystine. The animals were eviscerated or sterilized, in order to rule out any and all interference from the digestive tract bacteria. We also looked into the possibility of an exchange between the tagged sulfite and the sulfinyl group of cysteine sulfinic acid in the rabbit. We came to the conclusion that there was no clear-cut exchange, that the sulfite, but not the sulfate, is used for the synthesis of cysteine sulfinic acid, by a reaction which is the reverse of desulfinication, and that cystine is derived from cysteine sulfinic acid by reduction.

Original language: French.

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EXPERIMENTAL PART

The sulfite used comes either from Harwell or the radioelement department of the C.E.A. at Châtillon, where it is obtained by a reduction of a sulfate by red phosphorus. The radioactivity of the sulfite, as well as that of the sulfate, is so adjusted as to give 1.3 mc/400 mg of the crystallized product.

In order to rule out any influence from the bacterial flora of the intestine, the animals are either eviscerated or sterilized.

Preparation of the Animal by Sterilization

Three animals were treated as follows: a male rabbit aged 2 months, weighing 1300 gm. is put on a water diet for 48 hr, during which he receives, in four hypodermic injections, 100 ml of isotonic glucose serum, as well as 4 enemas, with 100 ml of 9% salt water every day, in order to hasten the evacuation of the content of the intestine. The animal then is anesthetized with Nembutal and, following laparotomy, the digestive tube is completely washed. To this end, two incisions are made, one in the stomach, and the other in the intestine. After the stomach has been emptied and washed with a Tyrode solution at 38°, catheters are inserted into the pylorus and rectum. Under weak pressure, about 2 liters of the Tyrode solution are then passed, which are evacuated by the incision in the big intestine. Once the solution is completely clear, the washing operation is suspended. A plain suture and a burying suture are used to close the two wounds. A 25 ml quantity of a solution which contains 0.5 gm of aureomycin and 2 gm of untagged Na_2SO_4 is injected into the stomach and at various points of the intestine. The abdominal wall and skin finally are sutured.

Two hours after the animal is awakened, a first intravenous injection of the tagged sulfite is made. After 9 hours and 18 hours respectively, more tagged sulfite is injected. The animal receives a total of 20 ml of an aqueous solution which contains 400 mg of Na_2SO_4, the radioactivity of which is 1.3 mc (125,000,000 counts). At the same time, a nutrient solution consisting of 200 ml of a solution of amino acids † and 75 ml of isotonic serum with glucose is injected hypodermically.

† Leucine 200 mg, isoleucine 100 mg, tyrosine 500 mg, tryptophane 400 mg, glutamic acid 1 gm, histidine 200 mg, proline 300 mg, glycine 1 gm, valine 200 mg, arginine 200 mg, threonine 200 mg, for 250 ml of water. Heating is in double boiler, at a boil for 10 minutes. After cooling off and passing through filter paper, solution is placed in ampules and sterilized by Tyndallizing.

Twenty-eight hours after the last sulfite injection, the animal is sacrificed. Following inoculation of a broth with a sample of the contents of the animal's intestine, and incubation at 38° for 48 hours, the culture medium is perfectly clear.

Preparation of the Animal by Evisceration

A rabbit which is identical to the one just mentioned, is placed on a fluid diet for 24 hours, anesthetized and subjected to artificial respiration. Following laparotomy, a catheter is inserted into the common bile duct of the animal in order to recover the bile. The intestinal vessels are ligated, section is made at the level of the pylorus, and the intestine is cut from the terminal end of the floating colon and taken out.

Following this operation, the animal receives 10 ml of an aqueous solution containing 400 mg of a tagged sulfite, corresponding to 1.3 mc for 40 minutes in continuous injection in the jugular vein and, at the same time, it receives 200 mg of glutamic acid and 100 mg of pyruvic acid in the vein of the ear, dissolved in 40 ml of water. Thirty minutes after the end of the injections, the animal is sacrificed.

This experiment was repeated five times.

COLLECTION AND PROCESSING OF ORGANS

In both cases, immediately after death, the skin is taken off, the hair is removed from it, and the thigh muscles are then pulled out, while the liver, kidneys, and other viscera, such as the lung, heart, empty stomach and brain, are put together. Except for the skin, the organs are cut up in pieces and immersed in boiling water for 10 minutes. An aqueous extract is made according to the Awapara technique,[8] in such a way that 1 ml of the extract correspond to 10 gm.

The skin and insoluble specimens taken out in the course of the above-described procedure are dehydrated by acetone, then dried and hydrolyzed for 12 hours against the current by 6*N* hydrochloric acid containing 10% formic acid in the amount of 25 ml of acid for 1 gm of dry tissue. The insoluble parts of the liver and kidney are put together for this treatment.

Four-fifths of the aqueous extracts from the kidney and liver are placed together and used for the isolation of free cystine according to the Dziewiatkowski technique, the remainder is analyzed by paper chromatography and ionophoresis.[6]

In the hydrolysates, the cystine is isolated by the same method.[3] The bile (5 ml) gathered in the course of the experiments on anesthetized rabbits is hydrolyzed by 6*N* hydrochloric acid for 12 hours at 100° in a sealed tube. The hydrolysate, following evaporation under vacuum, is hydrated again, and passed on an Amberlite IR4B (1 × 15 cm) column, first washed with *N* acetic acid and water. The filtrate is passed on a permutite 50 (1 × 15 cm) column, H form (HCL N). The filtrate contains taurine, which is identified by paper chromatography, in the following solvents: normal butanol, formid acid, water (75 : 10 : 15); phenol, water (80 : 20); and tertiary butanol, formic acid, water (75 : 10 : 15).

RESULTS

Cysteine Sulfinic Acid

In the rabbits sacrificed 28 hours after the end of the sulfite injection, we were unable to show the presence of cysteine sulfinic acid in aqueous extracts of the liver, kidney, muscle and brain.

In the animals which were sacrificed thirty minutes after the end of the sulfite injection, it was possible to show the presence of cysteine sulfinic acid in two rabbits out of five, in the aqueous extract of the liver and kidney by paper chromatography and ionophoresis.[6] The stains which correspond to that substance, in paper chromatography in normal butanol, formic acid and water, are eluted and subjected to paper ionophoresis at a pH of 2.7 for 5 hours.[6] The zone which corresponds to cysteine sulfinic acid is eluted and the eluate is used for the dosage of the cysteine sulfinic acid present, by the Moore and Stein method, as well as for measuring the radioactivity. The specific activity of cysteine sulfinic acid is 780 cpm/mg.

Taurine

Following purification, on an amberlite and permutite column, of the hydrolysate of the bile of the anesthetized rabbits, the filtrate which contains the taurine is subjected to preparatory chromatography on paper in phenol and water. Following elution of the zones of the paper which correspond to the taurine, this amino acid is dosaged by ninhydrine, and the radioactivity found is measured. Specific activity is 420 cpm/mg.

In the course of the experiments conducted with sulfate (1.3 mc/400 mg), we did not succeed in showing radioactive cysteine sulfinic acid or taurine.

Cystine

The specific activity of the cystine isolated from the various organs is given in Table I.

DISCUSSION

The possibility of bacterial syntheses in the digestive tract and of an exchange between the sulfinyl group of the cysteine sulfinic acid and the

Table I

Organ	*Cystine, specific activity in cpm/mg*		
	Following sulfate injection	*Following sulfite injection*	
	After 30 min	*After 30 min*	*After 28 hrs*
Skin	0.80	0.8	7
Viscera	—	—	17
Liver-kidney (protein)	0.6	8.6	20
Muscle	0.98	0.9	8.6
Liver-kidney (soluble extract)	5	100	—

sulfite having been ruled out, we show that the sulfur present in the sulfite is used by the rabbit for the synthesis of the cystine, while the sulfur present in the form of a sulfate, having the same specific radioactivity, is not incorporated by that amino acid. From a comparison between the specific activities of the cysteine sulfinic acid (700 cpm/mg), cystine (100 cpm/mg), and taurine (420 cpm/mg) which have been isolated, one can come to the conclusion that cysteine sulfinic acid is a forerunner of cystine and taurine. Accordingly, cysteine sulfinic acid may be reduced to cysteine in the system and the oxidation of the latter is reversible. The fact that the specific activity of the taurine found in the bile is greater than that of cystine can be explained by assuming, either that the decarboxylation of cysteine sulfinic acid and its subsequent oxidation is faster than its reduction to cysteine, or that taurine can be synthetized direct from the sulfate which comes from the oxidation of the sulfite brought in, or, finally, that the preformed taurine can exchange its sulfur against that of the radioactive sulfate present.

The results obtained by Machlin[9] after the injection of SO_4 to a chicken and fertilized egg seem to confirm the last-mentioned two hypotheses. But it is not possible to extrapolate the results found in the bird or rabbit, for it is not sure that the mammals and birds have the same sulfur metabolism.

While the utilization of sulfur, as a sulfite, for the synthesis of cystine, by way of a cysteine sulfinic acid stage, is beyond doubt, the total radioactivity found in these amino acids remains low as compared to the radioactivity introduced.

This can be explained by the weak specific radioactivity of the sulfite injected, and by the fact that the sulfite inserted can condense with an essential metabolite, by bisulfitic condensation, which removes this metabolite as well as sulfite, from the biological synthesis of cysteine sulfinic acid. On the other hand, the sulfite ion may behave as the sulfate ion which, while it diffuses rapidly in the interstitial fluids, is known to penetrate the cells but slowly. Thus, the sulfite can be oxidized to sulfate to a great extent[10] before it can be incorporated. Finally, it is probable that the activity of the synthetic system shown here is directed by the needs of the animal organism for sulfur bearing amino acids, and that the incorporation of sulfite must increase in very young animals or those suffering from a sulfur-containing amino acid deficiency.

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Investigation of the Incorporation of Amino Acids Into Proteins *In Vivo* and *In Vitro*

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The use of radioactive isotopes for the study of metabolic processes has rendered possible the solution of important questions in the field of biochemistry of the proteins, which could not be solved by many other methods. In spite of the large number of investigations devoted to the study of protein metabolism, such important problems as the pathways of syntheses and of conversions of proteins in the organism are still far from being solved. At present there is substantial disagreement among investigators as to the mode of synthesis of the proteins and the mechanism of their metabolic alterations in the organism. Some investigators believe that, beside the processes of degradation and synthesis of proteins, there also takes place in the organism a renewal of the constituent parts of protein molecules without marked degradation, i.e., by way of independent and non-synchronous incorporation and removal of different amino acids. Other authors assert that the incorporation of amino acids into proteins takes place only during the formation of new protein molecules. With the aid of radioactive isotopes it has become possible to decide which of these two concepts is correct.

Judging from investigations published by various laboratories in different countries one might conclude that no renewal of protein molecules takes place in the organism except during their synthesis *de novo*, but there are certain data which support the opposite viewpoint. Our investigations were undertaken to clear up this question.

We (V. Orekhovich, M. Levyant, T. Levchuk) studied the possibility of incorporation of amino acids into the proteins of the yolk, white and embryo of chicken eggs in the process of incubation. In our first series of experiments we studied the distribution of radioactivity in the above-mentioned proteins in eggs injected with tyrosine, containing radioactive carbon in the carboxyl (tyrosine-1C) group. Fertilized eggs were injected on the second, fourth, fifth, seventh, ninth and thirteenth day of incubation; they were taken for analysis thirty minutes, two hours, eighteen hours and twenty-four hours after introduction of the labelled amino acids. The results obtained in this series of experiments are given in Tables I and II.

It can be seen from these tables that the developing embryo takes up radioactive tyrosine very actively at all stages of development, whereas the proteins of the yolk and white of the egg do not incorporate radioactive carbon. As shown in Table II, the yolk and white protein contain no label as late as 72 hours after the introduction of radioactive tyrosine although the amino acid was injected direct-

Table I. Distribution of Radioactivity in the Proteins of Chicken Eggs at Different Periods of Incubation after Injection of Tyrosine-1C (2000 cpm/gm of egg)

Experiment No.	Time from start of incubation of egg until injection of tyrosine C^{14}, days	Time after injection of tyrosine C^{14}, hours	cpm/10 mg protein			
			White of egg	Yolk	Embryonic disk and yolk	Embryo
17	2	2	0	0	11*	—
1	2	18	0	1	20*	—
3	4	0.5	0	0	—	15
4	4	2	0	0	—	253
18	5	24	0	0	—	854
10	7	2	5	0	—	48
2	7	18	0	0	—	302
25	13	24	1	2	—	350

* In experiments 17 and 1 and in other experiments in which the proteins of the embryonic disk were investigated it was impossible, because of the small size of the embryonic disk, to avoid inclusion into the sample of fairly large amounts of yolk proteins. This led to a dilution of label in the "embryonic disk + yolk." Therefore, the actual radioactivity of the embryonic disk proteins is much higher than would appear from the figures in the table. In the following text all "embryonic disk and yolk" samples will be referred to as "embryonic disk."

Original language: Russian.

Table II. Distribution of Radioactivity in the Proteins of Chicken Eggs, Incubated for Seven Days, at Different Times after Injection of Tyrosine-C (4000 cpm/gm of egg)

Experiment No.	Time after injection of tyrosine C^{14}, hr	cpm/10 mg protein		
		Egg white	Yolk	7-day embryo
9	0.5	0	0	6
11	1	0	0	16
10	2	5	0	48
2	19	0	0	302
13	24	0	5	534
14	48	0	0	756
15	72	2	5	2253

ly into the white of the egg. As regards the embryo proteins, radioactive tyrosine appears in them as early as one hour after the injection of labelled tyrosine. These data permit us to assert that no proteins are synthesized anew in the white or yolk of an impregnated egg and that no processes of turnover take place in these proteins. We obtained similar data in experiments with other radioactive amino acids, e.g., glycine containing C^{14} in the carboxyl group and with S^{35} methionine (Tables III and IV).

Summing up the data presented in Tables I to IV we are justified in concluding that, in the absence of new formation of proteins, there is no renewal or turnover of the constituents of protein molecules. We obtain still more definite data in this respect in experiments with live chickens. Radioactive tyrosine was injected into a hen 18 hours before the laying of an egg, that is, at a time when formation of the egg proper is already completed, whereas formation of the egg white is only beginning. The results showed that in the first egg, laid 18 hours after the injection of radioactive tyrosine, the proteins of the yolk and of the embryonic disk did not contain tagged amino acids (Table V).

In contrast, the albumin of the white of the egg was highly radioactive. This distribution of labelled amino acid can be explained only by the fact that the oocyte had already been formed by the time the radioactive tyrosine was introduced and the synthesis of its proteins had practically ceased. The synthesis of the albumin of the egg white proceeded after the time of injection of radioactive tyrosine into the hen, and therefore the labelled amino acid was utilized in the synthesis of protein molecules. In the egg laid on the second day, radioactivity was

Table III. Distribution of Radioactivity in the Proteins of Chicken Eggs Incubated for Various Periods after Injection of Methionine

Experiment No.	Time from start of incubation of egg until injection of methionine S^{35}, days	Time after injection of methionine S^{35}, hr	cpm/10 mg protein		
			Egg white	Yolk	Embryo
19	5	24	0	0	1197
22	9	24	0	8	868
24	13	24	0	0	145

Table IV. Distribution of Radioactivity in the Proteins of Chicken Eggs Incubated for Various Periods after Injection of Glycine-1C^{14} (4000 cpm/gm of egg)

Experiment No.	Time from start of incubation of egg until injection of glycine C^{14}, days	Time after injection of glycine C^{14}, hr	cpm/10 mg protein		
			Egg white	Yolk	Embryo
31	4	24	4	0	—
35	6	24	4	4	1651
23	9	24	4	3	994
26	13	24	0	0	443
38	14	24	0	0	525
37	13	4	0	0	387

Table V. Distribution of Radioactivity in the Proteins of Eggs Laid after Injection of Radioactive Tyrosine into the Hen. (Tyrosine-1C^{14} Injected Feb. 24 at 18 hours, 2300 cpm/gm body weight)

No.	Egg laid		cpm/10 mg protein		
	Date	Time	Egg white	Yolk	Embryonic disk + yolk
1	Feb 25	1155	226	0	1
2	Feb. 26	320	271	6	55
3	Feb. 28	300	75	53	120
4	Mar. 20	1800	5	4	8
5	Mar. 22	1200		7	7
6	Mar. 24	1300	2	6	7
7	Apr. 7	1245	2	2	4

found in the proteins of the embryo, the yolk and the white of the egg. The explanation is that the embryo and the yolk proteins of this egg continued to develop while radioactive amino acid was circulating in the hen's body. The analysis of the proteins of a third egg, laid 90 hours after the injection of radioactive tyrosine, calls attention to the higher level of radioactivity of the proteins of yolk and embryonic disk, compared with the second egg.

This distribution of isotopic label in the proteins can be understood readily if we consider the course of development of the oocyte. From the above facts it follows that the incorporation of amino acids into proteins occurs during their synthesis and not as a result of renewal or turnover of their constituents.

That incorporation of amino acids does take place only during the synthesis of protein molecules is also evident from the data of our experiments (V. Orekhovich and L. Pavlikhina) on the transformation of procollagen into collagen. In order to elucidate this question we experimented on guinea-pigs. We gave healthy and scorbutic guinea-pigs subcutaneous injections of carboxyl-labelled radioglycine. At different time intervals after injecting the glycine, the animals were killed, procollagen and collagen were prepared from their skin, and the radioactivity of these proteins was measured (Table VI). Table VI contains the data showing that no C^{14}-glycine had been incorporated into the procollagen of scorbutic animals. This is due to the fact that the synthesis of procollagen is inhibited in scorbutic animals which is in agreement with the data obtained in investigations of the procollagen content of the skin in scorbutic and healthy animals (V. Orekhovich).

It had been shown that the skin of healthy guinea-pig contains about 10% of procollagen, while the skin of scorbutics contains less than 5%. It can be seen that this failure of procollagen synthesis is associated with a total lack of the incorporation of radioactive amino acids into these proteins. As our data indicate, there is no renewal of these proteins under such conditions. We wish to emphasize that the above-mentioned inhibition of incorporation in experimental scurvy is a specific feature of these particular proteins, since other proteins of several organs take up radioactive amino acids at the same rate as in healthy animals (as shown in Table VII at foot of this page).

We also studied (V. Orekhovich, T. Kurokhtina and N. Buyanova) the question of whether amino acids can be incorporated or turned over in the proteins of blood plasma *in vitro* to show, as opposed to the claims of other investigators, that no incorporation (and consequently no turnover) of radio-

Table VI. Rates of Incorporation of C^{14}-Glycine into the Procollagen and Collagen of Healthy and Scorbutic Guinea-Pigs

	Time, hr, after injection of amino acid							
	3	*6*	*10*	*18*	*24*	*120*	*168*	*384*
Procollagen								
Scorbutic animals	3	6	4	2	1	0	3	0
Healthy animals	45	134	140	192	190	122	89	70
Collagen								
Scorbutic animals	1	1	0	0	2	—	3	3
Healthy animals	9	12	13	12	16	—	23	25

Table VII. Rates of Incorporation of C^{14}-Glycine into the Proteins of Several Organs of Healthy and Scorbutic Guinea Pigs (cpm/10 mg protein) at Different Time Intervals after Injection of the Amino Acid

	Hours after injection of amino acid							
	3	*6*	*12*	*24*	*48*	*120*	*168*	*384*
Brain								
Scorbutic animals	3	8	10	10	20	7	6	5
Healthy animals	3	9	11	12	21	10	5	6
Muscle								
Scorbutic animals	19	13	14	26	60	15	18	12
Healthy animals	20	14	20	20	50	20	23	20
Spleen								
Scorbutic animals	60	90	119	117	187	46	47	12
Healthy animals	90	109	130	—	173	68	30	8
Kidney								
Scorbutic animals	72	72	172	110	70	—	62	18
Healthy animals	74	—	164	120	100	80	58	18
Liver								
Scorbutic animals	80	—	148	83	70	73	55	17
Healthy animals	107	—	185	130	110	61	47	20

active amino acids occurs in the proteins of blood plasma incubated *in vitro*.

The radioactivity of the proteins of blood serum noted by some investigators upon the addition of radioactive amino acids has been shown by A. Pasynsky, T. Pavlovskaya and M. Volkova to be due to the binding of these amino acids by proteins in the process of their denaturation. Experiments were performed on the blood serum of horses. To three milliliters of serum were added three milliliters of a phosphate buffer solution (*M* 15, pH 7.5) and an aqueous solution of methionine containing radioactive sulfur at a rate of 100,000 cpm/ml of the mixture. Then the mixture was subjected to ultraviolet or X-ray irradiation or to heating.

After irradiation by X-ray (800,000 r) the radioactivity of the protein was 942 cpm/10 mg of protein versus 80 cpm in the control sample. Extensive changes in radioactivity were also found after heating the mixture in a boiling water bath or after ultraviolet irradiation.

The effects noted in these experiments are due to the increased adsorptive capacity of the proteins upon denaturation, resulting from the release of new reactive groups in the protein molecule and not to the turnover of amino acid residues.

From all the data we have presented it follows that the incorporation of amino acids into proteins takes place only during synthesis of the proteins and not as a result of their "renewal". When the process of synthesis has ended, there occurs no further renewal of the constituent parts of the synthesized protein molecules. Thus, we are justified to a certain extent, to admit that protein turnover does not occur in the organism as a self-contained process, independent of the breakdown of protein molecules.

Pathways of Biosynthesis of Nucleic Acids

By George Bosworth Brown,* USA

There has been a tremendous stimulation of studies of the biochemistry of nucleic acids coincident with recent developments in atomic physics. To the chemist the name nucleic acid is by now merely that of a class of complex organic chemicals, although originally the name "Nucleinstoffe" was applied by Frederick Miescher,[1] in Basel, to one specific type obtained from cell nuclei. That type is now known as the desoxypentose nucleic acids (DNA) and much evidence has associated it with the morphologic forms recognized as the chromosomes. The bulk of the second type, the ribose nucleic acids (RNA), is found in the particulate matter of the cytoplasm of the cell, and plays an active role in the dynamic metabolic processes of the cell. The name, nucleic acids, is perhaps disconcerting at a conference such as this one, but from the broad definition of a nucleus—as the core of a matter —the nucleus of the cell is to the biologist what the nucleus of the atom is to the physicist, and the DNA, through its association with the genetic material, still merits the implications inherent in that name.

Two stimuli to studies of nucleic acids have come from the atomic energy programs. Firstly, the need for a better understanding of the connection between the influence of various types of radiation on the genetic characteristics of living matter has accelerated the search for clarifications of the long suspected relationship of at least the one type of nucleic acids to the carrier of hereditary characteristics. Secondly, the ready availability of radioactive isotopes has not benefited any biochemical problem to a greater degree than it has the study of nucleic acid biosynthesis and metabolism. In fact, it has made such studies possible. Living systems are able to synthesize their nucleic acids from the simplest materials and therefore classical biochemical and nutritional techniques, such as those of following growth or weight changes, could not be applied to studies of the origin of the nucleic acids. It was not until isotope tracer techniques became available that the origins of the nucleic acids could begin to be defined and their biosynthesis and metabolism to be described.

Both types of nucleic acids are macromolecules of at least the complexity of proteins; particle weights now being reported are far above the range associated with most proteins. The question of whether the active units of these materials in their native state in the cell are not some small portion of the aggregates isolated remains to be clarified. Figure 1 represents only the minimum amount of material necessary to depict four pyrimidines and purines and the covalent bonds involved in their attachments to the sugars and phosphates in a ribonucleic acid. Each purine or pyrimidine with its sugar and one of the phosphates to which the sugar is attached is referred to as a nucleotide, and the macromolecule is referred to as a polynucleotide. The complete macromolecule may have thousands of nucleotides. The 2′-desoxyribonucleic acids could be similarly depicted with the second carbon of the sugar carrying no hydroxyl group.

In the last few years many aspects of the metabolic origin of the polynucleotides have been learned through administration of isotopically labeled compounds, followed by the use of degradative procedures which permit analysis of individual moieties of the products which are derived from them. The purine adenine was the first organic compound found to serve as a precursor of the nucleic acids,[2] and it was also found to be converted into the guanine of the polynucleotides in the rat. The purine guanine is not utilized to any significant extent, although it can be utilized by other species. (In initial investigations[33] with guanine-N^{15}, and also[4] with hypoxanthine-N^{15}, and[3] uracil-N^{15}, the conclusions were reached that these compounds did not serve as polynucleotide precursors in the rat. However, in subsequent reinvestigations using the carbon-14 isotope, very small incorporations of each of those compounds have been detected.[5,6,7,8] These experiments are an illustration of the limitations inherent in the more limited dilution factors feasible with the natural "heavy" isotopes, and an excellent example of the value of the more sensitive radioisotopes.)

By now, most of the purines, pyrimidines, and their ribose derivatives (nucleosides) and the simple ribose phosphate derivatives (nucleotides) have been studied by the administration of samples which were isotopically labeled in the purine or pyrimidine moieties.[9] The present picture (Fig. 2) of the utilization of the various compounds by the rat can be briefly outlined as follows. Unlike adenine, neither of the pyrimidines, uracil nor cytosine (nor the thymine which is present in DNA) is utilized appreciably, but their nucleosides or nucleotides are very readily

* The Sloan-Kettering Institute for Cancer Research, New York City. Including work by A. Bendich, J. Fresco, P. M. Roll, and H. Weinfeld.

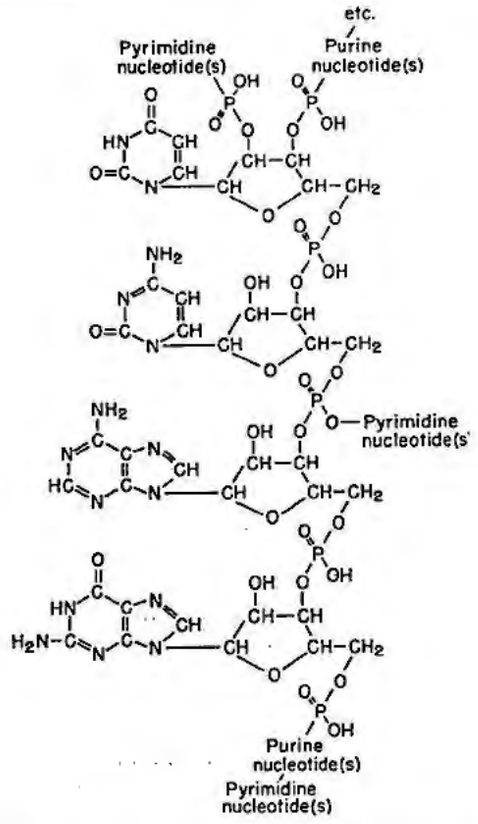

Figure 1. Fragment of a ribose polynucleotide, depicting one each of the nucleotide units derived from the two pyrimidines, uracil and cytosine, and one each from the two purines, adenine and guanine

utilized. The corresponding derivatives of adenine are, however, considerably less extensively utilized than is the free purine. The situation regarding guanine and its derivatives is entirely different from that with adenine, and it also differs from the situation with the pyrimidine derivatives. Neither guanine nor its nucleoside is incorporated to an appreciable extent. However, the guanine moiety of certain of its nucleotides[10,11] is quite extensively incorporated into the guanine of the nucleic acids. Since the nucleotide is the only guanine-containing compound serving as an effective precursor, it initially appeared[12] that the complete nucleotide might be the unit which was incorporated into the polynucleotide.

In order to ascertain how much of each nucleotide molecule actually reached the nucleic acids we have now carried out detailed studies with multiply labeled ribonucleotides, with which the fate of each major segment of the nucleotide could be followed.[13] These experiments involved samples of nucleotides of each of the purines and pyrimidines. These were obtained by biosynthetic methods: with N^{15} in the purine or pyrimidine, with C^{14} in both the purine or pyrimidine and in the ribose, and with P^{32} in the phosphate. (The ribose nucleotides with phosphate on the 2′ and 3′ hydroxyls of the ribose, the classical "yeast" nucleotides, are the only ones which have yet been so studied. The adenine nucleotide with the phosphate on the 5′ hydroxyl, or "muscle" adenylic acid, has been investigated with its adenine labeled and is a somewhat less effective precursor of PNA than are the 2′ or 3′ isomers.[11] Preliminary studies have shown that the 2′ and 3′ isomers of the nucleotides are readily converted, *in vivo* in the rat, into the 5′ nucleotides found in the "acid soluble" fractions from the tissues.)

None of the several nucleotides studied contributed its phosphorus to any specific position in the polynucleotides.[14] The P^{32} reached the inorganic phosphate, and that portion which did arrive in the polynucleotides was uniformly distributed among all of the phosphates of the macromolecule. Thus these mononucleotides were not the units from which the macromolecule was finally assembled.

After the administration of the labeled cytosine nucleotide, the polynucleotides contained a ratio of C^{14} in the cytosine and in the ribose which was attached to it which was identical with that in the administered sample. Thus the cytosine-ribose unit of the nucleotide was incorporated as a single unit, as had been shown for the nonphosphorylated cytosine-ribose unit.[15] A transformation of cytosine derivatives into uracil derivatives occurs to a small extent, and the uracil derivative obtained from the polynucleotides was also found to contain equal concentrations of C^{14} in its pyrimidine and ribose. Experiments involving the administration of nucleotides of uracil also indicated that its nucleoside portion was metabolized as an intact unit. In addition, the DNA's were investigated and the desoxyribose derivative of cytosine was likewise found to contain the same ratio of isotope in the desoxyribose and in the cytosine moieties. This suggests a conversion of ribose derivatives to desoxyribose derivatives by way of a reductive removal of the oxygen from the second carbon. In other words, in the rat, cleavage between the pyrimidine and the sugar did not appear to occur, and the pyrimidine nucleoside unit is maintained intact throughout the metabolic transformations (Fig. 3).

The results with the purine nucleotides were quite different. There was extensive incorporation of the

PURINE OR PYRIMIDINE	NUCLEOSIDE	NUCLEOTIDES
URACIL ±	+	+
CYTOSINE −	++	++
ADENINE ++	+	+
GUANINE ±	±	+

++ extensive utilization
+ moderate utilization
± trace utilization

Figure 2. Approximate extent of utilization of various purines, pyrimidines and derivatives by the rat, based primarily upon their incorporations into pentose nucleic acids

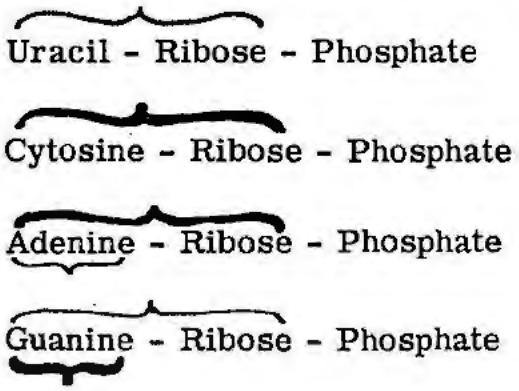

Figure 3. The moieties of each nucleotide which, in the rat, are incorporated into the ribonucleic acids. The units apparently incorporated to the greatest extent are indicated by the heavier brackets

ribose of the adenine nucleotides into the corresponding segment of the polynucleotide, but this was only about 80 per cent of that found for the purine moiety. Some portion of the nucleoside could have been incorporated with the glycosidic linkage intact, but there was obviously some additional purine transferred into the polynucleotide without its accompanying ribose (Fig. 3).

The results with the guanine nucleotides were even more striking. There was a proportionately smaller incorporation of its ribose; at most, 20 per cent of the guanine incorporated could have been accompanied by its ribose, and at least 80 per cent of that incorporated was transferred to a new ribose unit in the process. Consideration of these observations must take into account the fact that free guanine is not readily incorporated, so that the transfer to a new ribosyl moiety is probably accomplished in a single step. The transfer of purines to new glycosyl moieties is a type of transglycosidation[16] reaction, which, conversely, can be referred to as "transpurination" for emphasis.[17] This process (Fig. 4) is involved at some stage in the metabolic pathway via which purines are incorporated into the polynucleotides.

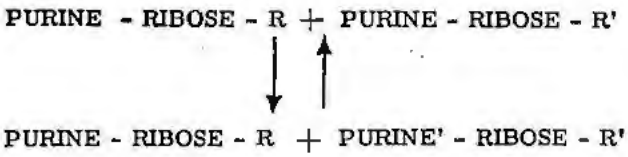

Figure 4. Generalized scheme for the transglycosidation type of reaction, the specific instance of which is referred to here as "transpurination". Purine' may be some other type of group

The question of whether a "transpurination" may occur between a nucleotide and a pre-existing polynucleotide cannot yet be directly answered. However, the postulation that the reaction may occur at that level may assist us in clarifying certain unexplained observations. These have arisen from many studies which have employed several of the now recognized nucleic acid precursors for comparisons of their relative incorporations into the two types of nucleic acids in different tissues and under various physiological conditions.

Studies with adenine[18] as the labeled precursor indicated very extensive renewals of RNA but very little renewal of DNA. During the rapid regeneration of liver following partial hepatectomy, with its accompanying increase in cell population, there was a very much greater incorporation of adenine into the DNA, however, there was little increase of that into RNA. When animals so treated were allowed to survive for 3 to 14 weeks it was found that the isotope which had been incorporated into the RNA disappeared rapidly, but that the isotope in the DNA was extensively retained. Such an apparent absence of dynamic metabolic equilibria indicate a biochemical stability[18] of the DNA in the non-dividing cells. These results with adenine have been difficult to reconcile[19,20,21,9] with results obtained on the incorporation of several compounds which are precursors of various portions of those purine molecules which arise by synthesis *de novo* in the animal. With such compounds, glycine,[22,19,20,23] serine,[19] and formic acid,[24,25] far smaller differences were observed between the renewals of RNA and DNA of the non-growing tissues; also, in tissues in which rapid cell division was occurring there was not as great a change in the relative renewals of the two nucleic acids.

To exclude questinns of variables in experimental conditions, experiments were undertaken which involved simultaneous administration of glycine and adenine,[26] and later of formate and adenine.[27] Each precursor was labeled with a different isotope (N^{15} and C^{14}) and the final metabolites were analyzed for each isotope. The results afforded direct confirmation of the existence of the disparities in the renewal data with precursors of different degrees of complexity.

One of the appealing speculations[23,26] as to possible reasons for the differences is that of more than one mechanism of synthesis. Any explanatinn based upon alternate pathways must be able to reconcile the fact that during rapid tissue growth there is a proportionately greater emphasis of the pathway by which exogenously supplied purine is incorporated into DNA. In considering the differences between the incorporations of the two types of precursors, a significant point is the very high absolute incorporations which can be observed with adenine. As much as 16 to 23 per cent of the adenine of rat liver RNA has been derived from administered adenine in 3 to 5 days.[18,26] The fact that adenine seems to "spare", or decrease, the incorporation of purine arising *de novo*[27] also suggests an efficient competition of exogenous adenine with that arising *de novo*. The incorporations of glycine or formate into nucleic acids via purines arising through synthesis *de novo* result in absolute renewals of the order of a fraction of a per cent, which is due in part to their equilibria with, and dilution by, other

sources and fates of one and two carbon units. However, regardless of the differences in the magnitudes of the absolute renewals observed, if the exogenous adenine and that arising by synthesis *de novo* were entering a single series of reactions leading to polynucleotides there should be no differences in relative renewals of different polynucleotides.

It has been established that nucleotide derivatives are formed in the course of the synthesis *de novo* of purines, and also that administered adenine is rapidly converted into nucleotide derivatives.[28,29,30] Thus it is not free purines but some ribose-phosphate derivatives thereof which must be considered as the "active" metabolites directly involved in the incorporations into polynucleotides. The postulation of the existence of a transfer of purines from some ribosyl derivative directly into the ribose-phosphate backbone of either of the nucleic acids (RNA or DNA), offers a mechanism for some direct incorporation of the adenine. Such an incorporation could be independent of the total synthesis of polynucleotides from other ribose containing units if an intermediate formed from exogenous adenine, but not identical with the intermediate involved in total synthesis, can be involved in the "transpurination" (Fig. 5). The existence of some incorporation of exogenous adenine via a "transpurination" in addition to via total synthesis of new macromolecules could help to explain some of the major differences observed between the incorporations of exogenous adenine and that of adenine derived from glycine and formate.

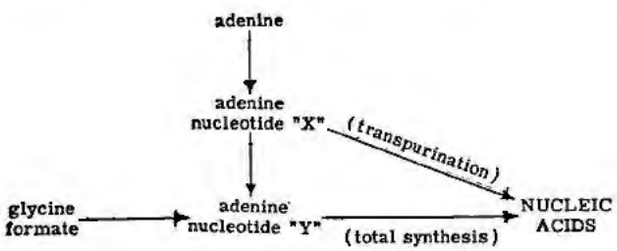

Figure 5. "Transpurination"

From a survey of the available evidence[9] it is apparent that, during the time when the new quota of DNA for the daughter cells is being created, an active synthesis of DNA occurs. If it is assumed that that highly active metabolic state includes the opportunity for "transpurination" as well as total synthesis, a disproportionate increase in the amount of adenine incorporated could be favored during that formative stage of DNA.

However, once the DNA is fully formed, and regardless of the source of the isotope which was incorporated during the formative stage, there is but a slow dilution of that isotope in tissue which is no longer rapidly growing, which indicates a long term retention of the DNA by the daughter cells. In several instances, with adenine,[18] with orotic acid[31,32] and, more importantly, by the parallelism, of the behavior of isotopes incorporated from a mixture of adenine and glycine,[33] it has been estimated that the small dilution of the isotope which is observed can be approximately accounted for by increases of the cell population. In experiments with formate[25] unequal apparent retentions of the bases were observed when values from the 1st and 24th days were compared. The heterogeneity of anabolism, which was demonstrated at the one day time,[25] undoubtedly influenced the values beyond the first day. Therefore the apparent retentions when the 24-day values were compared with the one day values were complicated by the continning anabolic phase. In the subsequent experiment involving glycine-C^{14} and adenine-N^{15} comparisons of the 1, 2, 3 and 4-month values completely avoided the anabolic phase and indicated very uniform retentions of the isotope.[33] Thus DNA retained in the new cells is no longer in a highly active metabolic condition, and any loss which occurs appears to be by complete catabolism.

It should be mentioned parenthetically that a considerable similarity, in the rat, of the renewals of nucleic acids by orotic acid to those observed with adenine suggests that a similarly efficient incorporation into the polynucleotides obtains for some "active" pyrimidine derivative arising from orotic acid, even though there is no evidence of such a transglycosidation from the cytidylic and uridylic acids which have been studied. (In several species of microorganisms extensive transglycosidation of pyrimidine derivatives does occur.[16,15] The present discussion is developed from resnlts obtained using the rat and, in view of the wide range of species differences in several phases of nucleic acid metabolism, may have to be modified for other species.)

The "transpurination" pathway discussed here can provide a possible explanation for the observations of Harrington and Lavik[34] on the influence of X-irradiation on DNA metabolism in rat thymus. They observed decreased incorporation of formate, orotic acid and phosphate into the DNA purines, but an unchanged or somewhat increased incorporation of adenine. (Those authors have also observed a similar continued incorporation of formate into thymine and have suggested a renewal of its methyl group independent of synthesis *de novo*. Such a possibility could further help in interpreting certain renewal data from one-carbon precursors.) If the effect of the irradiation was to decrease the total synthesis of DNA, but if the DNA in the cells which were preparing for mitosis was still in the metabolically active state and could still participate in "transpurination", the continued transfer of adenine into pre-existing macromolecules could explain the observed uptake of adenine.

This discussion is based upon concrete evidence that a "transpurination" to new ribosyl derivatives does occur in the rat, but the suggestions regarding transfer between a free ribosyl derivative and an intact polynucleotide are speculative. The Harrington and Lavik[34] data might also be interpreted as supply-

ing the only direct evidence suggesting such a transfer of purines into an intact polynucleotide.

The evidence indicating multiple mechanisms during the synthesis of DNA, and that indicating a prolonged and uniform retention of the DNA once it has been formed, can begin to be reconciled if it be assumed that the bulk of the DNA in non-dividing cells is present in such a condition that it is not involved in any metabolic changes. The new DNA which is forming, during the interphase of the cell which is to divide, is in a condition in which it is in a dynamic metabolic equilibrium. (It is not yet clear whether this involves the formation of one new complement of DNA, or whether two new complements of DNA are formed, one for each of the daughter cells, and the old one disposed of.) Once the complement of new DNA is fully completed, its condition must be altered, during the prophase of the cell, so that the bulk of it is excluded from further metabolic changes; either degradation, synthesis, or the "transpurination" reaction. Desoxyribonucleic acid which is to serve in the nucleus of the daughter cell can thus acquire a stability and permanence of character which is a desirable attribute [35,17,21] of the material unit which is to have the immutability associated with the concept of the gene.

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Nucleotide and Nucleic Acid Metabolism Studied with Adenine-C^{14}

By Edward L. Bennett,* USA

It is well established that nucleotides and nucleic acids are essential constituents of all cells. The nucleotides are fundamentally important as cofactors in many enzymatic reactions, particularly those reactions involving energy transformations. Desoxyribonucleic acids are intimately associated with processes of cell division and of self-duplication. They are constituents of chromosomes and viruses. Ribonucleic acids are believed to be associated with protein biosynthesis and may be important as templates or patterns for this synthesis. In order to understand cellular biochemistry, we need a knowledge of such things as the mechanisms of nucleic acid and nucleotide formation; the metabolic interrelationships between nucleotides, or between nucleotides and nucleic acids; and rates of renewal of nucleic acids. The nucleic acids have basic relevance to carcinogenesis. Knowledge of the effects of various agents such as carcinogens, anticarcinogens, and irradiation upon the formation and renewal of nucleic acids may aid us to understand both normal and abnormal cell division, and eventually may lead to effective treatment of malignant growths.

Adenine is one of two purine bases found in nucleic acids. It is also a constituent of the most abundant nucleotides found in cells, as well as a constituent of a number of coenzymes. Early tracer studies showed that adenine was utilized for the formation of nucleic acids in mice and rats.[1,2] However, the incorporation of adenine into the soluble nucleotides was not investigated in detail in the previous studies. In our studies, we have utilized adenine-4,6-C^{14} and have determined its total distribution in the different chemical fractions of the various organs of the mouse at numerous time intervals. In addition, we have determined the amount of radioactivity excreted in urine and breath. Thus, a knowledge of the fate of adenine, not just in one tissue or one type of compound, but in its over-all distribution has been obtained.

One of the more surprising early observations was the rapid and high degree of utilization of adenine for the formation of normal constituents of the animal cell.[3] Within two hours after intraperitoneal injection of adenine, about 50% of the adenine administered had been converted into compounds other than free adenine, and most of the remainder had been excreted in the urine. Time studies indicated that the amount of radioactivity found in ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) increased to a maximum 1 to 2 days after administration of the adenine, and compounds present in the cold acid-soluble fraction, presumably nucleotides, must have been utilized as precursors of nucleic acids. We have therefore made a detailed study in C_{57} mice of the low-molecular weight or acid-soluble compounds into which adenine is incorporated. This study has been greatly facilitated by the use of paper chromatographic and radioautographic techniques. At three time intervals, one-half hour, 2 hours, and 24 hours after administration of adenine, the acid-soluble fraction from the principal tissues in which adenine is utilized (carcass, small intestine, liver, and kidney) was isolated. The compounds present were separated by two-dimensional paper chromatograms and the radioactive compounds were located by radioautographs. The distribution of radioactivity is summarized in Table I. More than 80% of the radioactivity in the acid-soluble fraction had been incorporated into nucleotides of adenylic and guanylic acid within two hours after adenine injection. Within one-half hour after administration, at which time free adenine is still present, the specific activities of adenosine monophosphate, diphosphate and triphosphate are all equal in a given tissue. This indicates that these compounds, which play such a fundamental role in the energetics of living systems, are rapidly equilibrated. The specific activities of more complex nucleotides, such as diphosphopyridine nucleotide (coenzyme I) or triphosphopyridine nucleotide (coenzyme II), were significantly less than the activity of adenosine monophosphate at 2 hours, but were equal at 24 hours. Therefore these compounds are derived more slowly from the mononucleotides. In addition, radioactive guanosine mono-, di-, and triphosphate were found. The specific activities of the guanosine derivatives were $^1/_3$ to $^1/_{50}$ of that of the corresponding adenine derivatives, depending upon the tissue and the time after administration of adenine-C^{14}. This is the first demonstration that guanine nucleotides may be obtained from adenine at the nucleotide level. Previously, radioactive guanine had been found only in nucleic acids after

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adenine administration. The nucleotides of different tissues have very different specific activities, suggesting that nucleotides are synthesized within individual cells and are not rapidly translocated from one tissue to another. No evidence was found for mixed dinucleotides, i.e., nucleotides containing adenine and guanine, which might be considered as a building block or precursor for nucleic acid.

Experiments from which an apparent rate of renewal of nucleic acid can be calculated are limited and have usually been based upon rate of uptake of the tracer compound. When precursors of nucleic acids—such as adenine, orotic acid, or glycine—are administered, renewal rates based upon their rate of incorporation have several limitations. Assumptions must be made concerning the intermediates by which the precursor is incorporated, and the specific activities of these intermediates must be known. Most long-term experiments with adenine[4,5] and formate[6] in which incorporation has been determined at only two time intervals after administration, although permitting a comparison of "apparent retention" to be made in the tissues studied, do not yield sufficient data to calculate a valid "half-life".

The large and rapid incorporation of adenine into nucleotides, RNA, and DNA facilitates a determination of the minimum renewal rate of these classes of compounds in different tissues based upon the rate of loss of adenine-C^{14}, as shown for the small intestine in Fig. 1. By a study of the adenine specific activity at numerous time intervals up to 29 days after administration, we have found that 70 to 94% of the adenine incorporated into 5′-adenylic acid derivatives of all tissues (with the exception of carcass and heart) had an apparent half-life ranging from 18 to 28 hours. The remainder of the 5′-adenylic acid derivatives were renewed at a relatively slow rate with a half-life of 5 to 7 days. The tissues with the shortest half-life for the nucleotide-adenine, such as intestine and liver, had the highest specific activity shortly after administration.

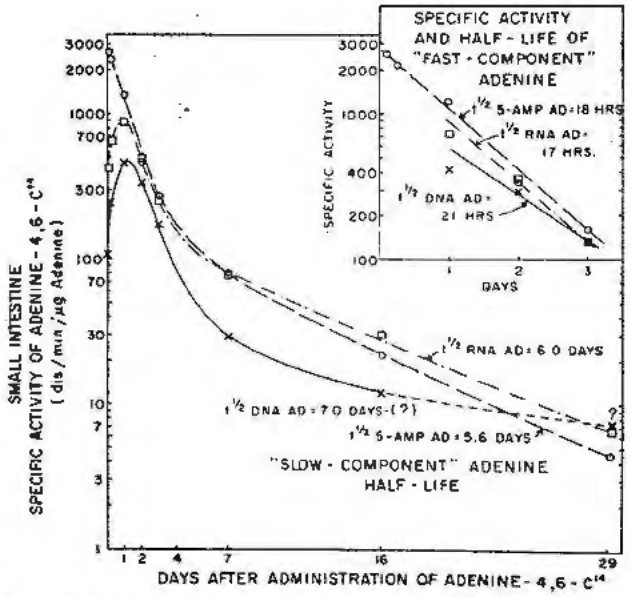

Figure 1. Specific activity and half-life of 5′-adenylic acid, RNA, and DNA adenine of small intestine of mice from 2 hours to 29 days after administration of adenine-C^{14}. Specific activity and half-life of "fast component" adenine shown in inset

Since each tissue has its own characteristic rate of cell renewal and metabolic processes in general, maximal specific activitiy and time of maximal activity in RNA and DNA are different for each

Table I. Distribution of Adenine-4,6-C^{14} after Administration to C_{57} Male Mice*

Fraction	*Small intestine*			*Liver*			*Carcass*		
	½ hr	*2 hr*	*24 hr*	*½ hr*	*2 hr*	*24 hr*	*½ hr*	*2 hr*	*24 hr*
	Distribution of injected radioactivity, %								
Cold TCA	9.1	8.8	3.1	8.4	10.7	5.0	25	10	8.0
RNA	0.4	1.7	2.3	0.16	0.7	1.7	0.3	1.0	1.2
DNA	0.05	0.45	1.2	<0.01	0.02	0.02	0.1	0.6	1.0
	Distribution of cold TCA soluble radioactivity, %								
Nucleotides	46	70	85	50	85	95	24	<90	<95
Adenine	32	3	0	34	1	0.5	65	1	0
Hypoxanthine	3	5	5	2	3	0.5	2	3	1
Allantoin†	5	2	1	3	2	0.5	3	2	1
"X-1"	3	8	8	2	2	1.5	1	0	0
Minor Cmpds.	11	12	1	8	6	2	5	0	0
	Distribution of nucleotide radioactivity, %								
5-AMP	33	44	43	20	33	23	13	15	10
DPN	1	4	8	4	5	9	2	6	8
ADP	25	22	22	34	27	30	20	26	26
ATP	30	20	19	33	20	21	53	40	47
GMP, GDP, GTP	1	3	6	2	4	10	1	1	2
IMP	9	6	4	7	10	8	10	11	8
TPN†	<1	—	—	<1	<1	<1	<1	<1	<1

* 1.3 mg of adenine-4, 6-C^{14}, specific activity 17,000 dis/μg was administered intraperitoneally to male C_{57} mice.

† Other compounds may be present in the radioactive allantoin area, particularly in the kidney. The TPN area had an R_f, less than ATP in both solvents and has not been otherwise identified except by its approximate correspondence to the R_f of an authentic sample of TPN.

tissue studied. The specific activity of adenine in RNA at 1 day was in the following decreasing order: large intestine, small intestine, stomach, kidney, lung, liver, and carcass. In the intestines, 83 to 93% of the adenine-C^{14} in RNA was renewed with an apparent half-life of 17 to 19 hours, and the remainder with a half-life of 5 to 6 days. The RNA-adenine of liver and kidney had a half-life of 5.5 to 7.0 days, while a half-life of 10 to 15 days was found for lung, carcass, spleen and testis.

According to current evidence, it is believed that precursors are incorporated intn DNA only in preparation for cell division, and hence the DNA half-lives presumably are a reflection of cell renewal in the tissues. The small intestine, large intestine, stomach and carcass (a composite sample including muscle and bone marrow) incorporated an appreciable amount (3.3%) of the administered adenine into DNA. In the first three tissues, a fast-component adenine with a half-life of 16 to 20 hours was found, while the carcass fast-component adenine had an apparent half-life of 35 hours. Each tissue had a slow-component adenine in the DNA with a half-life of 5 to 7 days. Seventy to ninety per cent of the adenine incorporated was renewed rapidly. It should be pointed out that the RNA/DNA-adenine-C^{14} ratio varies widely from tissue to tissue, and it also varies as a function of time. Thus, a comparison of ratios at a single time interval may be misleading and may account in part for the diverse RNA/DNA specific activity ratios foond in the literature.

Maximal specific activity of adenine in nucleic acids in all tissues, except carcass-DNA, occurs 24 to 48 hours after adenine has been administered. As previously discussed, adenine is essentially completely converted to nucleotides or to catabolites of adenine, principally hypoxanthine or allantoin, within 2 hours after administration. Catabolites of adenine are poor precursors of nucleic acids,[7] and thus it would appear that 5′-adenylic acid nucleotides are serving as precursors of adenine in both RNA and DNA. Additional evidence that 5′-adenylic acid nucleotides are direct precursors of RNA-adenine, perhaps sole precursors in intestines, is the striking correspondence of specific activity of RNA-adenine and nucleotide adenine in both small and large intestine subsequent to the second day after administration. In most tissues studied, a close relationship was found between the half-life of 5′-adenylic acid adenine and RNA-adenine. This suggests that adenine of RNA is derived directly from and equilibrated with soluble 5′-nucleotide adenine.

The high incorporation of adenine into nucleotides and nucleic acids also facilitates the study of factors that influence nucleic acid metabolism, such as irradiation, drugs, and pathological conditions.

One such study is the effect of X-irradiation upon the incorporation of adenine into nucleotides, RNA, and DNA of various tissues of A-strain mice. In this study, adenine was administered to mice at selected time intervals up to 48 hours after X-irradiation (800 r). Two hours after administration of adenine, the animal was sacrificed and determinations were made of the specific activities of adenine io nucleotides, RNA, and DNA found in small intestine, large intestine, carcass, liver, stomach, and spleen and gonads. In small and large intestine, a large initial inhibition of incorporation of radioactive adenine into DNA was observed. Two days after irradiation, the incorporation of adenine was several times the normal rate. In all the other tissues studied, incorporation of adenine into DNA was reduced to 25 to 40% of normal within 2 to 3 hours after X-irradiation and was further reduced to 5 to 10% of normal 48 hours after irradiation. Similar results were obtained with P^{32}-phosphate.[8] A relatively slight effect of irradiation upon the incorporation of adenine into RNA or 5′-adenylic acid nucleotides was observed; the largest change noted was a 35 to 50% decrease in incorporation of adenine into nucleotides and RNA of the carcass 48 hours after irradiation. At this time, adenine incorporation into DNA was 4% of normal. At the present time it is not known whether the observed inhibition of incorporation of adenine-C^{14} and P^{32}-phosphate into DNA is a consequence of cell death or represents a specific biochemical lesion produced by radiation.

To study the effect of aminopterin (a folid acid inhibitor) upon nucleotide and nucleic acid renewal, aminopterin was administered daily to C_{57} mice after a single adenine-C^{14} injection. The amount of radioactivity measured in the nucleotides, RNA, and DNA present in intestine, liver, carcass, and kidney was not significantly different from controls over a 16-day period, indicating that aminopterin did not affect the loss of adenine from these compounds once it had been incorporated.

Most of the available evidence indicates that the purine ring system is stable once it is formed. However, independent metabolism of several of the carbon atoms, particularly those in the 2 and 8 position of the purine ring, appears to be a possibility in view of our present knowledge of the mechanism of nucleotide biosynthesis. In pigeon liver preparations, it has been found that the carbon 2 of inosinic acid can exchange with formate in the medium, which suggests that this position may be labile.[9] At the present time, a comparative study of the metabolism of adenine-2-C^{14}, adenine-4, 6-C^{14} and adenine-8-C^{14} is in progress. In this experiment, a comparison is being made of the specific activities of the adenine of nucleotides and nucleic acids at numerous time intervals after administration. If any of the C^{14}-labeled positions of the adenine ring is labile in either the nucleotide or nucleic acid, the specific activity of the adenine labeled in this position should decrease more rapidly than that of the adenine-4,6-C^{14}. Preliminary results, however, do not indicate any differences in the specific activities of differently labeled adenines up to 28 days after administration. These results are in agreement with the concept of a stable purine ring.

It has been found that respiratory $C^{14}O_2$ is obtained after the administration of each of the labeled adenines. The rate of elimination of $C^{14}O_2$ is markedly different for each adenine-C^{14}. In 22 hours, 5% of the C-2, 7% of the C-4,6, and 1.7% of the C-8 position is obtained as $C^{14}O_2$. The respiratory $C^{14}O_2$ from adenine-4,6-C^{14} is believed to arise from the 6-position during the conversion of adenine to allantoin. Little $C^{14}O_2$ is obtained from adenine-2-C^{14} or adenine-8-C^{14} during the first hour or so after administration when a large amount of free adenine is present. Most of the $C^{14}O_2$ arises after the adenine has been incorporated into nucleotides, nucleic acids, and several minor unidentified compounds. These experiments indicate that either routes of adenine oxidation exist other than the known route to allantoin, or an exchange of the 2- and 8-positions of the purine ring does in fact occur in the mouse, particularly after the adenine has been incorporated into nucleotides or nucleic acids. Administration of A-methopterin or aminopterin, however, does not change the $C^{14}O_2$ excretion of adenine-2-C^{14} significantly, as would be expected if folic acid were involved in the formation of respiratory $C^{14}O_2$ from adenine-2-C^{14}.

In these studies, the primary emphasis has been upon normal metabolism because a basic knowledge of normal metabolism is necessary before extensive studies of abnormal conditions are undertaken. It is now planned to extend these investigations to a study of factors affecting the renewal of nucleotides and nucleic acids. In addition, it is planned to investigate in detail the incorporation and renewal of other precursors, such as orotic acid and 4-amino-5-imidazolecarboxamide, into nucleotides and nucleic acids.

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Absence of Amino Acid Incorporation into Antibody during the Induction Period

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The extent of antibody synthesis during the induction period forms the basis of the present study. The only previous study on this subject known to us is that by Green and Anker.[1] These authors first showed that the antibody concentration of carboxyl labeled C^{14}-glycine depended upon the isotope concentration of the non-protein glycine rather than upon the isotope glycine in the serum proteins. They then administered amino acids labeled with different isotopes at different times during the same secondary response to ovalbumin in rabbits. Thus, after a second injection of antigen, they gave C^{14} at 10 hours, N^{15} at 41 hours and H^2 at 89 hours. The induction period lasted about 3 days and peak titer occurred on the 5th day. They found that both C^{14} and N^{15}, which were administered during the induction period, appeared in the antibody on the 5th day. Furthermore, by comparison with the serum protein labels in this animal and in a second animal administered C^{14}- and C^{15}-labeled amino acids, they calculated that 31% of the antibody protein was synthesized during the first 60 hours after the injection of antigen, i.e., the approximate duration of the induction period, whereas the remaining 69% was synthesized in the following 72 hours during the rise of serum antibody. The discrepancy between the interpretation of these and our results will be considered in the discussion.

In the present work, we believe we have simplified the interpretation of the experimental results (*a*) by dividing the immune process between two rabbits at about the end of the induction period and (*b*) by limiting the administration of isotopically labeled amino acids to one of the rabbits. This division was effected by transferring spleen mince from a donor rabbit at the end of the induction period following a second injection of bovine serum albumin (BSA) to a normal recipient rabbit. The recipients of the spleen mince showed an early rise of serum-precipitating antibody with a peak titer on the second day. Such donors or recipients were administered yeast hydrolysate containing amino acids labeled with S^{35}. The antibody formed in the recipients (and in some donors after removal of the spleen) was then studied for radioactivity.

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MATERIALS AND METHODS

Spleen Mince Transfer

The secondary response of rabbits to bovine serum albumin (BSA) was used throughout the present investigation because the precipitin response was good, and the induction period and antibody rise to peak titer were short and definitely demarcated. The primary and the secondary responses were induced about a month apart by the intravenous injection of 30 mg BSA/ml 0.85% NaCl/kg rabbit.

Three days after the second injection of a donor rabbit (counting the day of the second injection as 0 day) at a time which usually preceded the appearance of precipitating antibody in the serum, the spleen was removed, weighed, minced in a Teflon tissue grinder in cold phosphate buffered 0.85% NaCl, put through a 100 mesh sieve and injected intravenously into one or more recipients either with or without washing. About four-fifths of the original spleen passed through the sieve. The washing consisted of suspending the screened material in 15 ml of the cold buffered solution, centrifuging at 1500 rpm for 10 minutes, discarding the supernatant and resuspending the splenic material in the cold buffered solution. The *in vitro* interval was 6 to 8 minutes without washing and 18 to 25 minutes with washing.

S^{35}-Labeled Amino Acids

The labeled amino acids were prepared by Abbott Laboratories, Chicago, and consisted of an acid hydrolysate of yeast to which was added unlabeled tryptophane in approximately the amount lost by the acid hydrolysis. They were always injected into the rabbits intravenously.

Specific Activity of Serum and Antibody Proteins

All specific activities of serum components are given as counts per minute per μg N (protein nitrogen).

Serum proteins were precipitated by adding 1 ml of an appropriate dilution of serum to 1 ml of 20% trichloracetic acid (TCA), washing with water and dissolving in 3 ml of 0.04 *N* NaOH. Duplicate samples of 0.05 ml were placed on oxidized copper planchettes for radioassay and similar amounts were used for determinations of protein N by a modified Folin-Cicolteau procedure.

Table I. Ratio of Specific Activity of Antibody Protein N to Serum Protein N in a Donor Receiving 12 Doses of 0.1 mc S^{35} at Approximately 12-hour Intervals, A*/S*

Day	cpm/μg N		A*/S*
	Antibody	Serum	
0		2.1	19.0
1		4.7	8.5
2		7.9	5.0
3		12.9	3.1
4		20.1	2.0
5	39.0	26.8	1.5
6	40.6	27.3	1.5

The equivalence ratio of antigen to antibody was determined by the method of Talmage and Maurer.[2] A sample of each antibody containing serum was mixed with BSA in the zone of slight antigen excess and, after incubation at 37°C for 30 minutes, was kept at approximately 5°C for 4 days. The precipitate was then centrifuged, washed twice in the cold with 0.85% NaCl solution and dissolved in 1.2 ml of 0.04 *N* NaOH. Of this material, 0.05 ml aliquots were used for protein and radioactivity determinations by the same methods outlined for the serum proteins.

Radioassay

Radioactivity was determined in a gas flow internal Geiger counter. In all cases duplicate radioassays were made and calculations were based on a total of at least 1280 counts. When necessary, the experimental counts were corrected for resolving loss and self absorption from empirical curves.

EXPERIMENTAL RESULTS

Stability of the S^{35} Label

The stability of the isotopic label was tested (*a*) by the passive transfer of antibody from a labeled donor to a normal recipient and (*b*) by the reverse experiment of the passive transfer of unlabeled antibody to a labeled recipient.

The donor rabbit was administered 0.1 mc S^{35} labeled amino acids at approximately 12-hour intervals for 6 days. On the 5th and 6th days, the specific radioactivity of the antibody was approximately 39.8 counts/μg antibody protein N (Table I).

The specific activity of this antibody, on the fifth day, when it was transferred to a recipient, remained at practically the same approximate value for a period of ten days.

The reverse transfer was made by injecting a non-immunized recipient intravenously with 11.5 ml of a high titered unlabeled anti-BSA serum. This rabbit showed 142 μg antibody N/ml serum 10 minutes later. It then received 0.2 mc S^{35} immediately after the injection of serum and 12 subsequent injections of 0.1 mc S^{35} at approximately 12-hour intervals through the succeeding 6 days. Radioassays on the 2nd, 5th and 7th days gave counts of 1.4, 2.2 and 1.4/minute/ μg antibody N as contrasted with counts of 36, 41 and 49 for serum protein N on the same days. Complement analysis of the serum showed that this antibody radioactivity was probably due to labeled complement precipitated with the antibody. These findings indicated that the antibody was not labeled with S^{35} after passive transfer.

S^{35} Labeled Donors and Unlabeled Recipients

The transfer of splenic materials from six S^{35} labeled donors into 11 unlabeled recipients resulted in the formation of antibody with a very low specific radioactivity (Table II, last column). In 9 of the 11 recipients, the spleen mince was injected as rapidly as possible into the recipient without washing. In recipients 6A and 6B, the splenic material was washed (*see* "Materials and Methods" above). These two rabbits gave the lowest radioactivity. In

Table II. Specific Radioactivity of Serum and Antibody Protein N Following S^{35}-labeling of Donor

Donor					Recipient					
		Antibody						Antibody		
Rabbit number	mc S^{35} administered	Serum count per μg N day 3	μg N per ml serum day 5	cpm/ μg N day 5	Rabbit number	Gram donor spleen per kg recipient	Serum cpm/ μg N day 2	μg N per ml serum	cpm/ μg N	Per cent labeled
1	0.2 × 6	30	987	60	1A	0.42	0.11	11.3	0.7	1.2
					1B	0.46	0.13	15.8	0.5	0.8
2	0.5 × 6	71	2625	102	2A	0.58	0.11	26.4	3.6	3.5
					2B	0.68	0.14	23.8	4.0	3.9
3	0.5 × 6	78	*	156†	3A	0.33	0.08	10.0	1.2	0.8
					4A	0.27	0.18	2.2	3.1	0.8
4	1.0 × 6	183	*	376†	4B	0.33	0.13	2.9	2.4	0.7
					5A	0.21	0.17	11.0	4.5	1.6
5	1.0 × 6	143	*	286†	5B	0.21	0.19	2.2	16.0	5.6
					6A	0.54	0.02	19.0	0.54	0.26
6	2: Day 2	70	*	210‡	6B	0.46	0.004	15.0	0.48	0.23

* Killed on 3rd day.
† Estimated value at 2 times the serum specific activity on day 3.
‡ Estimated value at 3 times the serum specific activity on day 3.

Table II, the percentage labeling of recipient antibody was calculated from the specific radioactivity of the donor antibody. This was determined directly by continuing the administration of labeled amino acids to donors 1 and 2 after splenectomy or was estimated indirectly from the specific radioactivity of the donors' serum proteins at the time of spleen transfer. The results are further explained in the following paragraphs.

Probably the most accurate method of estimating the percentage labeling of antibody in the recipient is that employed for labeled donors 1 and 2 and their recipients 1A, 1B, 2A and 2B. Each donor was labeled with 6 injections of S^{35} yeast hydrolysate at approximately 12-hour intervals for 3 days beginning immediately after the antigen injection. On the 3rd day, the spleen was removed and the mince from each spleen was divided between two recipients. Labeling of the donors at the same rate was then continued through the 6th day. This was done to get the specific radioactivity of antibody produced at this level of S^{35} administration. The level of specific radioactivity would not be expected to change under these conditions because antibody was being formed during a constant dosage schedule of labeled amino acids. Had all of the antibody appearing in the recipients been labeled in the donor in precursor form, the radioactivity of the recipient antibody should be identical with that of the donor. The ratio of specific antibody activity in the recipient to that in the donor gives the percentage of antibody in the recipient which was labeled during the induction period in the donor. There was no possibility that the antibody specific activity in the recipient could be lessened by a primary antibody response to antigen transferred in the spleen mince because a primary response does not begin until the eighth day or later.

The specific radioactivity of antibody in donor 1 was 60 cpm/μg N, whereas the specific activities of recipients 1A and 1B were 0.7 and 0.5 counts, i.e., labelings of 1.2% and 0.8%, respectively. No precipitating antibody could be detected in the donor serum on the day of splenic transfer although it contained coprecipitating antibody. The serums of recipients 2A and 2B possessed a higher radioactivity than recipients 1A and 1B, i.e., labelings of 3.5 and 3.9, respectively. These findings may probably be attributed to the passive transfer of a small amount of precipitating antibody in the spleen mince from donor 2 because the latter showed 81 μg precipitating antibody protein N/ml at the time of splenic transfer.

When donors were given repeated doses of S^{35}-labeled amino acids and were sacrificed on the 3rd day at the time of splenic transfer, their antibody labeling on the 5th day was approximated from the specific labeling of their serum proteins on the 3rd day by using a factor of 2. The rational for this procedure is shown in Table I. Administration of 0.1 mc at 12-hour intervals to the donor shown in this table resulted in a mean count of 39.8/minute/μg N for the antibody on the 5th and 6th days. This figure was 3.1 times the specific activity of the serum on the 3rd day. In two other similar experiments, antibody activities on the 5th day were 2 and 1.5 times the specific activities of the serums on the 3rd day. The factor of 2 is slightly lower than a mean of the three ratios.

Using this approximation for the donors, data from the 4 recipients, 3A, 4A, 4B and 5A, showed such low specific radioactivities for antibody N that only 0.7 to 1.6% of the antibody appeared to be labeled in the donor during the induction period. Data from recipient 5B indicated that 5.5% of the antibody was labeled in the donor. This high figure, we believe, is simply an expression of a low production of antibody (2.2 μg/ml as contrasted with 11 μg/ml in its companion recipient 5A). Both recipients, it should be pointed out, received about the same amount of spleen mince (Table II, column 7) from the same donor and, therefore, received passively about the same amount of labeled coprecipitating antibody.

A single dose of 2 mc S^{35} was given to donor 6–18 hours before the splenic transfer. At this time, the serum specific activity would be low as compared to the specific antibody label if the latter were being synthesized. As a conservative figure, we estimated the donor antibody on the 5th day to be 3 times the serum protein specific activity on the 3rd day. The spleen mince from this donor after being washed and injected into recipients 6A and 6B showed an estimated percentage labeling of only about 0.25%.

Therefore, eliminating the 3 highest readings because they probably were augmented by labeled coprecipitating or precipitating antibody transferred passively, the mean labeling of antibody was 0.8% during the induction period as indicated by the data from 5 donors and 8 recipients.

Unlabeled Donors and S^{35}-Labeled Recipients

Seven recipients received unlabeled spleen mince and were injected with labeled amino acids during the 2 days of their antibody rise to ascertain to what extent the resulting antibody was labeled. The specific radioactivity of these recipients was compared to the specific radioactivity of antibody from animals in which the entire immune process was completed in one animal and in which the labeling (*a*) was limited to a corresponding period, i.e., the 3rd and 4th day, or (*b*) was given throughout the induction period and antibody rise, i.e., for 5 days.

In comparing the various rabbits, probably the best criterion is the ratio of the specific radioactivity of antibody to that of the serum proteins. This value A^*/S^* varied over the narrow range of 0.9–2.5. The most satisfactory comparison involved labeling in the same manner for donor and recipient. Thus, donor 9 and its 2 recipients were all labeled beginning immediately after splenic transfer. In this case, the antibody in the donor possessed a specific radio-

activity of 17.4 cpm/μg antibody N and an A^*/S^* ratio of 1.3. The two recipients gave 22.3 and 18.9 cpm/μg antibody N and A^*/S^* ratios of 1.4 and 1.8, respectively. Such a close correspondence indicates that the incorporation of amino acids into antibody was not significantly modified by the method of transferring antibody-forming capacity with spleen mince. Other data corrohorated this conclusion. Variations in the A^*/S^* values were associated with variations in the method of administering the labeled amino acids, but all labeled recipients produced antibody with a high specific radioactivity.

DISCUSSION

We found (*a*) a low radioactivity of antibody in recipients receiving splenic cells from donors administered S^{35}-labeled amino acids and (*b*) a high radioactivity of antibody in recipients receiving themselves S^{35}-labeled amino acids during the rise of serum antibody. These results indicate that there is very little incorporation of amino acids into antibody during the induction period. The per cent labeled antibody in unlabeled recipients receiving spleen mince from labeled donors as shown in Table 2 indicates that there were the following two groups of animals: (*a*) 3 rabbits with high values (2A, 2B and 5B) ranging from 3.5 to 5.6% and (*b*) 8 rabbits with low values ranging from 0.23 to 1.6% (mean of 0.8% labeled. We have already given cogent reasons why the three high determinations were too high. Less than 1%, therefore, of amino acids were incorporated into antibody during the induction period under the conditions of our experiments. This amount is only about 3% that reported by Green and Anker.[1]

We do not at present know why our results differ so radically from those of Green and Anker[1] who concluded that 31% of the antibody was synthesized during the induction period. There may be some difference associated with the different antigens used in the two studies or the immune process may be somewhat deranged by our method of transferring the immune mechanism with splenic mince. More likely it is due to the fact that Green and Anker did not divide the immune process between two animals and did not take into account coprecipitating antibody or complement. These latter materials would have been labeled during the induction period in their work and would have been carried down in the immune precipitate on the 5th day when antibody was isolated for radioassay.

Taken at face value, our results indicate that almost all of the sulfur-containing amino acids present in serum antibody are drawn from the amino acid pool during the rise of serum antibody and not during the induction period. The lack of incorporation of amino acids into antibody during the induction period may indicate that synthetic enzymes are developed during the induction period which, once present, begin to form antibody. If very little time elapses between the appearance of the suggested enzymes and the synthesis of antibody, the rise of serum antibody should give an accurate approximation of the rate of antibody formation.

CONCLUSIONS

The secondary immune response to bovine serum albumin was divided between two rabbits. Thus, 3 days after a second injection of antigen into donor rabbits, the spleen was removed, minced and injected intravenously into normal recipient rabbits. At the time of transfer, there was some coprecipitating but usually no precipitating antibody in the donor's serum. After receiving the spleen mince, recipient rabbits showed an early rise of precipitating antibody in the serum which reached a peak on the second day. Either the donor or recipient was administered S^{35}-labeled amino acids and the specific radioactivity of the precipitating antibody determined in the recipient. These procedures led to the following conclusions:

When S^{35}-labeled amino acids (in single or repeated doses giving a total of 0.4 to 6 mc) were given to donor rabbits during the induction period (but not to their recipients), less than 1% of the total precipitin synthesized in the recipients was labeled at peak titer. Some coprecipitating antibody was synthesized during the first 3 days, but has not been adequately studied.

When S^{35}-labeled amino acids (in single or repeated doses giving a total of 0.3 to 1 mc) were given to recipient rabbits during the antibody rise, the precipitin synthesized at peak titer was labeled to the same degree as when the entire immune process took place in one rabbit and the labeled amino acids were administered during the rise of serum antibody.

Control passive transfer experiments indicated (*a*) that S^{35} was stably bound to labeled antibody when transferred to a normal "unlabeled" rabbit, and (*b*) unlabeled antibody did not acquire an appreciable radioactivity when transferred to a rabbit receiving S^{35}-labeled amino acids.

The results indicate that almost all of the sulfur-containing amino acids present in precipitating antibody are drawn from the amino acid pool during the rise of serum antibody and not during the first 3 days after the injection of antigen (induction period). They also (*a*) indicate that the rate of rise of serum antibody reflects the rate of antibody formation and (*b*) suggest that the induction period is concerned with the production of antibody-synthesizing enzymes rather than with amino-acid-containing antibody precursors.

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Dietary Factors in the Oxidation and Synthesis of Fatty Acid by Tissue Preparations

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Important information on the role of nutritional factors in fatty acid metabolism has been obtained, in this and in other laboratories, by experiments on isolated preparations of tissues from animals maintained under various dietary conditions. In such studies, the use of substrates labeled with radioisotopes is especially advantageous. Because of the sensitivity and specificity of radioactive measurements, synthetic processes can be detected even in the presence of active degradations resulting in a net decrease in the amounts of the products studied. Effects on the rate of the oxidation of the substrate by substances added *in vitro* are easily distinguished from stimulations or inhibitions of the overall metabolism. Moreover, since only minute amounts of substrates with high specific activity need to be used, the inhibitory action of fatty acids on their oxidation *in vitro* becomes negligible.

In previous experiments, it was shown that the *in vitro* oxidation of isotopic stearate or palmitate is depressed in the livers of rats, previously maintained on low-protein diets. Choline, added to the diets, or injected into the animals, restored the ability of the isolated tissue to oxidize fatty acids at a high rate.[1] Similar results have been obtained by others for the oxidation of non-isotopic octanoate.[2] These findings suggest that the lipotropic action of dietary choline is due, at least partly, to an enhanced oxidation of fatty acids in the liver. More recently we have found that this effect of choline, administered *in vivo*, is not specific for the liver, but can be demonstrated also in other tissues, such as kidney and heart.[3]

The diets used in our previous investigations contained little methionine (a major precursor of both choline and cystine) and minimal amounts of cystine. A study of the effects of supplementing these diets with cystine seemed of interest, in view also of other findings in the literature, such as the so-called "antilipotropic" effect of cystine, and its protective action against the massive liver necrosis, induced by diets poor in S-containing amino acids. Since a similar protection is exerted by tocopherol, the action of this vitamin was also investigated. Both the oxidation of long-chain fatty acids and their synthesis from acetate were studied, using isotopic substrates and homogenates, or slices, of rat liver.

EXPERIMENTAL

Male albino rats (initial weight, about 60 gm) were maintained for varying periods of time (mostly 2–4 weeks) on several diets, low in methionine and cystine and free from tocopherol and preformed choline (Table I). Other rats received the diets with supplements added in the following amounts (per 100 gm diet): cystine 1.5 gm, tocopherol 0.025 gm, methionine 1.0 gm, choline chloride 0.5 gm, guanidoacetic acid 1.0 gm. In each experiment, homogenates, or slices, of the livers of rats on one of the unsupplemented diets were incubated simultaneously with similar preparations from rats which had been maintained for the same length of time on the same diet with the added supplements. In the experiments on fatty acid oxidation, the incubation medium contained, in 6 ml of Ca-free Ringer-phosphate (pH 7.4), liver homogenate (in amounts corresponding to 400–500 mg tissue), penicillin G and dihydrostreptomycin 3 mg each, adenosine triphosphate 12 micromoles, and palmitate-1-C^{14}, or, less frequently, stearate-1-C^{14} (1–2 micromoles and 1–2 millicuries). After 3 hours of incubation in air at 37°, the $C^{14}O_2$ produced was measured. In the experiments on fatty acid synthesis, 500–700 mg of slices were incubated with penicillin and dihydrostreptomycin (3 mg each) and with acetate-1-C^{14} (2 micromoles, 1 microcurie) in 5 ml of Ringer-phosphate (pH 7.4). After 3 hours in air at 37°, radioactive measurements were carried out on the respiratory CO_2 and on the total fatty acids.

The isotopic values were corrected on the basis of the small amounts of C^{14} obtained from identical control flasks containing boiled homogenates or slices. On separate samples of the liver, total N, total lipides and lipide P were also determined analytically.

RESULTS

For the purpose of comparison, the experimental data (calculated either per mg of N, or for the whole liver of a 100 gm rat) have been expressed as "relative values", the value determined in each experiment on the liver of rats on the unsupplemented diet being made equal to 100. In all groups of ex-

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Table I. Composition of Experimental Diets

Diet no.	Alpha-protein, gm	Casein, gm	Total fat,* gm	Methionine cystine in diet,† gm
53 (52*)	8		5 (32*)	0.11
55 (54*)	15		5 (32*)	0.20
26 (28*)		5	5 (32*)	0.19
47 (46*)		8	5 (32*)	0.30

Other components: Salts 4 gm, Ruffex 2 gm, dextrin and sucrose to 100 gm. B vitamin mixture added twice a week.

* Crisco 4 gm, and cod liver oil 1 gm; or Crisco 30 gm, and cod liver oil 2 gm.
† High fat diets.

periments, the data exhibited rather wide variations. However, from a comparison of the averages, it is apparent that supplementation of the diets with cystine raised significantly the ability of the liver homogenate to oxidize the added fatty acid (Table II). This effect was of about the same degree as that obtained by supplementing the diets with choline, or with methionine (Table III). Tocopherol administration also enhanced fatty acid oxidation. No greater enhancements were observed, when both cystine and tocopherol had been added to the diets (Table IV).

In the experiments on the synthesis of fatty acids from acetate-1-C^{14}, the means of the relative values were not significantly different from 100. Accordingly, such means should be considered at present as merely suggestive. It would appear that, at least in the majority of our experiments, supplementation of the diets with cystine, or tocopherol, or both tocopherol and cystine, also enhanced fatty acid synthesis in liver slices (Table V).

In Table VI, a comparison has been made between the effects of choline, injected terminally in the animals, on the oxidation, or on the synthesis of fatty acids, respectively. In agreement with previous results 1–3 choline stimulated fatty acid oxidation in liver homogenates from rats which had been on a diet severely deficient in choline and did not receive cystine. The additive effect of the choline injection was also apparent in some, but not in all experiments on rats receiving cystine in the diet. On the other hand, the average values for lipogenesis in slices of rats, injected with choline, were mostly lower than in the livers of non-injected animals, whether the rats had, or had not, received cystine supplements.

DISCUSSION

In the present status of our knowledge, any attempt at interpreting the results summarized above must be largely speculative. Since fatty acid oxidation in liver homogenates is enhanced by supplementing the deficient diets with either choline or cystine, and since dietary methionine is a precursor of both substances, the possibility of a mutual sparing action of cystine and choline in maintaining high rates of fatty oxidation should be kept in mind.

In several of our experiments, the *in vitro* synthesis of fatty acids from acetate was also higher in

Table II. Oxidation of Palmitate-1-C^{14} in Rat Liver Homogenates. Effects of Cystine (1.5%) Added to the Diets

Diets	Dietary protein	Dietary fat, %	No. of exper.	$C^{14}O_2$ (Relative values*)	
				Per mg N liver †	Per whole liver 100 gm rat †
53-55	α-Protein	5	14	253	272
26-47	Casein	"	18	155	177
52-54	α-Protein	32	9	225	245
28-46	Casein	"	11	323	331

* Livers of rats on the unsupplemented diets = 100.
† Significantly different from 100.

the livers of the rats receiving cystine. These findings would be in line with results obtained in intact animals, fed D_2O and a low-protein diet.[4] More recently, it has been found that livers of weanling rats, fed a necrogenic diet (essentially similar to our Diet No. 55), exhibited decreased rates of lipogenesis and lower levels of coenzyme A.[5] Furthermore, a direct incorporation of cystine-S into liver coenzyme A was demonstrated.[6] Since this compound is involved in the synthesis as well as in the oxidation of fatty acids, it is tempting to postulate that the increased rates of both processes in the livers of rats on cystine supplemented diets is due to the maintenance of higher levels of coenzyme A in the tissue. On the other hand, choline, injected terminally in the animals, stimulated the oxidation of fatty

Table III. Oxidation of Palmitate-1-C^{14} in Rat Liver Homogenates. A Comparison of the Effects of Adding Cystine, Choline, or Methionine to Low-Fat Diets (Diets Nos. 53, 55, 26)

Dietary additions (per 100 gm diet)	No. of exper.	$C^{14}O_2$ (Relative values*)	
		Per 1 mg N liver †	Per whole liver 100 gm rat †
Cystine 1.5 gm	16	277	247
Choline Cl 0.5 gm	11	254	265
Methionine 1.0 gm	8‡	274	312

* Livers of rats on the unsupplemented diets = 100.
† Significantly different from 100.
‡ Including two experiments with cystine 1.5 gm and choline Cl 0.5 gm, added to the diets.

Table IV. Oxidation of Palmitate-1-C^{14} in Rat Liver Homogenates. A Comparison of the Effects of Adding Cystine and/or Tocopherol to the Diets (Low-Fat Diets, Nos. 53, 55, 26)

Dietary additions (per 100 gm diet)	No. of exper.	$C^{14}O_2$ (Relative values*)	
		Per mg N liver †	Per whole liver 100 gm rat †
Cystine 1.5 gm	11	194	237
α-Tocopherol 25 mg	10	361	322
Cystine 1.5 mg + α-Tocopherol 25 mg	6	211	269

* Livers of rats on the unsupplemented diets = 100.
† Significantly different from 100.

Table V. Fatty Acid Synthesis from Acetate-1-C^{14} in Rat Liver Slices. A Comparison of the Effects of Cystine, or Tocopherol added to the Diets (Low-Fat Diets, No. 53-55)

Dietary additions (per 100 gm diet)	*No. of exper.*	$C^{14}O_2$ *(Relative values*)*	
		Per mg N liver	*Per whole liver 100 gm rat*
Cystine 1.5 gm	8	243	326
α-Tocopherol 25 mg	6	322	347
Cystine 1.5 gm + α-Tocopherol 25 mg	5	717	1070

* Livers of rats on the unsupplemented diets = 100.

Table VI. Effects of Choline Injected Terminally on the Oxidation and Synthesis of Fatty Acids in Rat Liver Homogenates and Slices. Rats on Diet 26 - Choline Injected: 0.25 mM/100 gm Rat

Additions to diet (per 100 gm diet)	*Choline injected*	C^{14} *(Relative values*)*	
		CO_2 *from palmitate-1-C^{14}*	*Fatty acids from acetate-1-C^{14}*
Guanidoacetate 1.5 gm	—	100	100
Guanidoacetate 1.5 gm	+	301† (7)‡	87 (4)‡
Cystine 1.5 gm	—	240† (11)‡	233 (4)‡
Cystine 1.5 gm	+	468† (6)‡	117 (4)‡

* Livers of rats on diet 26 + guanidoacetate = 100. Values per whole liver of a 100 gm rat.
† Significantly different from 100.
‡ No. of experiments in parentheses.

acids, but not their synthesis. It seems therefore that choline and cystine act on fatty acid metabolism through mechanisms and at biochemical sites which are different for either of these factors.

While in most of our experiments the so-called antilipotropic effect of cystine was quite marked, in others the fat contents of the livers of animals receiving cystine were similar to, or lower than, those of the rats on the unsupplemented diet. Similar findings with levels of cystine above 0.5 per cent have been noted.[7] Moreover, an apparent "antilipotropic" effect of methionine, added to a threonine-deficient, choline-supplemented diet, has been described.[8] On the basis of our present results, the antilipotropic effects of S-containing amino acids may be ascribed to a stimulation of lipogenesis proportionally greater than that of fatty acid oxidation. At present, since quantitative data are lacking and in view of the number and variety of factors involved, the conditions under which such a disproportion occurs can only be determined empyrically.

The effects of tocopherol administration on both the synthesis and the oxidation of fatty acids were similar to those obtained by supplementing the diets with cystine. Such a similarity may be in line with a postulated role of alpha-tocopherol in the utilization of S-coataining amino acids for the synthesis of coenzyme A.[6]

SUMMARY

Rats were maintained on diets low in methionine and free from preformed choline and tocopherol, to which various supplements were added. The oxidation and the synthesis of isotopic fatty acids in liver preparations from animals on the unsupplemented, or, respectively, supplemented diets were investigated.

Supplementation of the diets with choline, cystine, methionine, tocopherol, or cystine and tocopherol, enhanced the oxidation of fatty acids in liver homogenates. In many experiments, addition of cystine, tocopherol, or cystine and tocopherol, to the deficient diets also increased the incorporation of isotopic acetate into long-chain fatty acids of liver slices. Choline, injected terminally into the rats, enhanced the oxidation, but not the synthesis of fatty acids *in vitro*.

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Mechanism of Incorporation of Labeled Amino Acids into Protein

By P. C. Zamecnik, E. B. Keller, J. W. Littlefield, M. B. Hoagland and R. B. Loftfield,* USA

Some years ago it was suggested by Lipmann[1] that energy rich phosphate compounds participate in protein synthesis. Since that time investigations using labeled amino acids have pointed in this direction 2, 3, 4 and model systems 5, 6, 7 have implicated the carboxyl group of the amino acid as a favored activation site.

By means of the gentle homogenization procedure of Bucher,[8] and the use of hexose diphosphate as an energy donor, the rate of incorporation of C^{14}-labeled amino acids into protein in a cell-free rat liver system was increased sufficiently to facilitate fractionation of the components of the complex homogenate.[23]

It also became possible to discard the mitochondrial fraction of the cell-free rat liver homogenate and to run the incorporation reaction anaerobically, using HDP as the energy source. At this point the incorporation system consisted of four components: (1) the labeled amino acid, (2) the energy donor, (3) the microsome fraction and (4) the 105,000 g supernatant fraction. Substitution of phosphocreatine and ATP-creatine transphosphorylase for the HDP-glycolytic enzyme energy generator provided a further simplification of the system, but did not eliminate the necessity for component (4). It was possible, however, to dialyze out small molecular weight components from this 105,000 g supernatant fraction, and in this way to demonstrate a requirement for added ATP. It was therefore reasonable to consider either that the microsomes or alternatively, the amino acids themselves, were being activated by ATP.

AMINO ACID ACTIVATION

In order to distinguish between these possibilities, the rate of exchange of PP^{32} into ATP was studied by Hoagland,[10] in the microsome and 105,000 g supernatant fractions. In the latter fraction, it was found that the addition of a complete complement of L-amino acids enhanced the rate of exchange of PP^{32} into ATP. This observation provided the encouragement to try to trap carboxyl activated amino acids, if such were being formed in this 105,000 g supernatant fraction in the presence of ATP. By the use of high concentration (1.2 molar) of hydroxylamine, aminoacyl hydroxamic acids were found. It was necessary to use salt-free hydroxylamine in order to prevent a high salt concentration from inhibiting the enzymatic reactions.

Interestingly enough, the extent of formation of hydroxamic acid was dependent on the number of separate amino acids added to the 105,000 g dialyzed supernatant. This finding pointed to multiple, separate activating enzymes (or multiple separate activation sites) rather than activation of all amino acids at a single enzymatic locus in the 105,000 g protein.[11] This latter fraction, containing all the soluble proteins in the liver cell, can be subjected to isoelectric precipitation at pH 5.2. The amino acid activating enzymatic fraction precipitates at this pH, and can be redissolved, with several fold enhancement of specific enzymatic activity.

A correlation between moles of hydroxamic acid formed and PP liberated in this reaction indicated a pyrophosphate splitting of ATP, with formation of an aminoacyl-AMP intermediate (Table I). Such a compound could be either bound to an enzyme, or else free in such small concentrations as to be as yet undetected.

THE PARTICIPATION OF GDP AND GTP INCORPORATION

When the microsome fraction was centrifuged out of four times the usual volume of suspending medium, and the pH 5.2 precipitated protein fraction was used in place of the entire 105,000 g supernatant fraction, it was found that ATP could no longer activate an otherwise complete system. We suspected that some

Table I. Stoichiometry of Hydroxamic Acid, P, and PP Formation*

Additions	*Hydroxamic acid* (μMg)	*P* (μMg)	*PP* (μM)	*PP + P/2*
0	0.1	1.3	0.3	
AA	1.2	1.5	1.3	
Δ	1.1	0.2	1.0	1.1

*7 mg pH 5.2 enzyme, 10 μM K_4ATP, μM $MgCl_2$ 1.2 mM NH_2OH, AA = 4 μM each of 12 l-amino acids, PP = inorganic pyrophosphate, P = inorganic phosphate. 1 ml final volume, incubated at 37°C for 60'. Under these conditions of a low Mg++/ATP ratio, pyrophosphatase activity is inhibited and pyrophosphate can therefore be detected. This is not the case where the Mg++/ATP ratio is around 0.5 (from Hoagland, M.B. *et al.*, unpublished data).

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other nucleotide might be involved. Dr. Rao Sanadi kindly furnished GDP prior to its commercial availability, and we found, under circumstances in which the protein fractions of the system were freed of the great bulk of accompanying nucleotides, that GDP or GTP played a special activating role independent of ATP.[12] These findings are illustrated in Tables II and III. In order to demonstrate this separate GDP effect, it was necessary to use ATP of high purity.

It is not clear how the GDP operates. There is a choice of considering that it serves as an intermediate aminoacyl transfer compound, operating between the ATP-amino acid activating system and the microsomes; or alternatively, that a particular amino acid is activated by GTP rather than by ATP. In reference to this latter less likely possibility, the activation of amino acids by the pH 5.2 precipitable fraction of the 105,000 g supernatant protein appears thus far to be specific for ATP. It should be mentioned that the GDP effect on incorporation into protein has been found for six separate labeled amino acids.

Table II. Specificity of GDP or GTP Requirement for Incorporation*

Non-adenine nucleotides 0.25 μM each	*cpm/mg protein*
None	17
CMP-5′	17
CDP	13
CTP	11
UMP-5′	19
UDP	13
UTP	19
IMP-5′	22
IDP	33
ITP	32
GDP	158
GTP	175
GDP, CTP, UTP, ITP	135
GDP, CTP, UTP, ITP	152

* Each flask contained microsomes (5.0 mg protein), pH 5.2 enzyme (1.5 mg protein), 0.02 mg pyruvate kinase, 10 μ*M* phosphoenolpyruvate, 0.1 μ*M* L-leucine-C^{14}, 82,000 cpm, in 1.0 ml final volume of medium. The homogenization and suspension medium consisted of 0.35 *M* sucrose, 0.035 *M* $KHCO_3$, 0.025 *M* KCl, and 0.004 *M* $MgCl_2$. Incubation 10 minutes at 37°C in 95 per cent O_2 — 5 per cent CO_2. Other experimental details as previously described.[9] (From Keller, E.B. *et al.*, unpublished data.)

Table III. Specificity of GDP or GTP Requirement for Incorporation*

Nucleotide 0.15 μM	*No ATP*	*0.5 μM ATP*
	cpm/mg	
None	2	18
GMP-3′	2	13
GMP-5′	1	60
GDP	7	188
GTP	4	144
GDP mannose	3	19
Xanthosine-5′-diphosphate	4	27

* Each flask contained microsomes (4.4 mg protein), pH 5.2 enzyme (1.2 mg protein), 0.02 mg pyruvate kinase, 10 μ*M* PEP, 0.2 μ*M* L-leucine-C^{14}, 164,000 cpm, in a final volume of 1.0 ml. Incubation 10 minutes. Other details as in Table II. (From Keller, E.B. *et al.*, unpublished data.)

In the cell-free system, once C^{14}-leucine or C^{14}-valine has become incorporated into microsome protein, it has not been possible to "wash it out" using large quantities of inert leucine or valine. The incorporation is thus a relatively irreversible step on the path to protein synthesis, and differs from the exchange process described by Gale and Folkes[15] for incorporation of C^{14}-glutamic acid into protein (and possibly into glutathione) in the fragmented Staphylococcus aureus.

FRACTIONATION OF MICROSOMES

Dr. Jerome Gross has taken electron micrographs of our microsome fractions, with the demonstration of two main particulate types: a pancake-like lipoprotein-rich particle, and a much smaller dense particle, rich in ribosenucleic acid.[13] If the microsome pellet is suspended in 0.5 per cent sodium desoxycholate, the lipoprotein fraction dissolves, but the ribonucleoprotein particles may still be collected by centrifugation, in a rather pure state.[14] These particles have a diameter, in unfixed unshadowed specimens, of around 240 Angstroms, and an RNA-to-protein ratio of 1. When microsomes, labeled either in a cell-free system or in the whole rat, are separated in this way, the preponderance of the radioactivity is found to reside in the ribonucleoprotein part of the microsome. In very short time *in vivo* experiments, with intravenous injection of C^{14}-leucine or C^{14}-valine of high specific activity, it is possible to remove lobes of the rat liver at time intervals of 3 to 20 minutes and to follow the specific activity of various centrifugally separable fractions of the liver. The highest specific activity early in the time course occurs in the ribonucleoprotein fraction of the microsomes.

This finding appears to provide evidence for the participation of ribosenucleic acid in protein synthesis. It seems, however, that only a very small fraction of the protein of the ribonucleoprotein takes part in this rapid synthetic process. When a small amount of radioactive leucine is injected into a whole rat, this ribonucleoprotein fraction becomes very rapidly labeled, then rapidly loses its radioactivity (Fig. 1). When a large amount of radioactive leucine is injected, with maintenance of a constant high level of intracellular radioactive leucine, the same small fraction of the ribonucleoprotein becomes labeled, and remains labeled by apparent continual synthesis and loss of this rapidly turning over protein or large peptide fragment (Fig. 2).

LOCATION OF RADIOACTIVITY IN LABELED PROTEIN

During the last few years the term "incorporation," as applied to amino acids and proteins, has begun to acquire a more precise meaning than it had in earlier days.[16,17] The finding of Peterson and Greenberg[8] of conversion of C^{14}-glycine to C^{14}-serine and formation

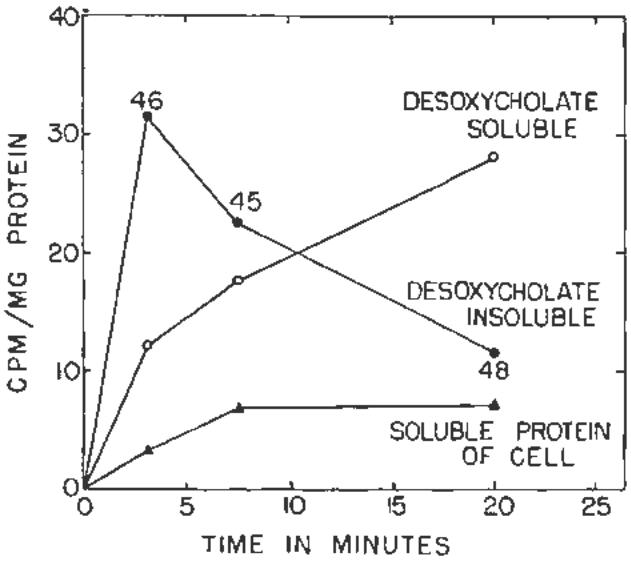

Figure 1. *In vivo* incorporation of a small dose of leucine-C^{14} into the two components of the microsomes and into the soluble protein of the cell. 0.25 μM DL-leucine-C^{14}, 2.3×10^7 cpm/mg in 0.5 ml isotonic saline was injected intravenously at 0 time into a 270 gm rat. The per cent RNA by weight of each desoxycholate-insoluble sample is indicated. The per cent RNA averaged 2.1 in the desoxycholate-soluble fractions of the microsomes and 1.7 in the soluble fractions of the cell. This per cent RNA by weight means per cent RNA in the combined RNA plus protein. Lipid, glycogen, and salts are not included

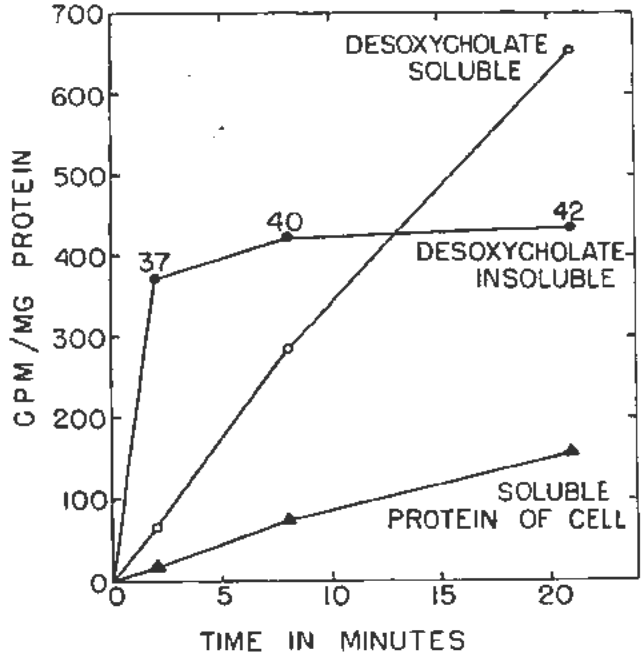

Figure 2. *In vivo* incorporation of a large dose of leucine-C^{14} into the two components of the microsomes and into the soluble protein of the cell. 100 μM DL-leucine-C^{14}, 2.2×10^6 cpm/mg, in 2.0 ml was injected intravenously at 0 time into a 300 gm rat. The per cent RNA by weight of each desoxycholate-insoluble sample is indicated. The per cent RNA averaged 2.3 in the desoxycholate-soluble fractions of the microsomes and 1.9 in the soluble fractions of the cell

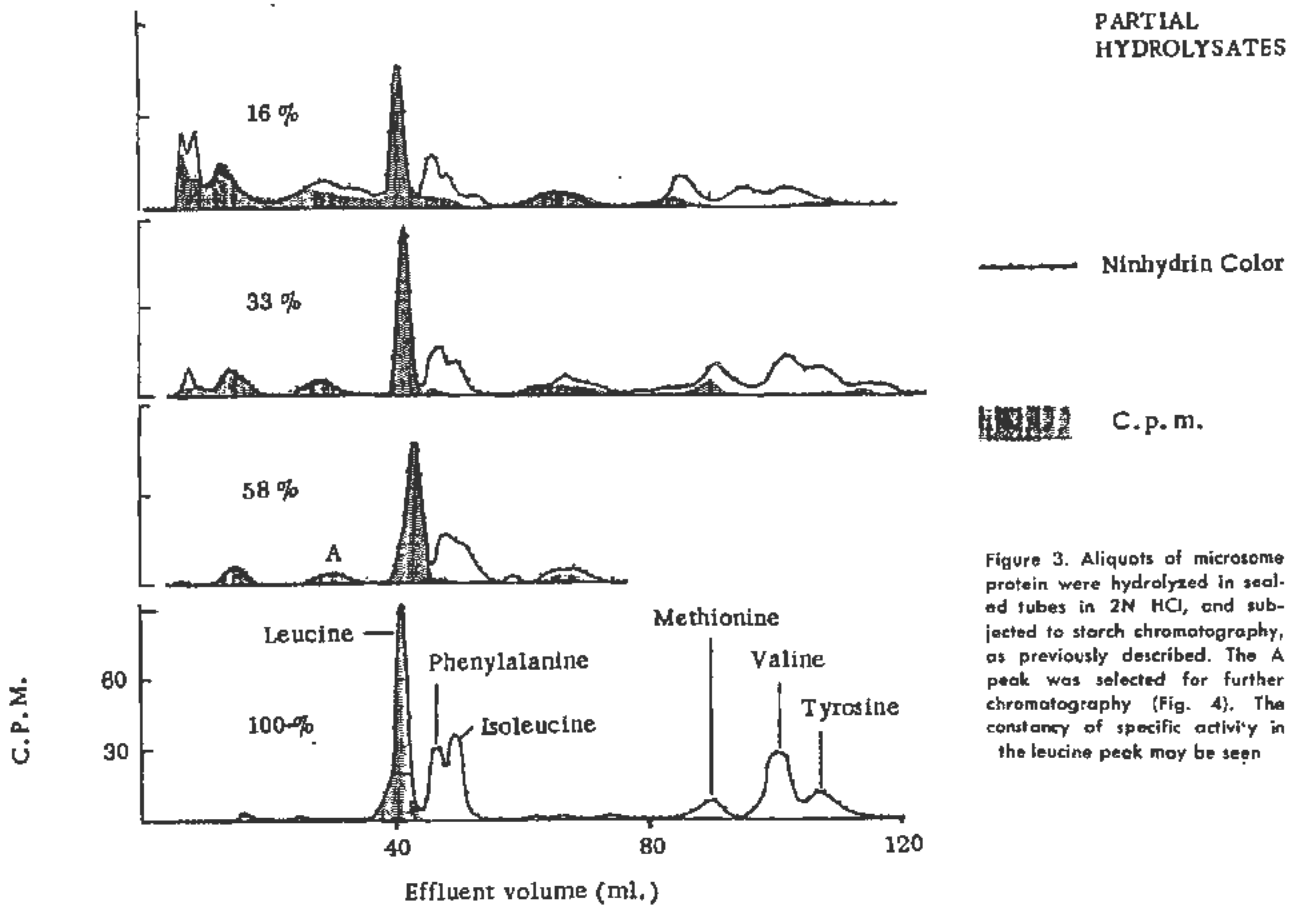

Figure 3. Aliquots of microsome protein were hydrolyzed in sealed tubes in 2N HCl, and subjected to starch chromatography, as previously described. The A peak was selected for further chromatography (Fig. 4). The constancy of specific activity in the leucine peak may be seen

of phosphatidyl-C^{14}-serine in homogenates has emphasized the importance of washing out contaminating lipid compounds from trichloracetic acid precipitated proteins. It has likewise been found necessary to wash such protein precipitates with mercaptoethanol or a similar sulfhydryl reducing compound in order to remove glutathione, a procedure particularly applicable where C^{14}-glutamic acid or C^{14}-glycine is being used.

The observation of Schweet and Borsook[20] that C^{14}-lysine is incorporated into guinea pig liver protein in a soluble cell-free system without added energy donors had also been a puzzle. It is now clear from partial protein degradation studies[21] that the lysine is bound to the protein by its E-amino group.

The best criterion that the labeled amino acid is combined in alpha-peptide linkage in the labeled protein is demonstration of radioactive alpha-peptides on partial hydrolysis of the protein.[18,19]

In this laboratory, the rate of release of C^{14}-leucine from microsome protein labeled in a cell-free system has been compared with the rate of release of the bulk of the protein leucine (Fig. 3). The specific activity of the leucine released by acid hydrolysis does not change, between 15 per cent and 100 per cent hydrolysis.[9] This finding is a strong indication that the labeled leucine is bound in microsome protein in the same type of linkage as is the unlabeled leucine, although it might still be in peptide linkages other than alpha. Following incomplete acid hydrolysis, peaks of ninhydrin color and radioactivity were found to appear at points different from the sites of emergence of known amino acids. One such peak, designated A on the curve for 58 per cent hydrolysis, was pooled, concentrated, and subjected to further chromatographic separation by Mrs. Anne Harris. It was found to consist of a mixture of closely related small peptides. In order to minimize the possibility of peptide rearrangements during hydrolysis of the protein,[22] with production of amino acid sequences not present as such in the original protein, milder hydrolytic conditions were employed in further studies. To facilitate identification of an individual peptide, a large batch of inert microsome protein was prepared. The A peak was chromatographed on a Dowex-50 column. From the Dowex-50 eluate, one sharp peptide peak was selected, and was found by use of DNP and paper chromatography to be isoleucylleucine (Fig. 4).

It may be concluded that the C^{14}-leucine in this cell-free system has become bound in alpha-peptide linkage in microsome protein.

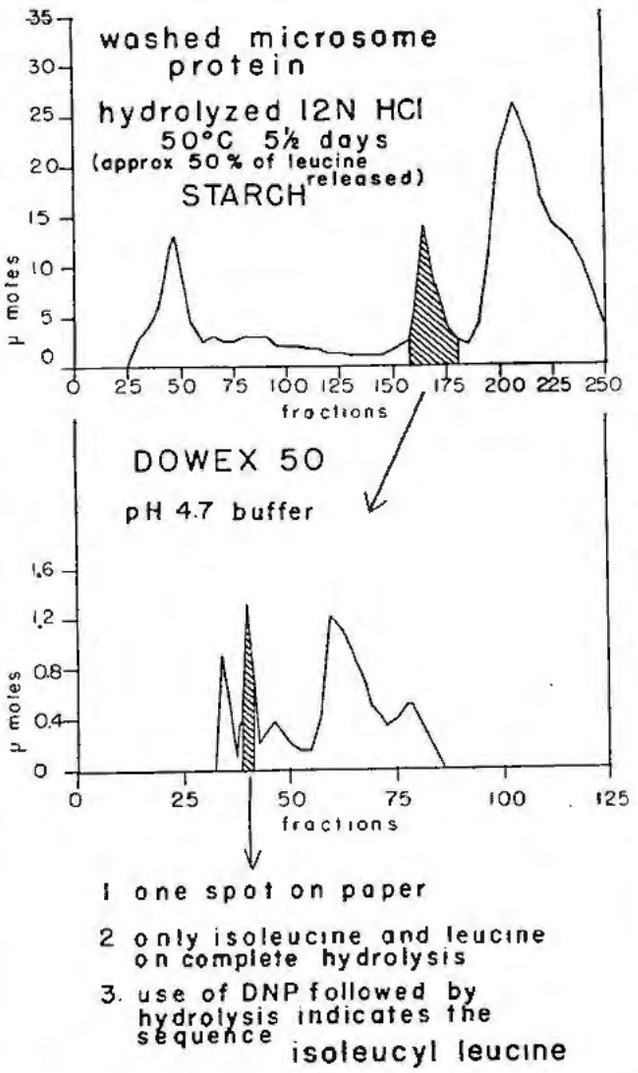

Figure 4. Further fractionation of the "A peak". The A peak is shaded in the upper figure. The shaded peak in the lower figure was concentrated, and chromatographed on Whatman No. 4 paper, using 2, 4, 6 collidine saturated with water (vertical direction), and 100 parts of secondary butanol to 40 parts of 3 per cent ammonia (horizontal direction)

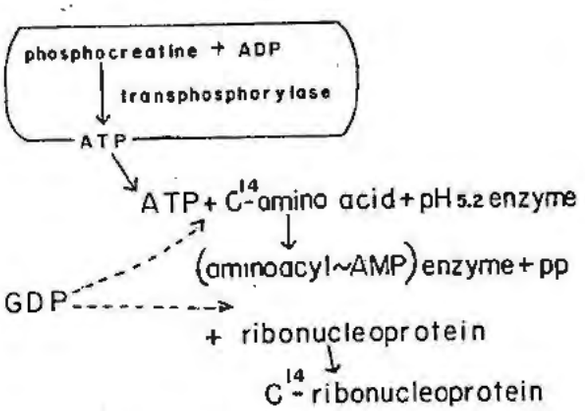

Figure 5. Schematic of results

SUMMARY

The above results may be summarized schematically, as in Fig. 5. In the rat liver homogenate, labeled amino acids appear to be activated by means of ATP and soluble enzymes to form aminoacyl-AMP compounds. The activated amino acids are built into proteins (or large peptides) in the presence of ribonucleoprotein particles located in the microsome

fraction. GDP, GTP, or a derivative thereof plays an essential role, in some unknown way, in this overall incorporation process. Once the C^{14}-amino acid is incorporated into protein, the process is not reversible in this system.

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Distribution of Radioactive Carbon Dioxide Incorporated into Rat Liver Glycogen; with Special Reference to the Role of the Oxidative Pathway

By P. A. Marks* and B. L. Horecker,† USA

Cumulative experiments of several investigators have demonstrated that labeled CO_2 administered to fasted rats[1] or incubated with liver slices from fasted animals[2] yields glycogen which is labeled in carbon atoms 3 and 4. In these positions 95 per cent or more of the tracer is present. These findings are consistent with the interpretation that CO_2 incorporation into glycogen proceeds by a reversal of the anaerobic glycolytic pathway. Recent studies, however, have established the presence in a variety of animal tissues of a cyclic process for the oxidative metabolism of glucose-6-phosphate,[3] distinct from the Embden-Meyerhof scheme. Intermediates in this alternative pathway have been identified as 6-phosphogluconate[4] ribose-5-phosphate, ribulose-5-phosphate[5] sedoheptulose-7-phosphate,[6] fructose-6-phosphate[7] and erythose-4-phosphate.[8]

This alternative oxidative route involves the early decarboxylation of 6-phosphogluconate to yield CO_2 and pentose-5-phosphate.[3] Bloom *et al.*[9] reported the occurrence of a non-glycolytic pathway in liver slices wherein CO_2 was preferentially formed from carbon atom 1 of glucose. This observation has been confirmed in a number of other laboratories,[10–13] but there is lack of agreement as to the quantitative importance of such a non-glycolytic pathway.[10]

The present studies have taken advantage of the fact that, with variously labeled precursors, the isotope distribution patterns in the carbon chain of glucose can be used as an indicator of the parameters of intermediary reactions involved in glucose and glycogen synthesis. These experiments were conducted at two levels of functional integration, the purified enzyme and the tissue slice. This report will first briefly review experiments carried out with C^{14} labeled ribose-5-phosphate with purified preparations of transketolase and transaldolase, enzymes of the oxidative pathway. It was found[14] that pentose phosphate-2, 3-C^{14} yields glucose-6-phosphate labeled in positions 1 through 4. Such observations provide a basis for prediction of the pattern of C^{14} distribution in glucose that might result if the reactions of the oxidative cycle contributed to glycogen synthesis.

The second portion of the paper presents observations on $C^{14}O_2$ incorporation into the glucose unit of glycogen of rat liver slices. Previous studies of labeled CO_2 incorporation into glycogen have generally been carried out with fasted animals. It has been demonstrated that fasting markedly affects overall hepatic carbohydrate metabolism.[15] The present studies attempt to ascertain the pattern of $C^{14}O_2$ incorporation into glycogen of rat liver slices from animals fed a high carbohydrate diet and from fasted animals.

HEXOSE MONOPHOSPHATE FORMATION FROM C^{14} LABELED PENTOSE PHOSPHATE WITH PURIFIED TRANSKETOLASE AND TRANSALDOLASE

Recently, studies were made of hexose monophosphate formation from pentose phosphate by crude rat liver preparations.[16] It was found that ribose phosphate-2,3-C^{14} yielded hexose-6-phosphate with nearly 50 per cent of the isotope in carbon atom 4, essentially equal labeling in positions 2 and 3 and 7 per cent of the tracer in carbon atom 1. Ribose phosphate-1-C^{14} gave rise to hexose-6-phosphase with 74 per cent of the isotope in position 1 and 24 per cent in position 3.

Experiments similar to those with rat liver extracts were carried out with preparations of transketolase and transaldolase. The results of incubation of ribose-

Table I. Degradation of Glucose Formed from Labeled Ribose Phosphate with Purified Transketolase and Transaldolase*

Glucose precursor	*Per cent of total radioactivity*					
	C 1	*C 2*	*C 3*	*C 4*	*C 5*	*C 6*
Ribose-5-phosphate-1-C^{14}	70	0	28	1	1	0
Ribose-5-phosphate-2,3-C^{14}	8	25	27	41	0	0

* Transketolase was purified from spinach[17] and transaldolase from yeast[18] R-5-P-1-C^{14} and R-5-P-2, 3-C^{14} were made as previously described.[16] The reaction mixture contained 20 μmoles R-5-P, 0.20 mg transketolase, 0.13 mg of transaldolase, 2.0 μmoles of Mg Cl_2 and 0.2 mole of thiamine pyrophosphate in a total volume of 1.0 ml. Incubation was carried out at pH 7.2, for 2 hours at 25°C. Glucose-6-phosphate was crystallized from the reaction mixture as the barium salt and enzymatically converted to glucose with potato phosphatase. Glucose was degraded by the modification[19] of the Leuconostoc mesenteroides fermentation method described by Gunsalus and Gibbs.[20] The reliability of this degradation procedure was established by degrading known glucose- 3,4-C^{14}, glucose-1-C^{14} and uniformly labeled glucose.[10]

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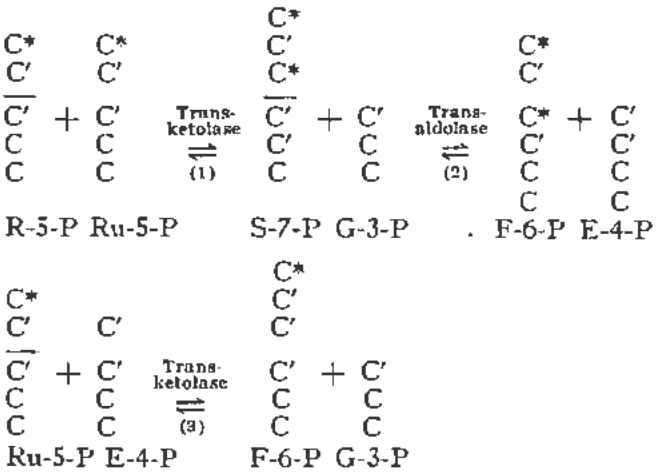
† National Institute of Arthritis and Metabolic Diseases.

5-phosphate-1-C^{14} and 2,3-C^{14} with a mixture of purified transketolase and transaldolase were very similar to those obtained with the crude preparations, and are shown in Table I.

The observed distribution of isotope from pentose to hexose can be accounted for by a series of reactions known to be catalyzed by transketolase and transaldolase[21] as shown below:



* Indicates course of label in ribose-5-phosphate-1-C^{14}.
' Indicates course of label in ribose-5-phosphate-2,3-C^{14}, all reactions proceeding to the right.
Abbreviations: R-5-P: ribose-5-phosphate; Ru-5-P; ribulose-5-phosphate; S-7-P: sedoheptulose-7-phosphate; G-3-P; glyceraldehyde-3-phosphate; F-6-P: fructose-6-phosphate; E-4-P: erythrose-4-phosphate.

Recently Racker *et al.*[22] have demonstrated the reversibility of reaction 3, which would lead to the movement of carbon atoms 1 and 2 of hexose to positions 1 and 2 of pentose. The reversal of reactions 2 and 1, would lead to position 3 of hexose becoming carbon atom 1 in pentose. Thus, these reactions would result in the distribution of label from positions 2 and 3 of pentose to positions 1 through 4 of hexose and from carbon atom 1 of pentose to the 1 and 3 carbon atoms of hexose. In view of the complicated pattern of interconversions catalyzed by transketolase and transaldolase, it is not possible on the basis of the present data, to assess the relative quantitative significance of the individual reactions.

RADIOACTIVE CO_2 INCORPORATION INTO GLYCOGEN OF RAT LIVER SLICES

Wood *et al.*[1] first demonstrated that labeled CO_2 incorporated into hepatic glycogen of fasted rats was distributed predominantly and equally into positions 3 and 4. In view of the above cited studies with purified transketolase and transaldolase, if the oxidative pathway were playing a role in glycogen synthesis, one would expect 2,3-labeled pentose phosphate, formed by the oxidation of 3,4-labeled glucose phosphate, to give rise to glucose with tracer in carbon atoms 1 through 4. The absence of such labeling in the experiment of Wood *et al.* is difficult to reconcile with evidence, obtained in various laboratories, for the participation of this oxidative pathway in hepatic glucose metabolism.[9–13]

The present investigation was designed to test the effect of diet as a factor in altering the pathways of glycogen synthesis. The pattern of distribution of $C^{14}O_2$ incorporated into glycogen of rat liver slices was studied with animals fasted and fed a high glucose diet.

Liver slices were obtained from rats fed a 58% carbohydrate diet for 4 days and from animals fasted for a 72-hour period prior to sacrifice. The liver slices were incubated in a Krebs-Ringer bicarbonate buffer in the presence of 600 μM of lactate or 300 μM lactate plus 300 μM of glycerol and $C^{14}O_2$. Incubation was carried out at pH 7.4, with an atmosphere of 95% oxygen and 5% carbon dioxide for 15 to 240 minutes. Glycogen was isolated,[23] and hydrolyzed to glucose.[2] Degradation of glucose was by a modification of the method of Gunsalus & Gibbs.[19] Radioactivity measurements were made with a gas flow counter. All CO_2 samples were converted to barium carbonate and counted at infinite thickness.

In the experiments with liver slices from both the fed and fasted rats, 86–99 per cent of the total C^{14} incorporated into glycogen entered positions 3 and 4 (Table II). Carbon atoms 1, 2, 5, and 6, however, were consistently labeled with specific activities reaching 5–10 per cent of those found in positions 3 and 4. This result may reflect the operation of the reactions catalyzed by transketolase and transaldolase. Other explanations for the distribution of $C^{14}O_2$ into carbon atoms 1, 2, 5 and 6 are available. $C^{14}O_2$ might be incorporated into position 1 by fixation to pentose phosphate.[24,25] The condensation of pyruvate and glyceraldehyde-3-phosphate by a reversal of the reactions described by Entner and Doudoroff[26] would result in tracer in positions 1 and 4.

At the present time, however, there is a complete lack of evidence for the occurrence of these reactions in mammalian tissues.

Whatever the mechanism, the isotope incorporated into positions 1, 2, 5 and 6 was small and variable compared to the labeling of carbon atoms 3 and 4. This finding suggests that, while under the conditions of the present experiment alternative routes of carbohydrate metabolism may play a role in glycogen synthesis, the glycolytic mechanism predominates. It would thus appear that the preferential oxidation of position one of glucose is not necessarily indicative of the role of the oxidative pathway in hepatic glucose and/or glycogen synthesis.

The pattern of isotope distribution in carbon atom 1, 2, 5 and 6 was similar in liver slices from fasted and fed animals. However, a striking difference between these two sets of experiments was apparent in the labeling of positions 3 and 4 when lactate was the only added substrate (Table II, experiments 1, 2, 6 through 12). In liver slices from fasted animals these positions were essentially equally labeled,

Table II. A. Distribution of C^{14} in Glycogen of Liver Slices from Fasted Rats*

Experiment No.	Incubation: Substrate	Incubation: Duration, min	Per cent of total activity: C 1	C 2	C 3	C 4	C 5	C 6
1	Lactate	30	1.1	1.1	47.4	48.0 (4020)	1.2	1.2
2	Lactate	120	1.8	1.0	47.6	48.6 (4140)	0.5	0.5
3	Glycerol + lactate	30	0.6	3.9	33.5	60.3 (1780)	0.3	1.4
4	Glycerol + lactate	30	2.4	4.1	29.5	56.8 (580)	4.0	3.2
5	Glycerol + lactate	30	1.1	1.9	32.0	63.5 (194)	1.1	0.4
	B. Distribution of C^{14} in Glycogen of Liver Slices from Fed Rats							
6	Lactate	15	4.5	0.5	43.2	50.5 (588)	0.8	0.5
7	Lactate	20	0.5	0.5	41.1	56.4 (1020)	1.0	0.5
8	Lactate	30	2.6	1.3	40.3	52.9 (1150)	2.4	0.5
9	Lactate	60	2.1	0.4	41.7	54.5 (6840)	0.3	1.0
10	Lactate	75	2.7	2.6	39.6	53.4 (1230)	1.3	0.4
11	Lactate	180	2.3	4.6	39.7	50.6 (4310)	1.6	1.2
12	Lactate	220	1.4	3.9	42.8	45.6 (7710)	4.1	2.2

* The materials and methods were as described in detail elsewhere.[18]

whereas in almost every experiment with liver slices from fed animals, position 4 had a higher specific activity than position 3. The lower specific activity of position 3 suggests that glycogen formation in fed rats involves a condensation of dihydroxyacetone phosphate derived from unlabeled endogenous precursors and newly formed 3-phosphoglyceraldehyde. The validity of this interpretation was tested by incubating liver slices from fasted rats with glycerol, a precursor of dihydroxyacetone phosphate, together with lactate and $C^{14}O_2$. This addition resulted in glycogen more heavily labeled in carbon atom 4 than carbon atom 3 (Table II, experiments 3 through 5). Fed rats, in contrast to fasted rats, have relatively large endogenous sources of dihydroxyacetone phosphate precursors, i.e., glycogen and glycerophosphate, and a similar condition arises when rat slices from fasted animals are incubated with unlabeled glycerol.

These findings complement those of Schambye *et al.*[27] who observed that the administration of glycerol-1-C^{14} to fasted rats resulted in glycogen which contained more tracer in position 3 than in 4. Recently, Dische and Rittenberg[28] reported asymmetric labeling of glucose carbon atoms 3 and 4 following administration of phenylalanine-4-C^{14} to fasted rats. These earlier observations of others and the present observations of unequal distribution of tracer into positions 3 and 4 suggests that, under certain conditions, the rate of condensation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate proceeds more rapidly than their isomerization. While this is not an exclusive explanation, it appears to be reasonable on the basis of the present data.

SUMMARY

1. Studies of the formation of hexose monophosphate from C^{14} labeled pentose phosphate by preparations of purified transketolase and transaldolase are described. Pentose phosphate-1-C^{14} yielded hexose monophosphate labeled 74 per cent in position 1 and 24 per cent in position 3. Pentose phosphate-2,3-C^{14} gave rise to hexose monophosphate labeled in positions 1 through 4. This isotope data is considered in terms of a series of reversible reactions catalyzed by transketolase and transaldolase, with sedoheptulose 7-phosphate as an intermediate, and one or more additional reactions involving the formation of hexose monophosphate from pentose phosphate and tetrosephosphate.

2. Studies are presented on the incorporation of $C^{14}O_2$ into glycogen of liver slices from fasted and fed rats. The tracer was predominantly distributed into positions 3 and 4. The small but reproducible quantities of isotope incorporated into carbon atoms 1, 2, 5 and 6 suggest that, while under conditions of the present experiments several pathways are present, the glycolytic scheme makes the major contribution to the synthesis of glycogen.

3. The labeling of positions 3 and 4 of glucose was essentially equal in liver slices from fasted rats, while with liver slices from fed animals carbon atom 3 consistently had a lower specific activity than

carbon atom 4. The possible interpretations of these observations are discussed.

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Studies on the Incorporation of Radioactive Precursors into the Nucleic Acids and Related Compounds in Living Cells

By R. M. S. Smellie and J. N. Davidson,* UK

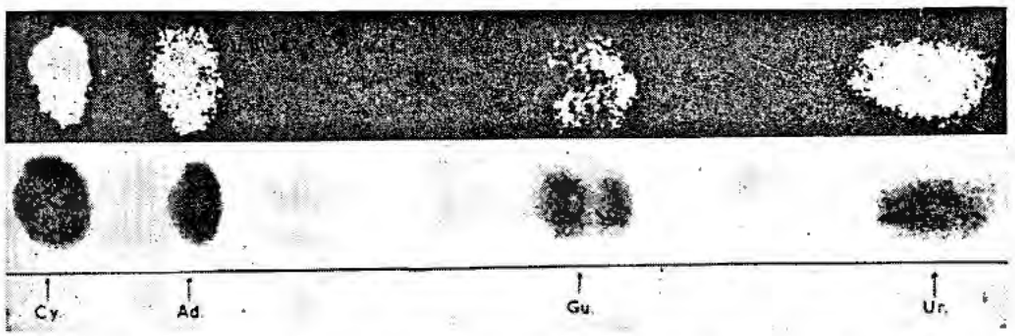

Figure 1. Ultraviolet print (above) and radioautograph (below) of an ionophoretic separation on paper of the ribonucleotides cytidylic acid (Cy), adenylic acid (Ad), guanylic acid (Gu) and uridylic acid (Ur) from the ribonucleotide fraction of liver tissue. The tendency for the isomeric guanosine-2′ and 3′ phosphate to separate from one another is seen on the autoradiograph

The advent within recent years of isotope techniques has given a great impetus to studies on the metabolism of the nucleic acids and their derivatives. While it has long been recognised that these substances can be synthesised in plant and animal tissues from small molecular precursors it was not possible to define these precursors until compounds labelled with C^{14} and N^{15} became available. Studies with such substances have revealed that ammonia, glycine, bicarbonate and formate are intimately connected with purine synthesis while, of these, only bicarbonate is utilised in the synthesis of the pyrimidine ring although the carbon of formate is incorporated into the methyl group of thymine.

Preliminary studies in Glasgow were directed at following the incorporation of P^{32} from inorganic phosphate into the nucleic acids of animal tissues coupled with simultaneous measurements of the nucleic acid content of these tissues. It soon became apparent that while reasonably accurate determinations of the nucleic acid content of tissues could be obtained from assays on relatively impure fractions, such fractious were entirely unsuitable for determination of radioactivity.[1] This difficulty however could be overcome by employing the technique of ionophoresis for the separation of ribonucleotides from other phosphorus containing compounds.[2,3]

This ionophoretic technique has been adapted for use with the mixture of ribonucleotides present in the ribonucleic acid (RNA) fraction obtained by a modification of the Schmidt and Thannhauser[4] procedure applied to the liver tissue of rats which had received P^{32}. Ultraviolet prints and radioautographs of short ionophoretic runs show that a number of components which exhibit radioactivity but no ultraviolet absorption are present in addition to the four mononucleotides. The specific activities of these substances, one of which is invariably inorganic phosphate, are generally higher than those of the mononucleotides which themselves account for not more than 75% of the total phosphorus of the fraction.

Ionophoretic runs of longer duration completely resolve the four nucleotides but do not completely free all the nucleotides from contamination. A further modification of the chemical fractionation procedures has therefore been adopted to yield material from which the nucleotides can be obtained in a pure state (Fig. 1).

The ionophoretic procedure may also be applied to the separation of the four deoxyribonucleotides present in enzymic hydrolysates of DNA. It has been observed that provided adequate precautions are taken to avoid contamination, the specific activity of isolated DNA is a true measure of the activity of the deoxyribonucleotides.

In one series of experiments, the turnover of rabbit liver DNA, nuclear RNA (nRNA) and the RNAs of the cytoplasmic fractions has been studied with the aid of P^{32}. The results of these experiments show that incorporation of P^{32} into the DNA is very low at all times; in contrast the activity of the nRNA

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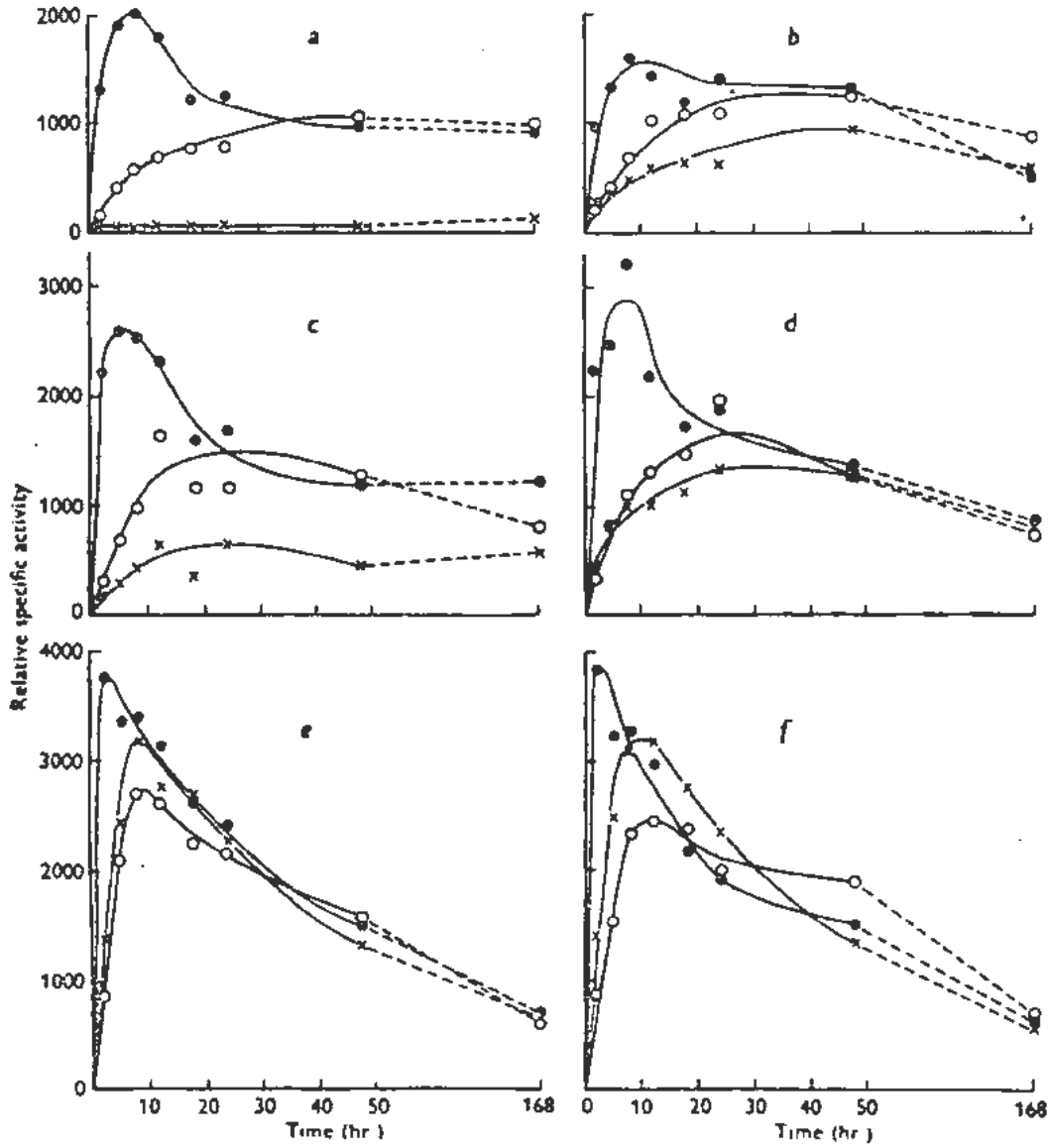

Figure 2. The relative specific activities of the DNA's (X), nRNA's (●) and cRNA's (O) of different rabbit tissues at intervals from 2 hr to 7 days after the intramuscular injection of 1 mc P^{32}. (a) kidney; (b) small intestinal mucosa; (c) spleen; (d) thymus; (e) appendix; and (f) bone marrow

rises very rapidly to a high value with a peak at about 15 hr. There is little difference between the curves for the different cytoplasmic fractions, the cell sap or non-sedimentable RNA always showing a slightly higher value than that for either of the particulate fractions. The time curves for the mitochondria and microsomes are so similar that it has not been possible to separate them but at the shorter time intervals the mitochondrial RNA usually exhibits a higher activity than does that of the microsomes.[5]

The incorporation of P^{32} into the DNA, nRNA and cytoplasmic RNA's of normal, regenerating and weanling rat liver and of normal, pregnant and foetal rabbit liver has also been studied (Tables I and II). In all cases the most rapid incorporation is observed in the nRNA while the cell sap RNA invariably gives higher values than are found for the RNA either of mitochondria or microsomes. Only in foetal rabbit liver does the activity of microsomal RNA exceed that of the mitochondrial RNA. Uptake of isotope by the DNA of regenerating rat liver and of foetal rabbit liver is much higher than in normal liver while in the livers of weanling rats and pregnant rabbits, values slightly higher than normal are obtained.

Similar studies have been carried out on the incorporation of C^{14}-formate and N^{15}-glycine into the nucleic acids of rat liver.[6] The results of these studies are shown in Table III. As with P^{32}, it has been found that the greatest uptake occurs in nRNA. The replacement of carbon and nitrogen is greater in the cell sap RNA than in the RNA's of the mitochondria or microsomes which are very similar to one another. The uptake of N^{15} and C^{14} by the DNA is very slow.

Table I. Specific Activities (Relative to the Tissue Inorganic Phosphate as 1000) of the Ribonucleotides from Cytoplasmic and Nuclear RNAs and DNAs of the Livers of partially Hepatectomised Rats (R) (Regenerating 26 hr), of Sham Operated Rats (C) and of Weanling Rats (W). 50μcP^{32}/100 gm Body Weight Were Administered 4 hr before Killing

	Adenylic acid	*Guanylic acid*	*Cytidylic acid*	*Uridylic acid*	*DNA*
Whole cytoplasm					
R	83	69	74	72	—
C	24	16	23	21	—
W	39	27	37	33	—
Mitochondria					
R	75	51	69	67	—
C	19	13	16	19	—
W	29	22	25	21	—
Microsomes					
R	74	64	59	62	—
C	16	12	14	12	—
W	33	21	20	20	—
Cell sap					
R	98	80	94	90	—
C	35	23	41	31	—
W	49	33	50	38	—
Nuclei					
R	590	606	590	702	43
C	361	318	320	308	3
W	370	349	338	380	14

Table II. Specific Activities (Relative to the Blood Inorganic Phosphate at 2 hr As 10,000) of the Ribonucleotides from the Cytoplasmic and Nuclear RNA and DNA of Rabbit Livers 4 hr after Administration of 50 μc P^{32}/100 gm Body Weight. (N, Normal Female; M, Maternal Female; F, Foetus)

	Adenylic acid	*Guanylic acid*	*Cytidylic acid*	*Uridylic acid*	*DNA*
Whole cytoplasm					
N	49	45	32	46	—
M	56	26	42	57	—
F	109	94	86	102	—
Mitochondria					
N	34	31	25	31	—
M	36	28	29	34	—
F	39	27	32	33	—
Microsomes					
N	21	16	15	15	—
M	13	13	21	15	—
F	63	54	56	70	—
Cell sap					
N	50	30	44	44	—
M	86	43	61	83	—
F	152	116	123	142	—
Nuclei					
N	599	489	567	453	27
M	916	866	796	882	60
F	897	744	758	1025	326

The pattern of P^{32} incorporation into the DNA, nRNA and cytoplasmic RNA (cRNA) of rabbit kidney, small intestinal mucosa, spleen, thymus, appendix and bone marrow at different intervals between 2 hr and 7 days after administration of the isotope has also been examined.[7] The results of these experiments (Fig. 2) show that the tissues may be divided into three categories according to their DNA turnover. In kidney where mitotic activity is negligible the DNA turnover is very low and is comparable with that observed in liver. In tissues of moderate mitotic activity such as intestinal mucosa, spleen

Table III. Incorporation of C^{14}-formate and N^{15}-glycine into the RNA and DNA of Resting Rat Liver. 80 μc C^{14}-formate Were Administered by Intramuscular Injection 4 hr Prior to Killing. N^{15}-glycine Was Administered by Intraperitoneal Injection 24, 22 and 20 hr before Killing in a Dose of 50 mg Glycine (20.92 atom % excess N^{15}) per Injection

		C^{14}-formate 4 hr cpm/100 μgP	N^{15}-glycine Atom % excess N^{15}
RNA	Whole cytoplasm	1976	0.166
	Mitochondria	1340	0.155
	Microsomes	1379	0.151
	Cell sap	2005	0.233
	Nuclei	3140	0.496
DNA		400	0.007

Table IV. The Specific Activities of the DNA, nRNA and cRNA of Different Rabbit Tissues 2 hr after the Administration of 1 mc C^{14}-formate, [8-C^{14}]-adenine or P^{32}

	C^{14}-formate			[8-C^{14}]-adenine			P^{32}		
	DNA	nRNA	cRNA	DNA	nRNA	cRNA	DNA	nRNA	cRNA
Appendix									
adenine	5460	3020	3410	10385	19600	13160	1373	3765	852
guanine	2420	3730	2220	462	1258	618	—	—	—
thymine	4080	—	—	—	—	—	—	—	—
Bone Marrow									
adenine	9080	7124	4075	14781	21700	10348	1289	3837	861
guanine	3345	4488	1970	2371	2600	422	—	—	—
thymine	10180	—	—	—	—	—	—	—	—
Intestinal Mucosa									
adenine	2400	3545	1650	4737	17700	12400	258	959	243
guanine	3045	4806	2340	1566	3470	410	—	—	—
thymine	3500	—	—	—	—	—	—	—	—
Kidney									
adenine	104	205	lost	0	14932	4760	81	796	138
guanine	140	490	lost	161	1411	0	—	—	—
thymine	129	—	—	—	—	—	—	—	—
Spleen									
adenine	722	927	lost	5800	18700	lost	154	2236	292
guanine	428	900	lost	2480	27500	1020	—	—	—
thymine	878	—	—	—	—	—	—	—	—
Thymus									
adenine	2445	1840	2500	4720	9831	5080	434	2229	310
guanine	2155	2360	1350	252	787	125	—	—	—
thymine	2100	—	—	—	—	—	—	—	—

Table V. The Effect of X-irrodiotion (1000 r) of Rabbit Abdominal Viscera and Femora on the Incorporation of P^{32} into the DNA, nRNA and cRNA of Rabbit Tissues. Each Animal received 1 mc P^{32} 2 hr before Killing

Period between irradiation and killing, hr	Erythrocytes as percentage of pre-irradiation values	Leucocytes as percentage of pre-irradiation values			Tissue	Specific activity as percentage of control values		
		Total	Granulocytes	Non-granulocytes		DNA	nRNA	cRNA
2	100	91	275	24	Appendix	41	62	75
					Bone marrow	56	56	50
					Kidney	77	146	154
					Thymus	119	110	112
2	100	103	213	56	Appendix	27	57	65
					Bone marrow	71	99	68
					Kidney	80	134	120
					Thymus	102	78	91
20	103	54	133	10	Appendix	17	31	32
					Bone marrow	23	24	17
					Kidney	92	75	87
					Thymus	37	52	46
49	87	68	276	28	Appendix	33	52	53
					Bone marrow	76	55	58
					Kidney	120	67	59
					Thymus	46	52	64

Table VI. The Effect of Hyperplasia of Rabbit Bone Marrow Caused by Haemolytic Anaemia Induced by Phenylhydrazine on the Uptake of P^{32} by the DNA, nRNA and cRNA of Different Tissues. 1 mc P^{32} Was Administered 2 hr before Killing. Red Cell Count 58% of Normal. Reticulocyte Count 85% of Total Red Cells. All Figures Represent the Mean Values of Two Experiments and Are Expressed As Percentages of the Normal Values

Tissue	*DNA*	*nRNA*	*cRNA*
Appendix	82	89	103
Bone marrow	245	116	192
Kidney	95	115	100
Spleen	980	131	242
Thymus	99	85	107

and thymus the rate of renewal of DNA is quite high, while in bone marrow where cell division is very rapid, the DNA activity is extremely high. The turnover of appendix DNA is also rapid and comparable with that in bone marrow.

In all these tissues the curves for the activity of the nRNA rise more rapidly and to a higher value than do the curves for either DNA or cRNA, the maximum in appendix and bone marrow probably occurring within 2 hr of P^{32} administration. The curves for cRNA fall into two groups (*a*) appendix and bone marrow where the maximum approaches that of DNA at 10 to 12 hr and (*b*) the remaining tissues in which the activity of the cRNA rises more slowly to a very broad maximum.

A general comparison of all these curves shows that by 48 hr the activities of the DNA, nRNA and cRNA of most tissues have reached a common level which is maintained up to 7 days.

In experiments with C^{14}-formate and [8-C^{14}] adenine the renewals of the guanine, adenine and thymine of the DNAs and of the guanine and adenine of nRNA and cRNA of rabbit appendix, bone marrow, intestinal mucosa, kidney, spleen and thymus have been examined. The results of these experiments (Table IV) show that certain similarities exist between the patterns of incorporation of adenine, formate and P^{32}. Thus in general the C^{14} content of the DNA bases is highest in appendix and bone marrow and lowest in kidney. A high level of uptake is discernible in the nRNA as compared with DNA and cNRA although in appendix and bone marrow the values for DNA, cRNA and nRNA are all quite close. It is particularly interesting to note that the activity of the DNA thymine after formate administration is always comparable with that of the purines despite the fact that formate contributes only to one carbon atom of thymine and to two carbon atoms in the purine ring. With C^{14}-adenine, the nucleic acid adenine is extensively labelled but conversion to guanine is in general poor.

The effects of X-irradiation on the metabolism of the nucleic acids of several rabbit tissues have also been examined (Table V). Exposure of the abdominal viscera and femora to approximately 1000 r has little immediate effect on the total blood erythrocyte or leucocyte populations. Closer examination however reveals that after exposure there is a rapid increase in the proportion of granulocytes and a corresponding fall in the non-granulocytes within 2 hr of irradiation. At longer time intervals the numbers of both erythrocytes and leucocytes decrease. The effects of irradiation on the uptake of P^{32} are most marked in the appendix and bone marrow DNA where the activities fall to about 35% and 60% respectively of the normal values after 2 hr. At longer time intervals (20 hr) an even greater depression of appendix and bone marrow turnover is apparent, while by 49 hr the activity figures showed signs of a return to the normal values. Renewal of thymus DNA is not greatly affected at first, but by 20 hr and 49 hr a marked decline in activity is discernible. In kidney a slight decrease in the activity of the DNA is observed but the normal level of P^{32} incorporation is so small (Fig. 2) that these changes are of little significance.

The effect of phenylhydrazine administration is to produce a haemolytic anaemia which results in hyperplasia of the bone marrow and enlargement of the spleen. In such circumstances, marked changes have been found in the uptake of P^{32} by the nucleic acids (Table VI). The activity of the bone marrow DNA is increased two- or three-fold while that of spleen DNA is increased tenfold. The activities of appendix, kidney and thymus DNAs are unchanged.

The uptake of P^{32} by the nRNA of these tissues is not so greatly affected by the phenylhydrazine treatment, the most marked effect being a 30% increase in the activity of spleen nRNA. The activities of bone marrow and spleen cRNAs are about twice the normal values while no changes are observed in appendix, kidney or thymus.

A comparison has been made of the composition of nuclei from a number of rabbit tissues isolated by the normal citric acid method and of nuclei isolated from the same tissues in non-aqueous media.[8] The results of such experiments show that the DNA phosphorus content of the non-aqueous nuclei (NAN) expressed as a percentage of the dry weight varies between 0.38 and 1.68 according to the tissue of origin. Variations in the RNA phosphorus are within a smaller range (0.27 to 0.48% dry weight). A rough estimate of the protein content of NAN based on the assumption that the main components

are protein and nucleic acids shows it to be between 79 and 92%.

Similar values determined for citric acid nuclei (CN) from rabbit appendix, intestinal mucosa and thymus show that the DNA phosphorus content as a percentage of the dry weight is much higher in NAN while the percentage of RNA phosphorus is lower. The protein content is also appreciably lower in CN than in NAN.

The activities of the DNAs from NAN and CN of appendix, intestinal mucosa and thymus after P^{32} administration are similar except in the case of intestinal mucosa 2 hr after P^{32} administration where it has been repeatedly observed that the activity of the DNA from NAN is higher than that from CN.

The specific activities of the nRNAs from CN in all these tissues are invariably higher than in the nRNAs from NAN. This difference is not observed at 18 hr and appears to be dne to heterogeneity of the nRNA in NAN some of which is lost in the preparation of CN. Extraction of NAN with dilute citric acid in conditions similar to those obtaining when CN are prepared removes appreciable amounts of RNA and protein. The activity of this extracted RNA is lower than that of the RNA remaining in the extracted NAN and this in turn is higher than that in the original NAN. After most careful studies on the purity of the NAN it has been concluded that the nRNA of appendix, intestinal mucosa and thymus is heterogeneous and that when nuclei are isolated from these tissues by conventional procedures employing dilute citric acid some of this RNA is lost from the nuclei.

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Application of C^{14}-Labeled Substances in the Study of Adipose Tissue Metabolism

By B. Shapiro and G. Rose,* Israel

In a review on the biochemistry of adipose tissue,[1] Wertheimer and Shapiro have concluded from the available experimental material that adipose tissue is not only a site of fat storage, that is, an inert tissue, but that it is endowed with specific metabolic abilities. An accurate evaluation of these abilities was hampered by the fact that preparations of adipose tissue contain only relatively small amounts of active protoplasm, though it has already been proven about 30 years ago that fat mobilisation by adipose tissue is contingent upon innervation.[2] This difficulty could only be overcome by the use of labeled, radioactive substrates. Thus it could be shown that most of the common enzymatic reactions are carried out by adipose tissue, but that its main activities are directed towards the metabolism of fat.

1. It has been found that uniformly labeled glucose, acetate-1-C^{14}, and pyruvate-2-C^{14} are oxidised by adipose tissue to $C^{14}O_2$. The conclusion is obvious that in the tissue the tricarboxylic acid cycle is active. This has been established unambiguously by the observation that malonate inhibits the $C^{14}O_2$ production from the above substances, indicating the central role of succinate in the process (Table I).

2. Adipose tissue synthetizes *in vitro* fatty acids from uniformly-labeled glucose, pyruvate-2-C^{14} and acetate-1-C^{14}. Under optimum conditions, the newly synthesized material per day amounted to 1% of the fresh weight of the tissue used. Acetate proved inferior to the other two substances; it appears that the tissue is better capable to "activate" higher than lower fatty acids. In one experiment with labeled glucose, *e.g.*,[3] the incorporation of C^{14} into fatty acids (cpm) was 40, 96 and 343 after 0.5, 1.5 and 4.0 hours: it proceeded linearly throughout the period of incubation.

The experiments dispose with the classical assumption that the liver is the sole site of fatty acid synthesis, and substantiate the findings of Stetten and Grail[4] that the quantity of fatty acids synthesized per day by the mouse is about 4 times the quantity present in the liver. The adipose tissue is likely to be responsible for at least part of this additional synthetic activity. This is in accord with the observation[5] that adipose tissue, incubated in serum containing heavy water, incorporates deuterium into its fatty acids. Although liver is more active than adipose tissue in renewing its fatty acids by synthesis, the adipose tissue has the advantage of the presence of a much larger amount of fatty acids, so that it may well account for a large part of the total quantity of fat synthesized by the body, in spite of the slower turnover.

The question arises whether the synthesis of fatty acids is due to the condensation of a C_2 unit onto an existing fatty acid chain, or to a synthesis *de novo*. The degradation of the fatty acids accumulating in the adipose tissue by the method of Anker[6] showed that the second alternative is correct. By this method, the silver salts of the fatty acids are treated with bromine; the reaction which takes place

$$R.CH_2COOAg + Br_2 \longrightarrow R.CH_2Br + CO_2 + AgBr$$

would give a ratio of 0 of the specific activities of $R.CH_2Br/CO_2$ if acetate-1-C^{14} is added to an existing fatty acid, and a ratio of 9 for complete at-random distribution of the radioactive carbon (for an average formula of C_{18} fatty acids). The value actually found[7] was 9.3.

3. Higher fatty acids are "activated" by adipose tissue, and not simply transported as such, by diffusion, from the tissue to the blood stream and *vice versa*. This could be demonstrated[7,8] by following the uptake of fat by somewhat fat-depleted adipose tissue from serum and other lipid media. Tables II and III show that (*a*) this uptake is inhibited by metabolic poisons such as fluoride or cyanide, (*b*) that only triglycerides and simple esters are assimilated, not, however, lecithine or esters of cholesterol. Although the uptake of the salts of free fatty acids is not affected by fluoride, one must not conclude that they penetrate the tissue by diffusion; their uptake is very susceptible to cyanide.

Additional experiments on the uptake of potassium stearate-1-C^{14} by adipose tissue *in vitro* confirmed these conclusions. The radioactive stearate was taken up at a high rate, but when the acetone extract of the tissue was passed through a magnesium oxide column, in order to separate the fatty acids from the esters, it was found that 90% of the activity was present in the *ester* fraction. The conclusion offers itself that on the external cell boundary an enzymatic

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Table I. Effect of Malonate on $C^{14}O_2$ Production*

Substrate	Rats	Malonate	% $C^{14}O_2$
Pyruvate-2-C^{14}	Starved	–	15.3
(0.01 *M*)		0.05 *M*	11.3
Pyruvate-2-C^{14}	Re-fed	–	21.8
(0.01 *M*)		0.05 *M*	14.2
Acetate-1-C^{14}	Re-fed	–	1.3
(0.02 *M*)		0.05 *M*	0.4

* Each incubation flask contained two halves of mesenterium of two rats; to one the labeled substrate was added, to the other an equivalent volume of saline. Total volume 1 ml.

exchange reaction takes place between free stearic acid of the medium and esters inside the tissue.[9]

That the enzyme involved is not lipase, follows from the observation, already mentioned, that the process is not affected by fluoride, a known lipase inhibitor. In fact, a dipose tissue contains a higher-fatty-acid-activating system, requiring ATP, magnesium ions and coenzyme A, similar to that discovered in liver by Kornberg and Price.[10] The activated acids can be trapped with hydroxylamine as hydroxamic acids (Table IV); thus the enzyme activity can be measured quantitatively. The activity was lost by dialysis and was not recovered by subsequent addition of ATP, magnesium ions and coenzyme A.

4. The processes of fat synthesis and fat assimilation by the adipose tissue discussed so far, are accompanied by combustion of the fatty acids supplied. Part of the radioactive carbon of stearate-1-C^{14} appears as $C^{14}O_2$; however, the rate of this process could not be measured accurately due to the large dilution of the radio-stearate by the fatty acids of the tissue.

5. A particular reaction occurring in adipose tissue is the formation of glycogen from glucose, demonstrated by the use of the labeled sugar. It is worthy of note that this reaction is also linked to the synthesis of fatty acids; indeed, the experiments with adipose tissue illustrate admirably the interrelationship between the pathway of fat and carbohydrate metabolism.

It had been recognized for some time[11,12] that adipose tissue of starved and of re-fed rats behaves differently. In the adipose tissue of normally fed animals, no glycogen is found; when they were starved and afterwards supplied with a diet rich in carhohydrates, the polysaccharide is formed; after a period of normal feeding, it disappears again. These processes parallel the fat metabolism in adipose tissue: the deposition of glycogen precedes the deposition of fat and terminates when formation of the latter is at its maximum.[13,14] Furthermore, fasting decreases the capacity to synthesize fat almost to zero (an ohservation which has also been made in the case of liver slices,[15]) and re-feeding restores the synthesis of fat.

The syntheses of glycogen and fat are not only parallel reactions; they are interdependent. Glycogen-containing adipose tissue has a higher fat-synthesizing activity than tissue free of glycogen,[5] and in tissue from starved animals the uptake of stearate-1-C^{14} is less rapid.[9] Moreover, in this case, only about one third of the radioactivity present in the tissue is derived from the ester fraction. One is forced to conclude that glycogen serves as precursor of fat synthesis in adipose tissue. On the other hand, it has been shown[16] that the incorporation of radiocarbon from glucose into glycogen is not diminished by starvation, whilst the utilisation of glucose for fat synthesis is decreased by 30% and the combustion of the sugar to CO_2 by 90%. This may mean that in starved tissue the carbohydrate metabolism is blocked

Table II. Uptake of Fatty Acids (micro-equ per cm^3) from Serum by Adipose Tissue *in vitro*

Experiment	From total esterified fatty acids			From phospholipids			From cholesterol esters		
	Initial	After 4 hr	Uptake	Initial	After 4 hr	Uptake	Initial	After 4 hr	Uptake
1	14.0	12.0	2.0	3.3	3.3	0	1.9	2.15	-0.25
2	7.0	5.7	1.3	2.1	2.3	-0.2	1.2	1.4	-0.2
3	11.8	9.2	2.6	3.0	2.9	0.1	2.2	2.4	-0.2
4	16.7	12.5	4.2	5.7	5.1	0.6	6.5	6.8	-0.3
5	14.9	11.9	3.0	5.0	4.9	0.1	5.4	5.4	0
6	14.6	11.3	3.3	5.1	5.2	-0.1	5.8	7.3	-1.5

Table III. Uptake of Various Fatty Acid Compounds by Adipose Tissue

Compound	Number of experiments	Mean "initial" concentration (meq/cm^3)	Uptake (meq/cm^3 medium)
Triolein	25	15	1.8 ± 0.22
Triolein + *M*/20 NaF	16	15	1.04 ± 0.27
Lecithin of egg	11	12	-0.2 ± 0.2
Sorbitan-monolaurate	9	12	4.3 ± 0.44
Sorbitan-monopalmitate	19	14	3.6 ± 0.4
Sorbitan-monopalmitate + *M*/20 NaF	15	14	1.2 ± 0.35
Sorbitan monooleate	11	9	2.3 ± 0.35
Sorbitan monooleate + *M*/20 NaF	9	9	0.8 ± 0.3
Sodium oleate	9	12	2.4 ± 0.53
Sodium oleate + *M*/20 NaF	5	12	2.9 ± 0.88

Table IV. Fatty Acid Activation (Klett Units) by Adipose Tissue*

	Higher fatty acid Hydroxamate		*Lower fatty acid Hydroxamate*
1. *Enzyme precipitate*			
Complete system	61	70	
–CoA	35		
–Mg++ and ATP	36		
– stearate	62		
2. *Enzyme: aqueous extract*			
Complete system	152		42
–CoA and ATP	77		22

* 50 units CoA, 10 μM ATP, 10 μM $MgCl_2$, 2 μM K-stearate, 100 μM potassium phosphate buffer (pH 7), 4.100 μM hydroxylamine (pH 7.4), 20 μM cysteine, + enzyme in 1 cm^3. Incubated in N_2 at 37°C for 2 hr.

beyond the glucosephosphate step. In normally fed animals, glucose (and, *via* glucose, glycogen) is degraded to pyruvic acid and thus transformed into fat or burnt to CO_2; in a period of starvation, the metabolism of glucose proceeds to the phosphate stage, giving rise to an increased glycogen production. Another explanation which cannot be excluded is that the uptake of glucose is necessary to supply the energy for the synthesis of long-chain fatty acids from C_2 units, and that it is this uptake of glucose which is inhibited by starvation. This explanation has been advanced by Lyon, Masri and Chaikoff[17] for fat synthesis in liver. Of course, the synthesis of fat may be linked both chemically and energetically to the catabolism of sugar.

In a classical essay, Rudolf Schoenheimer[18] has postulated the dynamic state of body constituents, amongst them body fats. The experiments reported here have shown that even in adipose tissue, regarded as a mere depository of fat, such a dynamic state exists; in fact, this tissue, due to its large mass, may well be the most important site for the synthesis and the catabolism of fat in the body.

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Metabolism of Radioisotope-Labeled Drugs

By P. K. Smith, H. G. Mandel and C. Davison,* USA

A vital part of any understanding of drug action is the knowledge of what happens to drugs in the body. Therapeutic agents are frequently so altered in structure and physical properties in the course of their metabolism that their degradation products may be recognized only by means of isotopic labeling.

The synthesis of drugs with radioisotope labels in known positions provides a powerful tool for investigating their degradation in the animal body. In these laboratories this has required the synthesis of several well known drugs with radiocarbon or radiosulfur labels, their administration to animals, patients and human subjects and determination of their metabolic products by suitable analytical procedures, including countercurrent distribution, column and paper chromatography.

The identification of the necessarily small amounts of such labeled derivatives is ordinarily accomplished by isotope dilution techniques designed to demonstrate their identity with synthetic substances of known structure. These conventional procedures offer difficulties in work with drugs, since the structure of the unlabeled model compounds which must be prepared are unknown and must be surmised. Some of these difficulties have been circumvented by letting the animal prepare radioactively labeled metabolites which may be used individually as indicators to guide the direct isolation of identifiable quantities of metabolites, with no prior assumptions about their structure. The indicators are prepared as radiochemically pure entities by fractionation on the micro scale of the labeled metabolites obtained after administering a radioactive precursor of high specific activity to an animal. After the addition of a single indicator to urine or other large pools of degradation products from a number of animals receiving unlabeled parent compound, the radioactivity may be osed to guide the isolation of sufficient material for identification by chemical means. As in the conventional isotope dilution procedure, the isolation of radioactive material satisfying the criteria of homogeneity demonstrates the derivation of the product from the precursor. This approach offers particular promise for the identification of metabolites of the more potent pharmacological agents which can be administered only in minute amounts. Although very large pools of excreta or tissue extracts would be required to provide identifiable quantities, suitable modifications of the countercurrent distrihution technique permit these large pools to be processed with the aid of relatively little radioactive indicator. This approach to the use of isotopic labeling in metabolic experiments is, of course, not limited to studies of new drugs and offers promise for the investigation of physiologically active agents generally.

METABOLISM OF SALICYLATES

One of the first groups of radioisotope labeled drugs investigated was the salicylates (Figs. 1, 2, 3). The program was initiated by the synthesis of C^{14} carboxyl labeled salicylic acid by modification of the methods of Kolbe and Schmitt. Anhydrous sodium phenolate was reacted in a pressure flask with $C^{14}O_2$ released by the action of sulfuric acid on barium-C^{14}-carbonate. By reacting the sodium phenolate with an excess of solid carbon dioxide in a metal bomb at several atmospheres pressure and heating at 120° to 130° for several hours, an almost quantitative yield of salicylic acid was obtained. The resulting salicylic acid was purified and administered to human subjects for metabolism studies. After the administration of the labeled drug, urine was collected and the various components were separated by countercurrent distribution. By determining various chemical properties of the labeled components and comparing the relative distribution of authentic compounds in the countercurrent system, the drug metabolites were identified. It was observed that approximately one-tenth of the total dose of administered salicylic acid was excreted unchanged; one-third formed the glucuronates; and one-half the salicylurate. There also was a small amount of gentisate. The glucuronate fraction consisted of an ether and ester type. These results showed that most of the expected metabolites were produced and that gentisate formed only a very minor metabolite of salicylate. This makes it quite improbable that the analgesic effect of salicylate is due to its conversion to gentisate. A similar experiment carried on in the rat indicated that no decarboxylation took place, since the expired carbon dioxide, after the administration of the labeled drug, contained no C^{14}. No other metabolites could be observed.

In order to determine whether salicylamide also acted as an analgesic because of its conversion to

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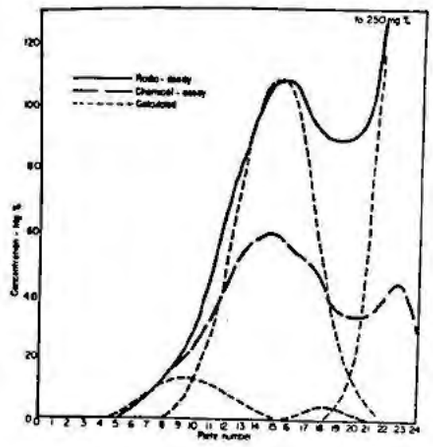

Figure 1. A 24-plate countercurrent distribution of urinary products in patient after 1 gm of C^{14} carboxyl salicylic acid (countercurrent distribution, human salicylate urine-1)

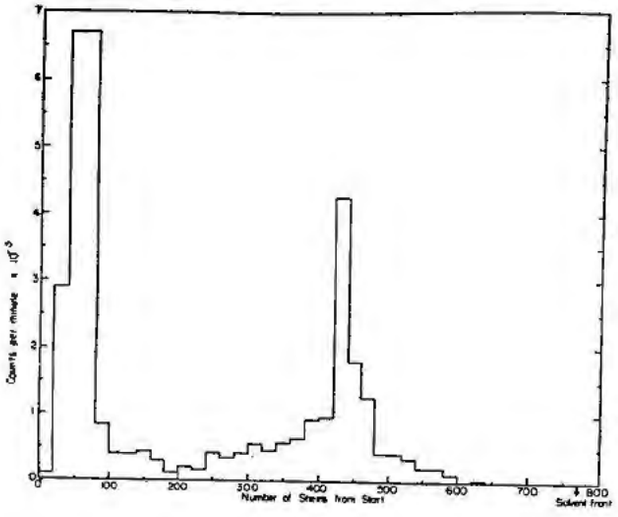

Figure 2. Evidence for two salicyl glucuronides in human urine (chromatogram-salicyl glucuronate fraction from human urine)

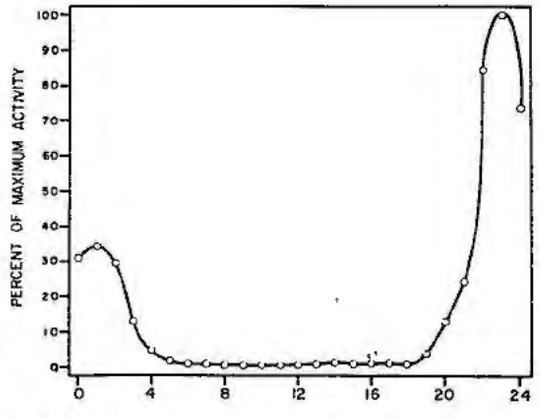

Figure 3. Evidence for free and conjugated C^{14} salicylamide in human urine (C^{14} salicylamide countercurrent distribution of urinary metabolites, patient No. 1)

salicylic acid, a similar experiment was carried out with this drug labeled with C^{14}. After the administration of the drug to patients and analyses by the same procedures, non of the usual metabolites of salicylic acid could be found, indicating that the compound was not converted to salicylic acid, and, therefore, was biologically active on its own accord. The metabolite formed after this drug included principally a glucuronate derivative as well as a small amount of the original drug.

The presence of aspirin in human plasma after oral administration has not been proved unequivocally. Formerly the presence of aspirin was indicated indirectly by elimination of other known metabolites. Experiments were devised to decide definitely whether this drug was absorbed as such (Figs. 4, 5). By labeling aspirin separately in either the carboxyl group or on the acetyl portion and then carrying out the usual separation and identification procedures, it was possible to determine definitely that the suspected compound was aspirin. When aspirin labeled in the carboxyl group was administered, the presence of salicylic acid plus aspirin could be detected in the plasma. Aspirin labeled in the acetyl portion was observed in the plasma of patients receiving this drug. No other metabolites were observed. Aspirin was found to be present in the plasma of humans for approximately two hours after administration at levels of less than two milligrams per cent. Calculations reveal that at least a major portion of the salicylate found in the plasma had been formed by the hydrolysis of aspirin after absorption.

METABOLISM OF PENTOBARBITAL

As a first application of the technique of biologically prepared radioactive indicators, Titus and Weiss studied the metabolism of radioactive pentobarbital in the dog. This has thus far resulted in evidence for the existence of nine metabolites of pentobarbital which together account for all of the administered radioactivity. One of these has been isolated and characterized as 5-ethyl-5-(1-methyl-3-carboxypropyl)-barbituric acid. Another has been shown to be a glucuronide of d-5-ethyl-5-(3-hydroxy-1-methylbutyl)-barbituric acid, one of the two diastereoisomeric forms of this alcohol which are also metabolites of pentobarbital. These products account for 80 percent of the administered drug. Four unknown metabolites, accounting for 17 per cent, remain to be identified. Urea, which accounts for 2.6 per cent of the administered radioactivity, is the only metabolite thus far identified in which the barbiturate ring is opened.

EFFECT OF RADIATION ON THE BIOSYNTHESIS OF NUCLEIC ACIDS

It has been observed that ionizing radiations interfere with the biosynthesis of nucleic acids and produce differences in the pattern of incorporation of nucleic acid precursors. If this interference were

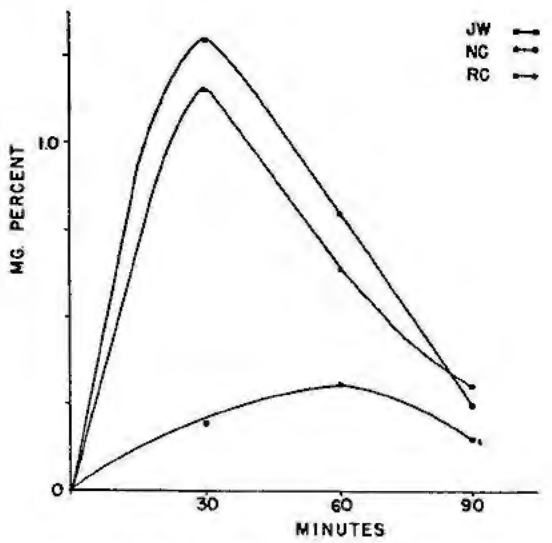

Figure 4. Plasma levels of aspirin after administration to human subjects

specifically with the enzyme systems involved in synthesis, it seemed reasonable that an appreciable change would be found in the incorporation pattern after X-irradiation of the host. Because of the association of nucleic acids with chromosomes, mitosis and tissue growth, one of the promising approaches for the control of neoplasia has been thought to be the disturbance of nucleic acid metabolism. Ionizing radiations have been shown to cause nuclear damage, chromosomal fragmentation, mutation, and inhibition of mitosis. Various experiments were carried out in this laboratory to determine the effects that various conditions might have on the incorporation of various purines and pyrimidines and their precursors into nucleic acids. The precursors that have been studied so far include 4-amino-5-imidazolecarboxamide, adenine, and ureidosuccinic acid.

Studies were made of the delayed effect of X-irradiation on the incorporation of radiocarbon from 4-amino-5-imidazolecarboxamide-C^{14} into nucleic acids of livers of mice. The 4-amino-5-imidazolecarboxamide, labeled in the C_4 position, was synthesized from $NaC^{14}N$ by the method of Shaw and Woolley, modified for use with radioactive materials. After the conversion into ethyl cyanoacetate in a yield of 86 per cent, the imino ethyl ether hydrochloride was produced in a 92 per cent yield, which was then converted to the malonamamidine hydrochloride in an 88 per cent yield. After the diazotization, reduction, formylation, and ring closure to 4-amino-5-imidazolecarboxamide, an over-all yield of 45 per cent based on $NaC^{14}N$ was achieved. The substance was shown to be of good purity by ultraviolet spectroscopy, descending paper strip chromatography in a system of *n*-butanol-water, and by countercurrent distribution in a system of sodium phosphate buffer at pH 6.5 and *n*-butanol. The melting point of the hydrochloride was 257°. The specific activity was 0.07 μc per mg. The results of these investigations, demonstrating the incorporation of ingested 4-amino-5-imidazole carboxamide into the purines of nucleic acids of the mouse, add another species to the list of organisms which can utilize this compound for synthesis. Previous reports have indicated that in the rat the purineless mutant of *E. coli* and in yeast 4-amino-5-imidazolecarboxamide can serve as a source of nucleic acid purines.

To determine the quantitative difference, if any, in the incorporation of 4-amino-5-imidazolecarboxamide into the nucleic acid purines of the livers of normal and X-irradiated mice, CAF_1 mice were arranged into two groups so that each group had identical weight and sex distribution. The experimental group received a total body radiation of 400 roentgens at a rate of 79 roentgens per minute. Twenty-four hours after the irradiation, the experimental and the control groups were administered a total of five doses each of 25 mg per kg of 4-amino-5-imidazolecarboxamide by intraperitoneal injection at 12-hour intervals. Twenty-four hours after the last injection the animals were sacrificed, the livers were removed and degraded to nucleic acid purines, and were assayed as described above. This irradiation experiment was repeated under similar conditions on another group of experimental animals. The average PNA: DNA ratio was found to be approximately 11:1 for the X-irradiated mice and 7:1 for normal mice. The findings of other investigators that X-irradiation apparently inhibits the synthesis of DNA purines is confirmed by the present results. It would seem, therefore, that the effect of X-irradiation on the incorporation of 4-amino-5-imidazolecarboxamide does not specifically involve the conversion of that precursor into purines but is more generally related to nucleic acid metabolism. Furthermore, this investigation shows that in spite of the time lag between the irradiation and the first adminis-

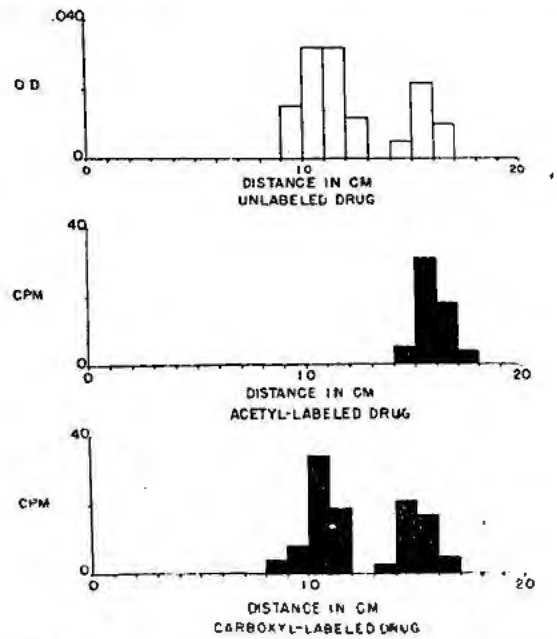

Figure 5. Evidence for presence of aspirin in human subjects (chromatograms of plasma samples after aspirin administration)

tration of the precursor, these effects are still observable after twenty-four hours.

The effects of X-irradiation on the incorporation of radioactive adenine into the adenine and guanine fractions of nucleic acids were examined. Adenine-8-C^{14} was synthesized from 4,5,6-triaminopyrimidine formed by the condensation of malononitrile and thiourea, as described by Traube and Hoffer. The triaminopyrimidine was condensed with an equimolar quantity of sodium-C^{14}-formate in the presence of HCl. The addition of 1.3 milliequivalents of HCl per mole of formate, produced 4,6-diamino-5-C^{14}-formamidopyrimidine in a yield of 95 per cent. This compound was converted to adenine-C^{14} by condensation with diethanolamine in an oil bath under a nitrogen atmosphere. Adenine was precipitated from the reaction mixture by the addition of silver nitrate to form the silver salt of the purine. The recovered adenine-8-C^{14} was then purified by chromatography on Dowex-50 resin. The final product had a specific activity of 0.67 μc per mg and was formed in a yield of 62 per cent based on formate-C^{14}. Groups of CAF_1 mice were injected intraperitoneally with adenine-8-C^{14} five times at twelve-hour intervals, beginning twenty-four hours after they were exposed to 400 roentgens of total body X-irradiation. Pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) were isolated from liver tissues according to the procedures of Bendich *et al.* The nucleic acids were hydrolyzed to the purine bases with 1*N* HCl, purified on Dowex-50 resin, plated directly, and radioassayed in a gas-flow proportional counter. In all cases the liver PNA purines showed higher specific activities than those of the liver DNA. X-irradiated mice showed a slightly higher incorporation into the PNA purines of liver tissue compared to the corresponding values of control mice. There was an increased incorporation of adenine into the tumor PNA purines of X-irradiated mice compared to the controls while the incorporation into tumor DNA purine was essentially the same. This lack of effect of X-irradiation on the relative specific activities of DNA purines in Sarcoma-37 was surprising. One might expect that such a rapidly growing cellular tumor would show more marked changes, after X-irradiation, in the incorporation into DNA than would a relatively non-growing adult tissue such as the liver. It is possible that the recovery periods of the various tissues from the effects of X-irradiation show wide time variations, and that the DNA metabolism of the tumor had recovered sufficiently from the ionizing radiations in the twenty-four hours before the adenine administration so that an effect no longer could be demonstrated.

It has been found that ureidosuccinic acid is a precursor of orotic acid in certain bacteria and others have shown that orotic acid is a precursor of nucleic acid pyrimidines in various organisms. It seems likely, then, that ureidosuccinic acid may serve as a precursor of nucleic acid pyrimidines by way of orotic acid, and it was therefore of interest to investigate the behavior of ureidosuccinic acid *in vivo*, with particular reference to its incorporation into nucleic acids. Ureidosuccinic acid was synthesized labeled with C^{14} in the ureido carbon, according to the method of Nyc and Mitchell. Urea-C^{14} was condensed with potassium carbonate to give labeled potassium cyanate, and this was reacted with dl-aspartic acid to give C^{14}-ureidosuccinic acid. The over-all yield was 50 per cent based on urea. Increasing the time of interaction of potassium cyanate with aspartic acid to forty-eight hours and acidifying at a lower temperature made the yield more reproducible. The purity was checked by paper chromatography in several solvent systems. The specific activity of the C^{14}-ureidosuccinic acid was 0.12 μc per mg.

Excretion studies using CAF_1 mice showed very rapid urinary elimination of about half the injected radioactivity. Apppreciable activity was also found in the expired CO_2. A survey of tissue distribution and a detailed investigation of incorporation into nucleic acids were carried out with normal mice and with mice bearing sarcoma-37. Ureidosuccinic acid was incorporated into all the nucleic acid pyrimidines. A much higher incorporation into liver PNA than into tumor PNA was found, and the activity in the acid-soluble fraction of liver was much above that of tumor. The incorporation into uracil was greater than into cytosine in liver cytoplasm PNA but not in tumor cytoplasm PNA. There were no significant differences in uptake between individual pyrimidines in nuclear PNA or in DNA. The uptake into PNA was very much higher than into DNA in liver. The turnover of both PNA and DNA was higher in liver of tumor-bearing mice than in that of normal animals. The turnover of tumor DNA was somewhat higher than that of liver DNA. There were no great differences between cytoplasm and nuclei in PNA turnover.

The pattern of excretion and distribution of ureidosuccinic acid observed seemed to follow that found by Hurlburt and Potter for orotic acid in tumorbearing rats. The incorporation picture also agrees with our present knowledge on orotic acid as a nucleic acid precursor and is in agreement with a conversion of ureidosuccinic acid to orotic acid and incorporation by this pathway.

An attempt was made to determine the effect of X-irradiation on the incorporation of ureidosuccinic acid into nucleic acids of livers and spleens of CAF_1 mice. One group of 25 mice had previously received a total body X-irradiation of 400 r at a rate of 79 roentgens per minute. Twenty hours after the X-irradiation, the mice were injected intraperitoneally with five doses of this compound, 30 mg per kg body weight at twelve-hour intervals. The pyrimidines were isolated from nucleic acids of liver and spleen tissues. The results indicate that X-irradiation produced an increased incorporation of this compound into the PNA pyrimidines and a slightly

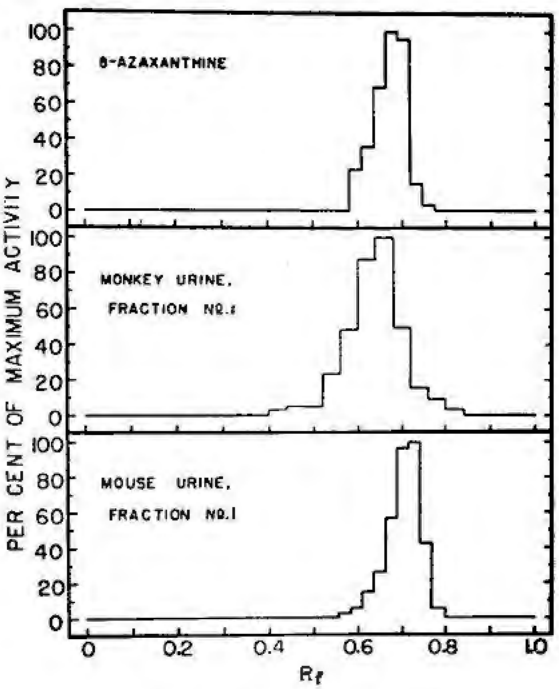

Figure 6. Evidence for the identity of 8-azaxanthine with the principal metabolite of 8-azaguanine

decreased incorporation into DNA pyrimidines of both liver and spleen as compared with the values of control mice.

Experiments are in progress to investigate the incorporation of 8-azaguanine labeled with C^{14} into the nucleic acids of a microorganism which is inhibited by this drug. Methods are being worked out for the optimum incorporation followed by the degradation into the nucleic acid portions and the isolation of azaguanine-containing nucleotides. Preliminary results indicate that approximately two per cent of the administered azaguanine may be taken up by the bacterium and radioactivity appears in one particular fraction of the nucleic acid hydrolysate. The purification of this compound is still in progress. The biological activity of these azaguanine-containing nucleotides will be investigated, since the possibility exists that azaguanine is active by the formation *in vivo* of such intermediates.

METABOLIC ALTERATION OF RADIOMIMETIC COMPOUNDS

The similarity of biological action of nitrogen mustard and ethylenimino compounds led to the clinical use of triethylenemelamine (TEM) in malignant neoplastic disease. It has been postulated that in body fluids the nitrogen mustard compounds are transformed into the reactive ethyleneimonium cations which subsequently react with functional groups of cell proteins or with essential enzymes to produce cytologic changes resulting in mitotic inhibition. To gain a better understanding of their probable mechanisms of action, it became necessary to study their metabolic transformations in tumor-bearing and control animals. A study was made of the influence of tumors of varying degree of susceptibility on the pattern of distribution and excretion of TEM. Since the high toxicity of ethylenimino compounds limits the dosage which may be safely administered to experimental animals, the application of radioactive techniques employing C^{14} labeled TEM of high specific activity was suitable for the purpose.

Triazine ring labeled TEM was synthesized from urea-C^{14} with a specific activity of 7.6 mc per mg. A study was made of the excretion and tissue distribution of radioactivity following intraperitoneal administration to control mice and mice bearing lymphosarcoma-1, sarcoma-37 and leukemia-1210. The exhaled CO_2 contained little radioactivity. A major portion was excreted in the urine within twenty-four hours as cyanuric acid. No striking differences were observed between the uptake of radioactivity by organs of any of the tumor bearing groups and control groups. No selective localization of radioactivity was observed as compared to other organs. From previous results it appeared likely that TEM exerted its biological effects through the small proportion of the intact drug retained by the body or through the ethylenimino moiety split off from the triazine ring during its metabolism. Ethylene labeled TEM was synthesized from ethanol amine by conversion to ethylenimine through the B-aminoethyl sulfuric acid stage. The ethylenimine was reacted with cyanuric chloride to form triethylenemelamine, symmetrically labeled in the three ethylenimino rings, with a specific activity of 1 μc per mg. Preliminary studies on the metabolism of this compound indicates that only one to two per cent in normal mice of the label is oxidized to CO_2 and approximately 70 per cent is excreted in urine within twenty-four hours. Paper chromatography and ion exchange columns are being employed in isolating and identifying the urinary metabolites.

BIOLOGICAL ACETYLATION OF DRUGS

The problem of acetylation and deacetylation of drugs within the animal body is being investigated by the use of acetyl-labeled *N*-acetyl-*p*-aminophenol

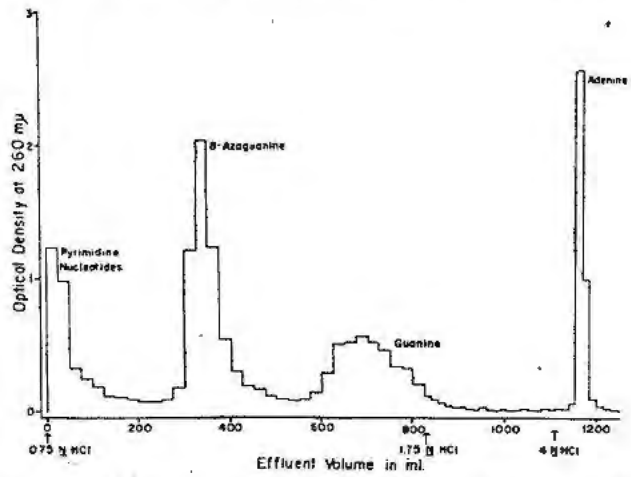

Figure 7. Separation of 8-azaguanine from other purines and pyrimidine nucleotides (resinogram of PNA hydrolysate)

(NAPA). The general plan is to administer the phenol and isolate its chief urinary metabolite, NAPA glucuronide. The relative amount of radioactivity retained in this compound is a measure of the extent of de- and re-acetylation. Comparisons are being made in a number of vertebrate species.

N-acetyl-*p*-aminophenol was synthesized from *p*-aminophenol and carbon-14-labeled acetate. According to literature reports, the major excretion form of this substance is as the glucuronide, and, therefore, a column chromatographic system of separation of this substance was worked out involving adsorption on Dowex-1 anion exchange resin and elution with 0.04*N* hydrochloric acid. It was found that urine could be chromatographed without previous purification and that the NAPA glucuronide could be readily separated in a satisfactory state of purity. Since suitable samples of pure NAPA glucuronide could not be obtained, our product was identified by ultraviolet absorption spectrum, by colorimetric determination of the glucuronide and *p*-aminophenol, and chromatographic identification of the latter, by preparation of the methyltriacetyl derivative of the conjugate, and by radioactive content.

Comparable doses of about 200 mg per kg of the labeled NAPA were administered orally to five animal species, including rabbits, monkeys, dogs, and cats. About one-tenth this dose was administered to two human subjects. Urine was collected for twenty-four hours and lyophilized. The resulting concentrate was chromatographed and those samples bearing the characteristic ultraviolet spectrum of the NAPA glucuronide were pooled, analyzed quantitatively by ultraviolet absorption, and a suitable aliquot was radioassayed. The ratio of the molar radioactivity of the isolated metabolite to that of the administered NAPA gave a measure of the de- and re-acetylation occurring during the metabolism. No more than traces of unconjugated NAPA have been observed. While the animal variation was large, rabbits and cats and one human subject showed little evidence of appreciable exchange of acetyl groups. The overall amounts isolated of glucuronide found, however, were low, indicating either that there is no re-acetylation mechanism, or that other degradation mechanisms take precedence rather than de-acetylation. The other species, including preliminary data on dogs, show considerable exchange with unlabeled acetate, indicating the presence of both de- and re-acetylases.

METABOLISM OF PARA-AMINOBENZOIC ACID

While the metabolism of para-aminobenzoic acid (PAB) has been investigated in a number of microorganisms which require it, its metabolism has had less study in organisms which do not need this vitamin. While *Escherichia coli* ordinarily do not require PAB, the induction by X-irradiation of a mutant requiring it had been reported. The metabolism of radioactive PAB was undertaken in this mutant.

The PAB used was labeled in the carboxyl group with carbon-14. The microorganisms (*E. coli*, ATCC No. 9723a) were grown on a defined medium of inorganic salts plus glucose with minimal amounts of PAB. After twenty-four hours growth, the cells were filtered off and were found to contain some 80 per cent of the total radioactivity. Almost all of this activity could be extracted by acetone-water and water, and these pooled extracts were studied almost exclusively. The radioactivity remaining in the supernatant from the cells was largely present as the original substrate. Although paper chromatography of the extracts revealed that the cell extracts contained essentially no PAB and a large amount of new substances, the poor definition of spots obtainable necessitated a change in separation technique. It was found that chromatography on Dowex-50 cation exchange resin separated four components in the extracts, none of them consisting of the original PAB. For comparison a series of pure compounds were subjected to the same separation procedure. Folic acid and a number of its derivatives were tested as well as various obvious hydroxylation, deamination and decarboxylation products of possible PAB metabolism. None of these were identical to any of the isolated radioactive fractions. Acidic and basic hydrolysates of all four fractions were prepared and re-chromatographed and PAB was obtainable from all. It also seems quite likely that the four fractions are closely related to each other, possibly as artefacts of isolation, for on re-chromatographing of the single fractions, some of the other fractions were formed. In an effort to determine whether certain of these fractions were active coenzymes or inactive degradation products, the growth-promoting ability of each for the mutant was determined. Two of the fractions, denoted as A and D, were found to promote growth at a concentration comparable to that required for PAB, while fractions B and C were at least ten times less potent. The folic acid and citrovorum factor activity of the fractions was also investigated by their growth-promoting ability for *Streptococcus lactis* and *Leuconostoc citrovorum*, respectively. Fractions C and D were growth factors for these microorganisms of a low potency compared to the natural factors, while the other two had no detectable activity. However, C and D have definitely been distinguished from folic acid or citrovorum factor by chromatography. The evidences suggest considerable similarity exists between certain of the metabolites and the citrovorum factor. These metabolites may consist of labile conjugates of some sort. It remains for further purification and isolation of larger amounts to elucidate their structure and possible function.

METABOLISM OF CYSTEAMINE

Among the most effective compounds opposing the acute effects of ionizing radiations is cysteamine

(*beta*-mercaptoethylamine). Studies are in progress to investigate the metabolism of this drug and to determine alterations in its metabolism which may be induced by X-irradiation. These experiments are designed to reveal the mechanism of action of such a compound.

Radioactive cysteamine was synthesized by reaction of ethyleneimine with radioactive hydrogen sulfide (H_2S^{35}). The unstable free amine was converted to the stable salicylate or benzoate salt and used as such in metabolic studies. Complete analyses of urinary metabolites have been carried out in the C_3H mouse, using a cation exchange resin system of separation. Comparative studies were made in both sexes of rate of excretion and pattern of metabolites in normal mice and in animals subjected to a lethal dose of X-irradiation of 900 roentgens and having the drug injected immediately before. The normal mice excrete almost all the radioactivity during the first eight hours, with the remaining few per cent emerging in the next sixteen hours. Irradiated mice exhibited a delayed excretion with only some 72 per cent excreted in eight hours. Males eliminated the remainder in sixteen hours, while females retained about 20 per cent for more than twenty-four hours. The somewhat delayed excretion of radioactivity may be simply a result of anuria. During the first eight hours some forty per cent of the cysteamine was found unchanged in the urine of the male with or without irradiation. A small portion has been identified as cystamine and the disulfide of cysteamine, but most of the remainder is present in oxidized forms. Sulfate and taurine are present in large amounts, while there are lesser amounts of two other metabolites identified tentatively as the sulfinic acid of cysteamine, hypotaurine, and as cystamine disulfioxide. The male animals exhibited no striking changes in this metabolism with radiation. Normal female animals, on the other hand, while showing

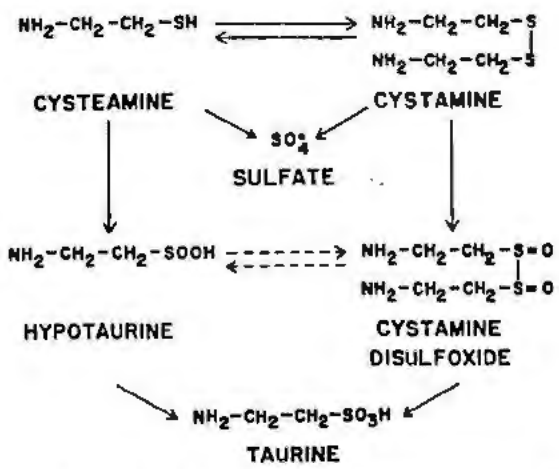

Figure 8. Proposed path for metabolism of cysteamine in the human

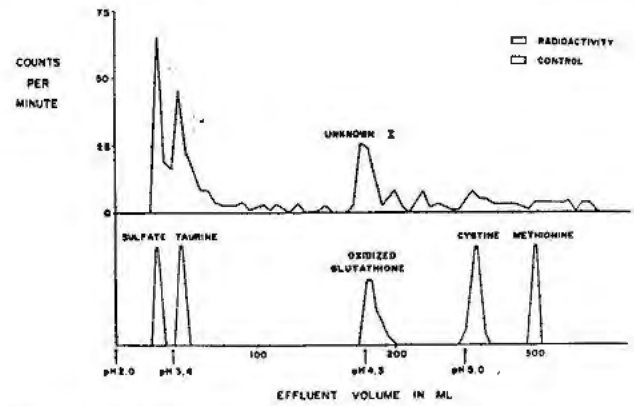

Figure 9. Evidence for metabolites of cysteamine in the human (chromatogram of urinary sulfur compounds)

qualitative similarity in excretion pattern, excreted less unchanged cysteamine and more taurine. Moreover, radiation increased the formation of oxidized metabolites even further.

Investigations are in progress to determine the metabolism of cysteamine in the human (Figs. 8, 9). Three normal male subjects were given fixe microcuries of cysteamine benzoate orally. Urine collections were made for some days and chromatography of the urinary metabolites is in progress. The rate of elimination of cysteamine is delayed as compared to the mouse. In contrast to the complete excretion seen in twenty-four hours in the latter, our subjects eliminated about forty per cent of the activity in eight hours and approximately 50 per cent in the first day. The remainder is found in the urine in the course of the next week, suggesting that a portion of the compound is rather firmly bound to some bodily constituent. The metabolites of cysteamine in the human are quite similar to those observed in the mouse. Sulfate and cysteamine have been definitely identified, while there are comparatively small peaks which are apparently taurine, hypotaurine, and cystamine. No cystamine disulfoxide could be detected. Two small peaks have not been identified. They are quite acidic in nature and might represent compounds such as cysteic acid, guanidotaurine or carbamidotaurine.

It is planned to complete these identifications and to investigate the mechanism by which cysteamine protects against X-ray. Since the metabolism of cysteamine does not appear to be markedly altered in irradiated animals, it may be that it has no specific mechanism other than offering a protecting environment for some important sulfhydryl moiety of the body. Such studies may involve comparison in irradiated animals, with and without cysteamine protection, of blood sulfhydryl, blood glutathione, tissue non-protein and protein sulfhydryl, and tissue Coenzyme A levels.

Isotopic Studies in Cholesterol Metabolism

By M. W. Biggs,* USA

Tritium-labeled cholesterol has been used in our laboratory in a series of investigations on cholesterol metabolism in atherosclerosis. In the course of these studies, tritium-cholesterol has been fed to 18 patients, some with atherosclerosis and others with no disease. The rate of appearance of tritium-cholesterol in the plasma has been studied as a function of time following a tritium-cholesterol meal. We know from these studies that the dynamics of exogenous cholesterol metabolism vary from individual to individual. The differences which we have observed involve the quantitative aspects of the partition of newly absorbed tritium-cholesterol into the free and esterified cholesterol pools of the serum. The normal patient shows a serum free-cholesterol specific activity which is higher than the esterified cholesterol specific activity for 1 to 2 days following a tritium-cholesterol meal. Other patients, however, show the reverse with the esterified cholesterol specific activity higher than the free-cholesterol specific activity during this period.

The different types of serum cholesterol specific activity absorption curves which we have observed in our series of patients can be correlated in a highly significant way with the beta-lipoprotein spectra as defined in the ultracentrifuge according to the method of Gofman and coworkers.[1] The different types of serum cholesterol specific activity absorption curves do not show a significant correlation with total serum cholesterol values. Details of this correlation and data on the first 10 patients studied have been published elsewhere.[2]

To illustrate the different serum cholesterol specific activity absorption curves found in different patients Figs. 1 and 2 contain data for two additional cases studied with tritium-cholesterol. In Fig. 1 the esterified cholesterol specific activity is greater than the free-cholesterol specific activity for 4 days following the tritium-cholesterol meal. In Fig. 2 the free-cholesterol specific activity is higher than the esterified cholesterol specific activity for 2 days. The first is a patient with angina pectoris and xanthoma tuberosum who shows a markedly pathological beta-lipoprotein spectrum. The second patient is clinically well and has a more normal beta-lipoprotein picture. Patients with xanthoma tuberosum and those with a pathological beta-lipoprotein spectrum such as illustrated by patient No. 1 have an increased incidence of atherosclerosis.[3,4] It has been a working hypothesis in our laboratory that when the differences in the dynamics of cholesterol metabolism in these 2 patients is understood we will have increased our insight into the pathological physiology of at least one type of human atherosclerosis.

In conjunction with the human studies some fundamental aspects of normal exogenous cholesterol metabolism have been investigated in the rat. These experiments have been designed to help in the interpretation of the human data. It is now known that newly absorbed cholesterol enters the systemic circulation via the lymph of the thoracic duct.[5,6] Newly absorbed cholesterol in thoracic duct chyle is contained in lipoproteins that flotate in the ultracentrifuge with a flotation rate greater than 400 S_f units. The ultracentrifugal techniques and nomenclature of Gofman and coworkers[1] have been used in this paper. It is probable that newly absorbed cholesterol is contained in those lipoprotein molecules commonly designated as chylomicrons.[7] The newly absorbed tritium-cholesterol in chyle is contained in both free and esterified forms. The esterified cholesterol carries quantitatively the larger portion of the newly absorbed cholesterol, and the esterified cholesterol specific activity is higher than the free-cholesterol specific activity in chyle.[6]

The present experiment was designed to investigate the normal metabolic fate of exogenous cholesterol following its entrance into the systemic circulation. For this purpose chyle containing tritium-cholesterol has been injected intravenously into a series of normal rats, and the distribution of tritium-cholesterol in the plasma and liver has been studied as a function of time. The data indicate that following its entrance into the systemic circulation chyle cholesterol enters a specific metabolic pool which is rapidly and differentially removed from the circulating plasma. The principal site of this removal is the liver. Following this hepatic "clearing" a large portion of the chylous cholesterol esters are rapidly hydrolyzed in the course of normal hepatic metabolism.

EXPERIMENTAL

Tritium-Labeled Cholesterol

Tritium-labeled cholesterol was prepared according to the method of Bloch and Rittenberg[8] with a few minor changes.[9] The tritium-cholesterol was purified via the dibromide and the final product had a melting point of 148°C. The specific activity was 1.81 μc of

* Indiana University School of Medicine.

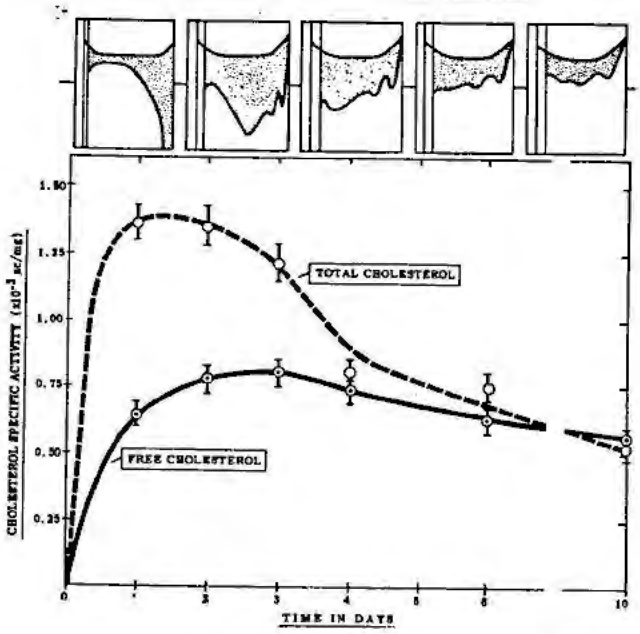

Figure 1. Patient 1. The graph shows the changes in serum cholesterol specific activities as a function of time following a single feeding of 0.103 gm of tritium-cholesterol (specific activity = 1.81 μc/mg) in a fatty meal. A tracing of the patient's beta lipoprotein spectrum as defined in the ultracentrifuge heads the figure. It shows the following concentrations: S_f0–12 = 219 mg %, S_f12–20 = 116 mg %, S_f20–100 = 716 mg %, and S_f100–400 = 644 mg %. The total serum cholesterol was 666 mg%

tritium per mg of cholesterol. Tritium-cholesterol so prepared contains 46% of the label in the vicinity of the Δ5-3-hydroxy system and 54% in the isopropyl group of the side chain according to Fukushima and Gallagher.[10,11] The tritium label occupies stable positions in that there is no reduction of specific activity on refluxing in 30% potassium hydroxide in 50% alcohol for 12 hours. Tritium was chosen as the labeling isotope because its weak beta radiation and relatively short biological half-life make it particularly suitable for studies in human subjects.

Assay of Tritium Radioactivity

Tritium was assayed in the gas phase in ionization chambers with a vibrating reed electrometer and suitable potentiometer. Biological samples of cholesterol prepared as the digitonide were burned in a suitable combustion train. The water so obtained was allowed to react with lithium aluminum hydride, and the hydrogen liberated was used to fill the ionization chambers. Details of the method have been published elsewhere.[12,13]

Cholesterol Specific Activity Determinations

Plasma ur serum total cholesterol specific activity measurements were carried out as follows: the serum sample, from 0.5 to 3.0 ml, was extracted in a large centrifuge tube with 30 volumes of cold alcohol: acetone (1 : 1) with vigorous stirring. The tubes were centrifuged at 1000 rpm for 10 minutes and the supernatant decanted off. The protein residue was extracted a second time with 15 volumes of alcohol: acetone and the extracts combined. No attempt was made to make this extraction quantitative. The combined alcohol: acetone extracts were made 0.2*N* to potassium hydroxide by the addition of the necessary amount of 2*N* aqueous potassium hydroxide. The saponification mixture was heated to 65°C for two hours and then allowed to stand overnight. The solution was acidified with 10% hydrochloric acid and made up to a known volume, usually 50 ml. Two quantitative portions of this volume were set aside for duplicate cholesterol determinations as decribed below, and a third aliquot was used for tritium assay. To this aliquot for tritium assay was added 60.0 mg of carrier cholesterol. The mixture was heated to boiling and 35 ml of 0.5% digitonin in 80% alcohol was added. The cholesterol digitonide precipitate was filtered with suction and washed with ether: acetone (2: 1) and then with anhydrous ether. A quantitative recovery of the cholesterol digitonide is not necessary. The samples were placed in an 80°C oven until combusted for tritium assay.

The specific activity of a given cholesterol sample was calculated from the rate of the electrometer drift and the known dilution factors introduced by the addition of carrier and precipitation with digitonin. A cholesterol-digitonide empirical formula of $C_{83}H_{138}O_{30}$ was used. The standard deviation of the cholesterol specific activity measurements in the range of the activities of this paper is ± 6%.

Plasma or serum free cholesterol specific activity measurements were made as above with the exception that the saponification step was omitted. The alcohol: acetone extract was made up to a known volume,

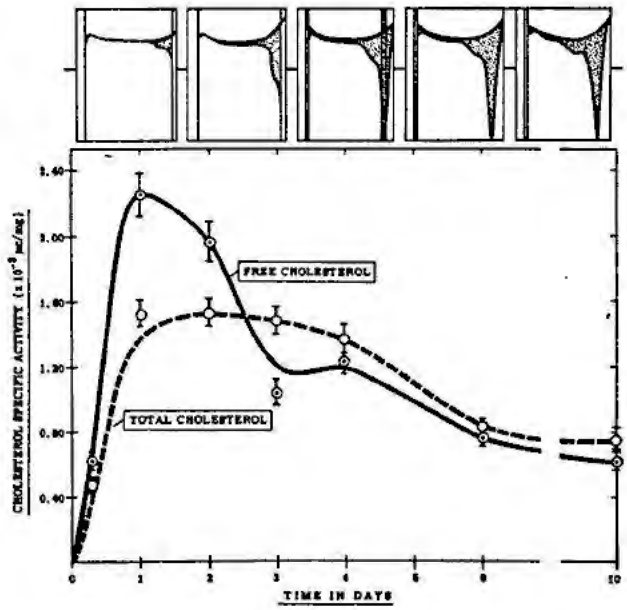

Figure 2. Patient 2. The graph shows the changes in serum cholesterol specific activities as a function of time following a single feeding of 0.115 gm of tritium-cholesterol (specific activity = 1.81 μc/mg) in a fatty meal. A tracing of the patient's beta lipoprotein spectrum as defined in the ultracentrifuge heads the figure. It shows the following concentrations: S_f0–12 = 604 mg %, S_f12–20 = 117 mg %, S_f20–100 = 125 mg %, and S_f100–400 = 52 mg %. The total serum cholesterol was 293 mg %.

and measured aliquots were taken for duplicate cholesterol determinations and tritium activity measurement as above.

Liver cholesterol specific activity measurements were made as follows: The livers were minced, frozen, and lyophilized. The dried tissue was extracted with boiling alcohol: ether (4 : 1) for 9 hours. The extracting mixture was renewed at 3-hour intervals. The extracts were combined and the ether removed on a hot plate. The alcoholic extract was made up to exactly 100 ml. Aliquots from this 100 ml volume were used for both free and esterified cholesterol specific activities as described below.

Duplicate portions of this volume were taken for free cholesterol determinations. A third aliquot was used for free cholesterol tritium assay following the addition of carrier cholesterol and precipitation with digitonin.

Duplicate portions of the original extract were taken for total cholesterol determination and another aliquot for total cholesterol tritium activity measurement. The samples for cholesterol analysis and that for tritium assay were saponified in identical fashion. The solutions were made (1 : 1) alcohol: acetone. Sufficient 2*N* aqueous potassium hydroxide was added to make the mixture 0.2*N* to potassium hydroxide. The saponification mixtures were maintained at 65°C for 2 hours with frequent shaking. Then the samples were acidified with 10% hydrochloric acid. Carrier was added to the sample for tritium assay and digitonin precipitation was carried out as above. All cholesterol determinations were done as indicated below.

Cholesterol Determinations

The cholesterol method used in our laboratory is that developed by Coleman.[14] It is fundamentally that first described by Schoenheimer and Sperry[15] and modified by Sobel and Mayer.[16] All cholesterol determinations were done in duplicate, and the reported values are the average of two determinations.

Collection of Thoracic Duct Lymph

Male rats of the Wistar-Purdue strain, 350–400 gm in weight, were used as donor animals. A polyethylene tube (od 0.060 in.) was placed in the abdominal portion of the thoracic duct according to the technique described by Bloom *et al.*[17] A second polyethylene tube was fixed in the stomach by a purse string suture to facilitate the administration of tritium-cholesterol. After recovery from the ether anesthesia, the animals were maintained in restraining cages without anesthesia throughout the lymph collections. Approximately six hours after surgery if the lymph flow was good, each animal was given 5 ml of whole milk plus 30 mg of tritium-cholesterol (specific activity equal to 1.81 μc/mg) dissolved in 1 ml of cotton-seed oil. The lymph subsequently collected was delivered to a flask containing Acid Citrate Dextrose Anticoagulant to prevent clotting. Each 100 ml of this solution contains sodium citrate USP-2.06 gm, citric acid 0.75 gm, and dextrose 2.29 gm.) The lymph used for the injection experiments was collected as soon as the lymph became pure white and chylous. The lymph was stored at 4°C and was used within 48 hours of collection.

ANIMAL STUDIES

All of the experimental animals were of the Wistar-Purdue strain, female and weighed between 200 and 225 gm. All animals were allowed Purine Rat Chow *ad lib* up to the beginning of the experimental period. Each experimental animal received 0.5 ml of chyle containing tritium-cholesterol intravenously by tail vein. At a specified time after the chyle injection each animal was killed by exsanguination from the abdominal aorta under ether anesthesia. The blood was collected in a syringe containing a drop of heparin solution, and the plasma was separated from the red blood cells immediately by centrifugation. Immediately following exsanguination the liver was excised, minced, and frozen for lyophilization.

RESULTS

The first series of 15 animals received 0.5 ml of chyle containing 28 mg% free cholesterol (specific activity equal to 0.426 μc/mg) and 83 mg% total cholesterol (specific activity equal to 0.543 μc/mg). The animals were killed in groups of 3 at 30 minutes, 45 minutes, 1 hour, 2 hours, and 4 hours after the chyle injection.

At 30 minutes following the chyle injection, over 90% of the chyle cholesterol has been removed from the circulating plasma. At this time over 80% of the injected chyle cholesterol is to be found in the liver. The total serum cholesterol values for each rat and the percentage of injected chyle cholesterol found in the plasma and liver at each time interval is recorded in Table II. For these calculations the plasma volume for each rat has been estimated at 10 ml.

During the period between 30 minutes and 1 hour following the chyle injection the specific activities of the liver cholesterol rose to levels greater than 5 times that of the corresponding plasma cholesterol specific activities. The plasma total cholesterol specific activities and the liver total cholesterol specific activities are recorded in Table I. The average values for the plasma and liver specific activities at each time interval are plotted in Fig. 3 to give a qualitative representation of the changes of these values as a function of time. Table I also gives the free, total, and esterified cholesterol values for the whole liver as well as the specific activities for these metabolic pools. The esterified cholesterol specific activity is a calculated value from the free and total cholesterol specific activities and is therefore less certain. The average values for free, total, and esterified cholesterol specific activities at each time point have been plotted in Fig. 4 and give a qualitative picture of the changes in free and esterified cholesterol specific activities in the liver as a function of time.

In the original chyle injected there was 0.060 μc

Table I. Plasma and Liver Cholesterol Specific Activities Following the Intravenous Injection of Chyle Containing Tritium-Cholesterol

Rat number	Time after chyle injection	Plasma total cholesterol specific activity ($\times 10^{-3}$ μc/mg)	Liver cholesterol			Liver cholesterol specific activity		
			Free mg	Total mg	Ester mg	Free	Total ($\times 10^{-3}$ μc/mg)	Ester
1	30 min	1.4	12.3	15.6	3.3	6.9	12.9	35
2	30 min	2.6	12.2	14.3	2.1	6.6	12.8	49
3	30 min	1.9	18.4	21.1	2.7	4.6	9.1	40
4	45 min	2.2	12.1	15.1	3.0	8.5	12.1	27
5	45 min	2.3	13.3	15.6	2.3	7.2	10.9	32
6	45 min	1.5	13.4	16.5	3.1	8.4	11.0	22
7	1 hr	1.9	12.1	16.6	4.5	7.9	10.5	17
8	1 hr	1.9	14.4	16.8	2.4	8.2	10.4	24
9	1 hr	2.4	13.7	16.0	2.3	9.9	10.7	15
10	2 hr	1.8	13.6	16.8	3.2	3.7	4.9	10
11	2 hr	–	–	–	–	–	–	–
12	2 hr	3.5	12.3	15.1	2.8	7.7	6.9	3
13	4 hr	2.5	16.1	19.4	3.3	3.8	4.0	–
14	4 hr	2.9	17.0	20.0	3.0	4.1	4.1	–
15	4 hr	3.1	13.8	15.0	1.2	4.5	4.9	–

of tritium in free cholesterol. At 30 minutes following the chyle cholesterol injection there was on the average 0.083 μc of tritium in free cholesterol in the liver; at 45 minutes there was 0.104 μc in free cholesterol; and at 1 hour there was 0.116 μc in free cholesterol in the liver.

A second series of 6 rats was studied using a different lymph collection. The lymph used for injection of these animals contained 33 mg% free cholesterol (specific activity equal to 0.42 μc/mg) and 72 mg% total cholesterol (specific activity equal to 0.52 μc/mg). These animals each received 0.5 ml of lymph by tail vein and were killed serially at 15 minutes, 29 minutes, 45 minutes, 1 hour, 3 hours, and 8 hours. The data for this series of animals are given in Table III.

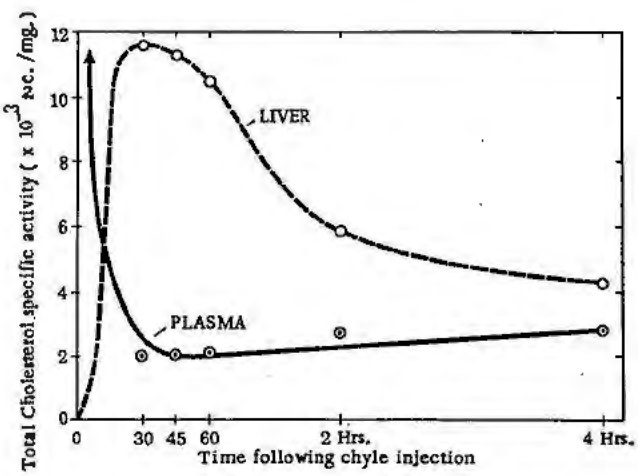

Figure 3. The average specific activity values for liver and serum total cholesterol plotted as a function of time following the intravenous injection of chyle-containing tritium-cholesterol

DISCUSSION

During the period 30 minutes to 1 hour following the chyle injection the liver total cholesterol specific activity has risen to some 5 fold the specific activity of the plasma total cholesterol. This clearly indicates that chyle cholesterol is in a separate metabolic pool which does not mix appreciably in a metabolic sense with the rest of the plasma cholesterol. Chyle cholesterol is specifically and rapidly removed from plasma, and the predominant site of this removal is the liver. Thus it can be shown that cholesterol entering the circulation from the intestine travels a specific metabolic pathway. Eventually this cholesterol undoubtedly becomes indistinguishable from cholesterol endogenously synthesized, but at first it is separate and travels a separate metabolic path. Positioning of this fact into our present day ideas of the respective roles of "endogenous" and "exogenous" cholesterol in atherosclerosis pathogenesis must await further studies.

In Fig. 4 we see that there is a definite trend during the period 30 minutes to 2 hours for the esterified cholesterol specific activity to fall. This could result from the return of cholesterol esters to the serum or rapid hydrolysis of cholesterol esters in the course of normal hepatic metabolism. The data support the latter explanation for some 0.116 μc of tritium cholesterol was found in the liver in the free cholesterol pool at the end of 1 hour while only 0.060 μc of tritium cholesterol was contained in the free form in the 0.5 ml of chyle injected. Hence, appreciable amounts of tritium-cholesterol introduced in the chyle in the esterified form appears in the liver in the free form at one hour.

The height of the rise of liver esterified cholesterol specific activity at 30 minutes indicates that hepatic "clearing" of chyle cholesterol probably does not involve appreciable hydrolysis of cholesterol esters as a plasma event prior to hepatic uptake.

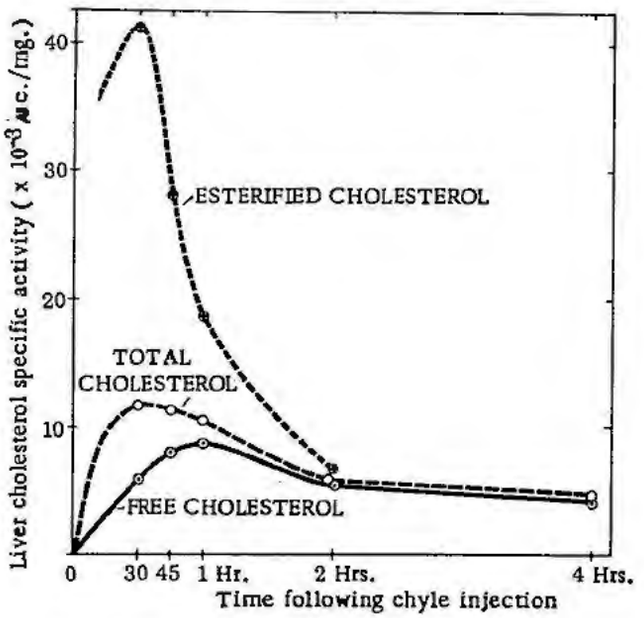

Figure 4. The average specific activity values for the liver free, total, and esterified cholesterol are plotted as a function of time following the intravenous injection of chyle-containing tritium-cholesterol

Interpretation of the human data in light of the present studies is undoubtedly premature. However, as a working hypothesis, we believe that in the normal metabolism of chyle cholesterol a large portion is "cleared" into the liver, possibly into other tissues as well, and here appreciable quantities of the cholesterol esters are hydrolyzed. The return of free cholesterol from the liver into the plasma produces the cholesterol specific activity absorption curves seen in Fig. 2. If the hepatic metabolism of chyle cholesterol is faulty, if the chyle cholesterol is not efficiently "cleared" from the plasma, and if the hepatic hydrolysis of cholesterol esters does not occur properly, cholesterol specific activity absorption curves such as that illustrated in Fig. 1 may result. There are other possible explanations of these human curves of course. The possibility that chyle in different patients differs markedly in its cholesterol make-up will have to be considered.

SUMMARY

Previous studies with tritium-cholesterol feedings to normal and to atherosclerotic patients have demonstrated quantitative metabolic differences in cholesterol metabolism in various individuals. These differences concern the quantitative aspects of the partition of newly absorbed cholesterol into the free and esterified cholesterol pools of the plasma. The type of cholesterol specific activity absorption curve obtained can be correlated in a highly significant way with the beta-lipoprotein spectrum as defined with the ultracentrifuge.

The present study was designed to help explain the metabolic differences seen in the human subjects. Chyle containing tritium-cholesterol has been administered intravenously to 21 healthy rats. The partition of the tritium-cholesterol in the plasma cholesterol and in the liver free and total cholesterol as a function of time has been studied.

Within 30 to 60 minutes following the chyle injection over 90% of the chyle cholesterol has been removed from the plasma. Examination of the liver shows from 70 to 80% of the injected dose is to be found in this organ at this 30- to 60-minute time period. The specific activity of total cholesterol in the liver rises to greater than 5 times that of the plasma total cholesterol specific activity, clearly indicating that chyle cholesterol is differentially removed from the plasma by liver.

Studies of the free and total cholesterol specific activities in the liver support the belief that a large portion of the newly absorbed chyle cholesterol esters

Table II. Percentage of Chyle Cholesterol in the Plasma and the Liver

Rat number	*Time after chyle injection*	*Serum total cholesterol (mg %)*	*Per cent of injected T-cholesterol in plasma*	*Per cent of injected T-cholesterol in liver*
1	30 min	58	4%	89%
2	30 min	77	9%	81%
3	30 min	82	7%	85%
4	45 min	77	8%	81%
5	45 min	64	7%	76%
6	45 min	52	4%	81%
7	1 hr	84	7%	77%
8	1 hr	77	7%	78%
9	1 hr	58	6%	76%
10	2 hr	88	7%	37%
11	2 hr	60	Lost	Lost
12	2 hr	52	8%	46%
13	4 hr	53	6%	34%
14	4 hr	64	8%	36%
15	4 hr	60	8%	33%

Table III. Plasma and Liver Cholesterol Specific Activities Following the Intravenous Injection of Chyle Containing Tritium-Cholesterol

Rat number	*Time after chyle injection*	*Plasma total cholesterol specific activity ($\times 10^{-3}$ μc/mg)*	*Liver total cholesterol specific activity ($\times 10^{-3}$ μc/mg)*	*Per cent of injected T-cholesterol in plasma*	*Per cent of injected T-cholesterol in liver*
16	15 min	4.06	2.70	24	49
17	29 min	0.57	4.12	3	66
18	45 min	0.58	6.00	3	72
19	1 hr	0.76	6.80	5	73
20	3 hr	0.98	2.70	5	40
21	8 hr	1.47	1.54	9	24

undergo rapid hydrolysis in the course of normal hepatic metabolism.

The significance of these studies in the interpretation of the human data has been discussed briefly.

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Isotopic Studies of Steroid Metabolism in Man

By Leon Hellman,* R. S. Rosenfeld,* David K. Fukushima,* H. Leon Bradlow,* T. F. Gallagher,* R. Gordon Gould† and George V. LeRoy‡

The metabolism of body fat was the first aspect of biochemistry to be studied by isotopic methods. The earliest report,[1] which concerned the use of deuterium as an indicator in the study of intermediary metabolism, appeared just 20 years ago. The procedures developed for administration of stable isotopes to small laboratory animals have been adapted to studies of human metabolism using both stable and radioactive isotopes. Radioactive carbon (C^{14}) and radioactive hydrogen (tritium) have replaced carbon-13 and deuterium for most tracer studies of lipids and steroids. The radioactive isotopes are preferred because the methods of assay are simpler and more sensitive than is the case with stable isotopes (Appendix A). The low energy of the beta radiation of carbon-14 and tritium and the relatively rapid rate of biochemical turnover of substances containing these isotopes permit the use of tracer doses with enough radioactivity for accurate assay without hazard to the recipient.

This report will be confined to studies of the metabolism of cholesterol, coprosterol, bile acids, and steroid hormones in man, and only data obtained by use of carbon-14 and the two isotopes of hydrogen will be presented.

ENDOGENOUS SYNTHESIS OF CHOLESTEROL FROM ACETATE

The cholesterol of the body is of dual origin; a portion is derived from cholesterol ingested with the diet, and the remainder is synthesized in various tissues. There is general agreement that the principal precursor of endogenous cholesterol is a two-carbon compound similar to or identical with acetic acid.

In order to study cholesterol synthesis from acetate,[2] 200 microcuries (μc) of labeled sodium acetate were administered to human subjects, free and ester cholesterol were separately isolated from the plasma,[3] and the radioactivity of these products was measured at frequent intervals over a prolonged period of time. The data from a typical case indicating the changes in the specific activities of free and ester cholesterol with time are shown in a semi-logarithmic plot in Fig. 1. The maximal value for the specific activity of free cholesterol had been reached by the time the initial sample of blood was obtained; thereafter the specific activity of this fraction declined rapidly. The ester specific activity was at its minimal value with the earliest sample of blood, rose to a peak at about 2.1 days and at that point intersected the specific activity curve of the free cholesterol. After this point of intersection, the ester cholesterol maintained a slightly higher specific activity than that of the free cholesterol for the duration of the period of observation.

At 0.3 day after the administration of acetate, a point closely approximating the maximum value of the specific activity of free cholesterol, from 0.05 to 0.22 per cent of the acetate administered had been incorporated per millimole plasma free cholesterol. Corresponding values for plasma ester cholesterol at this point ranged from 0.02 to 0.07 per cent millimole. At the crossover point of free and ester cholesterol (about two days) where both fractions possess the same specific activity, from 0.03 to 0.12 per cent of the administered dose was present per millimole of sterol. Labeled acetate is rapidly dissipated by many competitive metabolic processes, such as oxidation to carbon dioxide,[4] conversion to fatty acids, etc., within one to two hours after administration. Therefore, the maximal specific activity is attained within that period because the precursor has disappeared through metabolic transformation to longer-lived compounds or by elimination from the body. Many distribution and metabolic reactions of cholesterol are reflected in the disappearance curves derived from the specific activity values. For these reasons the physiological reactions involved in the turnover of plasma cholesterol demand additional information which must be supplied by further studies.

CHOLESTEROL DERIVED FROM THE DIET

In order to compare the metabolic behavior of plasma cholesterol derived from the diet with that of cholesterol synthesized *in vivo* from acetate, cholesterol labeled with either isotopic carbon or hydrogen was administered orally to human subjects and the plasma cholesterol was studied for an extended period.[5] In certain instances, both endogenous and exogenous cholesterol metabolism were examined simultaneously by the use of appropriately labeled acetate and cholesterol. These techniques were also applied to an examination of the behavior of plasma cholesterol in four patients with hypercholesterolemia.

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The data obtained for four and a half days following the simultaneous administration of sodium acetate-2-H^3 and cholesterol-4-C^{14} to a normocholesterolemic subject are plotted in Fig. 2. As shown on the lower right portion of this figure, the specific activity of free cholesterol synthesized *in vivo* from acetate was at a maximum with the earliest sample and then declined rather rapidly. The ester cholesterol derived from acetate was at a minimum value with the first sample and then gradually rose until it intersected the curve of the free cholesterol at approximately three days, after which the specific activity of the ester cholesterol exceeded that of the free. For comparison with these data obtained by the use of tritium, the specific activity curves from a patient who received C^{14}-labeled acetate are shown in the upper right portion of Fig. 2. The metabolism of cholesterol synthesized from acetate was similar for both isotopes, and these data with others to be presented, demonstrate the interchangeable use of carbon and hydrogen isotopes for biochemical study of the cholesterol molecule.

The activity of plasma free cholesterol originating in the diet was at a minimum value with the 3-hour sample, as illustrated on the left portion of Fig. 2. The specific activity then rose to a peak at about 1.5 to 2 days and then declined. The radioactivity of the "exogenous" plasma ester cholesterol was also at a minimum with the first sample, increased less rapidly than that of the free sterol, and reached a peak value at about 2.5 days, having intersected the free curve at about 1 day. After this crossover point, the ester specific activity was greater than that of the free for the duration of the period of observation.

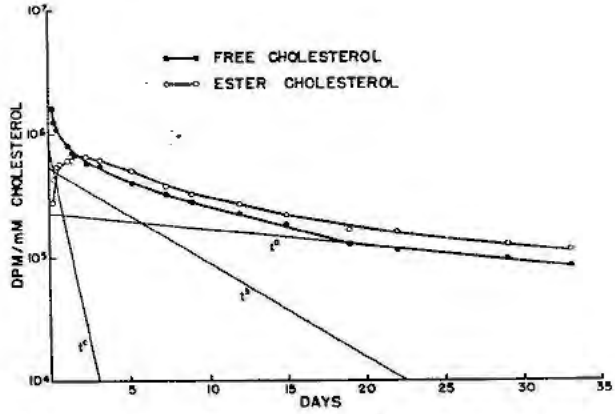

Figure 1. Incorporation of acetate-2-C^{14} into plasma cholesterol

Figure 3 compares the incorporation of dietary cholesterol-4-C^{14} into plasma cholesterol of a subject with a normal cholesterol level with a patient who had hypercholesterolemia accompanying the xanthoma tendinosum syndrome. It can be seen that the mode of rise of free and ester cholesterol, the points of intersection, and the subsequent rate of decline over a ten-day period, are virtually indistinguishable. The specific activity of the plasma cholesterol in the normocholesterolemic patient was higher than that of the hypercholesterolemic subject. However, this difference may be related to individual variation in the quantity of labeled cholesterol absorbed or to dilution of the labeled sterol by the larger plasma cholesterol pool in the hypercholesterolemic.

A subject who had hypercholesterolemia associated with the xanthoma tuberosum syndrome received

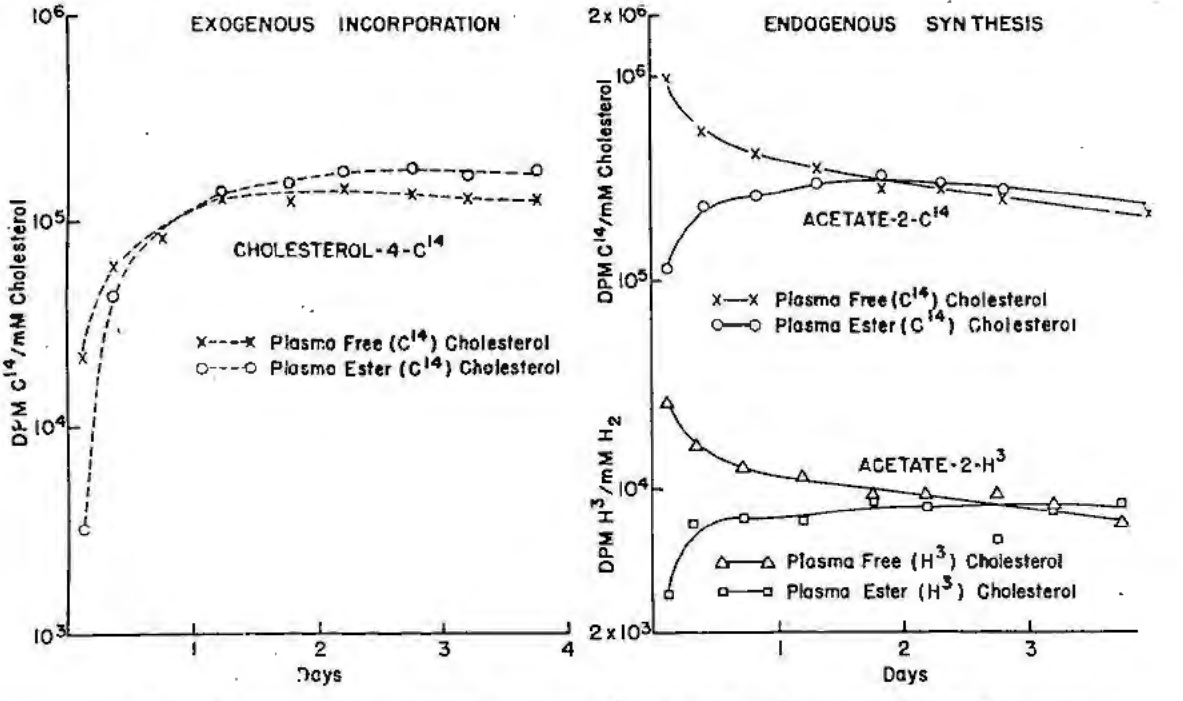

Figure 2. Simultaneous utilization of dietary cholesterol-4-C^{14} and acetate-2-H^3 for plasma cholesterol synthesis compared with incorporation of acetate-2-C^{14}

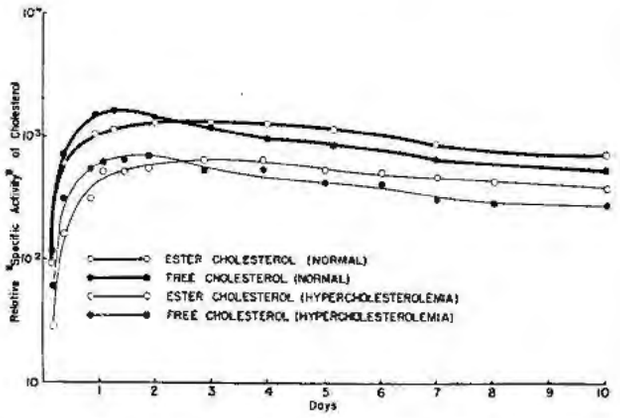

Figure 3. Incorporation of dietary cholesterol-4-C^{14} into circulating cholesterol in normal and hypercholesterolemic man

cholesterol-H^3 and acetate-2-C^{14} simultaneously. The patient was studied for six months and Fig. 4 is a comparison of the behavior of cholesterol arising from these two sources for the first 43 days. The findings in this patient differ in two respects from those obtained in normocholesterolemic subjects and the patient with the xanthoma tendinosum syndrome. The rising specific activity curve of plasma ester cholesterol synthesized *in vivo* does not intersect the free curve until about the sixth day. In all patients previously studied, this point of intersection occurred at two to three days. The plasma cholesterol derived from the diet exhibited another difference in that the ester cholesterol specific activity was initially at a higher level, rose more rapidly and exceeded the specific activity of the free cholesterol throughout the experiment. Since the free cholesterol specific activity was always below the value for ester cholesterol, the two curves do not intersect. The latter parts of the curves in Fig. 4 show rates of decline of cholesterol radioactivity parallel to those observed in other patients with hypercholesterolemia as well as patients with normal plasma cholesterol levels. These data derived after the simultaneous administration of labeled acetate and cholesterol affirm the conclusion previously drawn that either labeled compound yields identical results concerning the long term aspects of the behavior of plasma cholesterol.

Cholesterol-4-C^{14} was also given to a patient who had hypercholesterolemia accompanying the nephrotic syndrome. The radioactivity of the plasma cholesterol behaved in a similar fashion to that observed in the previous subject with xanthoma tuberosum.

When labeled cholesterol is fed daily for several weeks the specific activity of plasma cholesterol reaches a steady state in 3–4 days. Equilibration of the cholesterol of the different tissues of the body occurs at rates that are peculiar to the individual tissues. Comparable data have been obtained using dogs and rats.[6]

The radioactivity excreted in feces of patients who received C^{14} cholesterol varied from 14 to 28 per cent. A much smaller proportion, from 0.35 to 1.76 per cent, of the administered radioactivity was excreted in the urine during the same time interval. Expired carbon dioxide after administration of

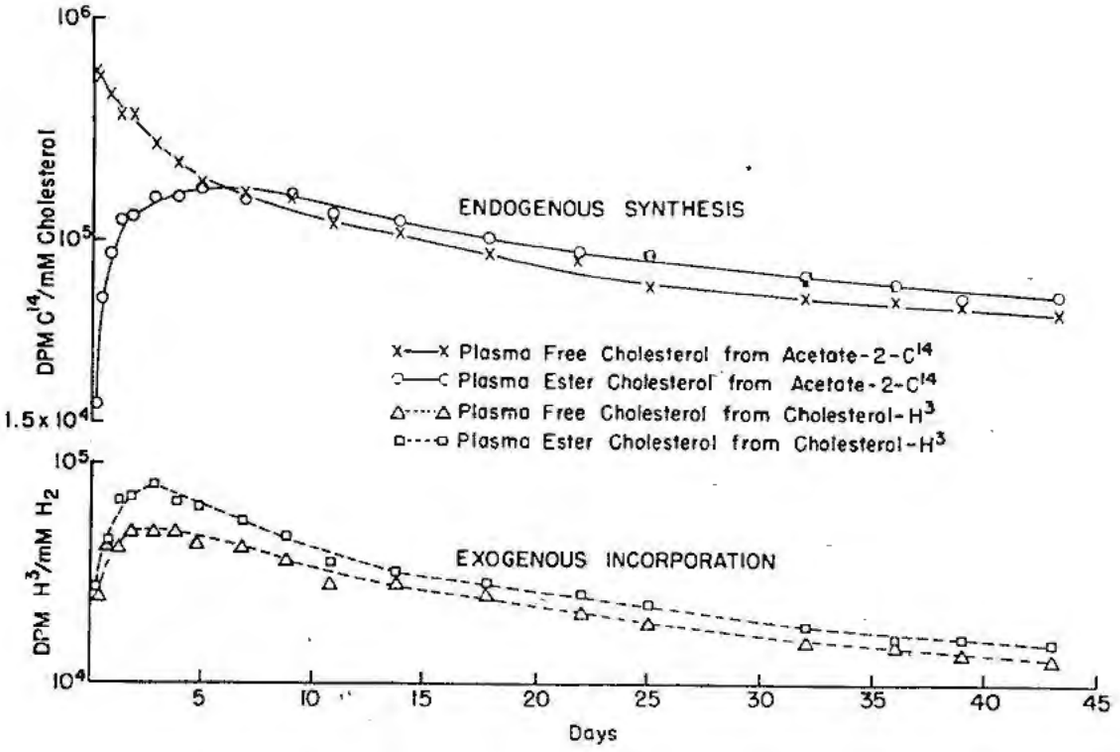

Figure 4. Simultaneous incorporation of dietary cholesterol-H^3 and acetate-2-C^{14} into plasma cholesterol in hypercholesterolemia

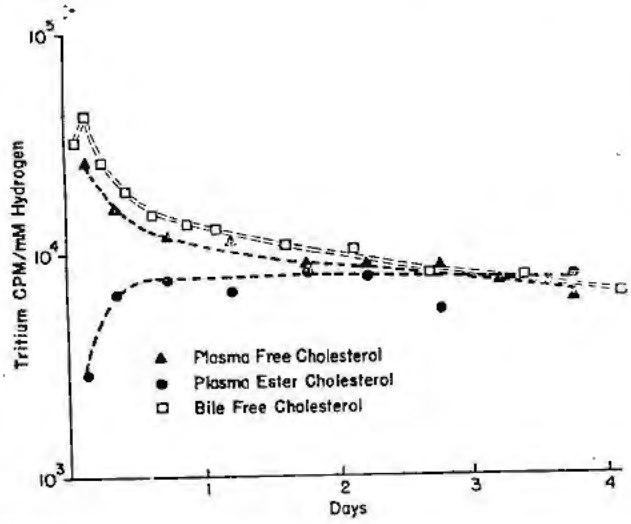

Figure 5. Incorporation of acetate-2-H^3 into plasma and biliary cholesterol

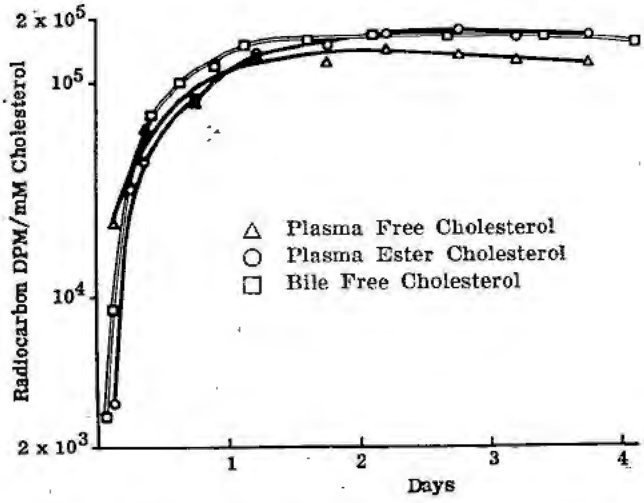

Figure 6. Incorporation of oral cholesterol-4-C^{14} into plasma and biliary cholesterol

cholesterol-4-C^{14} had no measurable radioactivity; as little as 0.1 per cent of the administered dose could have been detected each day by the method used.

Dietary cholesterol and cholesterol made *in vivo* from acetate differ in metabolism only during the immediate post-absorptive and post-synthetic period in that biosynthetic cholesterol rapidly appears in the blood while the peak circulatory level of dietary cholesterol is at a later time. However, the radioactivity data indicate conclusively that the two "species" of plasma cholesterol merge by the third or fourth day following synthesis or ingestion and are then indistinguishable except for the isotope used to label each precursor. In addition it seems important to call attention to the possibility that fed and biosynthesized cholesterol may be transported by different means immediately after either formation or absorption. It is likely that biosynthetic cholesterol is secreted into the circulation from the site of synthesis in combination with lipoprotein. The molecular form in which dietary cholesterol enters the circulation via the lymph is poorly characterized and may be different in physical properties from lipoprotein-bound endogenous cholesterol. Since the physiological processes that give rise to both "species" are continuous, both forms are present in the circulation at the same time, and the possibility remains that the immediate post-absorptive phase is critical for the study of pathological cholesterol metabolism. Since biosynthetic cholesterol is constantly secreted in the bile and mixes in the intestine with cholesterol present in food, preformed or biosynthetic cholesterol is always available for absorption and both may contribute to the critical nature indicated above for the post absorptive phase.

CHOLESTEROL SYNTHESIS BY VARIOUS TISSUES

The cholesterol content of human tissues ranges from 0.12 per cent in the case of the red cells to as much as 7.5 per cent in the case of the adrenal glands. Equilibration of plasma and tissue cholesterol proceeds at varying rates, and in the case of some tissues, such as the adult nervous system, probably never occurs. Taken together with the fact that tissues other than the liver are able to synthesize cholesterol, the organ content of this sterol is the resultant of the contributions of either or both of these mechanisms. Cholesterol synthesis from acetate has been demonstrated to occur *in vivo* in skin, in normal human adrenal tissue, in adrenals stimulated by ACTH, in the corpus luteum of menstruation, in the corpus luteum of pregnancy, in the ovary during pregnancy, in the fetal portion of the placenta, and in tumor tissues, including adrenal and breast cancer. These results are in agreement with animal studies, although fewer human tissues have been examined. The extent to which cholesterol synthesized in tissues other than the liver exchanges with plasma cholesterol has not been established and thus presents an important area for further study.

BILIARY CHOLESTEROL AND BILE ACIDS

The endogenous and exogenous precursors of biliary cholesterol and cholic acid have been studied in patients with complete bile fistulas after the simultaneous oral administration of tritium labeled sodium acetate and cholesterol-4-C^{14}.[7,8] As shown in Fig. 5, biliary cholesterol, synthesized *in vivo* from acetate, was of higher specific activity than plasma "free"

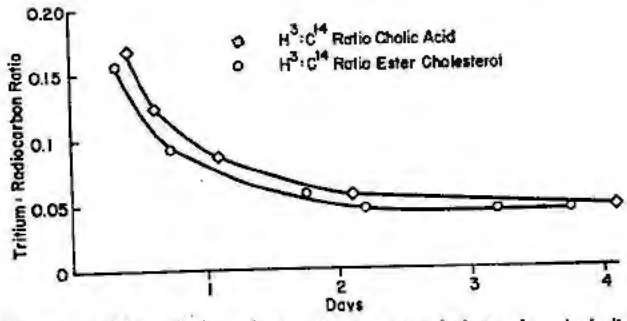

Figure 7. Tritium: Radiocarbon ratios in ester cholesterol and cholic acid

cholesterol during the first 8 hours of the experiment; thereafter the specific activities of plasma "free" and biliary cholesterol were identical over a period of 5 days. The curves describing the appearance in plasma and bile of cholesterol derived from ingested labeled sterol (Fig. 6) were similar for 2 days. After the second day, the specific activity of the biliary "free" cholesterol derived from preformed dietary cholesterol exceeded that of plasma "free" cholesterol. The specific activity of cholic acid was always lower than that of circulating cholesterol. The finding that the isotope ratio of C^{14}:H^3 was identical in cholesterol and cholic acid as can be seen in Fig. 7 indicates that the sterol was a direct precursor of the bile acid.

THE TRANSFORMATION OF CHOLESTEROL TO COPROSTANOL

The conversion of cholesterol to coprostanol (coprosterol) has been studied in an attempt to define the mechanism of this transformation.[9] Two principal hypotheses have been advanced to explain this reaction: (1) a direct stereospecific reduction of the double bond and (2) a three stage conversion from cholesterol involving the intermediates delta^4-cholestenone and coprostanone, accomplished with loss of the hydrogen at C-3 in cholesterol. After feeding cholesterol-3-H^2, 4-C^{14} or incubating this double-labeled compound with human feces, the conversion to coprostanol occurs in large part by a mechanism in which the hydrogen at C-3 remains intact. Therefore, it is unlikely that coprostanol arises from cholesterol principally by the second route (hypothesis (2) above). For this to occur, the deuterium atom at C-3 would be completely lost and the coprostanol would have contained C^{14} but no deuterium.

Coprostanol derived from the incubation of cholesterol-3-H^2, 4-C^{14} *in vitro* with human feces, contained deuterium both at C-3 and elsewhere in the molecule. Stepwise removal of deuterium at C-5 and C-6 showed that these carbons alone contained the extra deuterium. The evidence is clear that a portion of the labeled cholesterol was used as the deuterium donor in the conversion to coprostanol.

CONVERSION TO STEROID HORMONES

The presence of isotope in urinary steroid hormone metabolites of gonadal and adrenal origin after the administration of either labeled acetate of cholesterol or both, as well as the similar specific activity of certain urinary metabolites and the plasma cholesterol, clearly indicate the interrelation of cholesterol metabolism with that of the steroid hormones.

When isotopically labeled acetate is administered, the tracer has been found in certain steroid hormone metabolites isolated from the urine.[10,11] Since acetate is a known precursor of cholesterol, such experiments indicate that cholesterol, as such, is a permissible if not an obligatory precursor for the steroid hormones. When ring A labeled cholesterol is fed, or injected intravenously, the tracer label of the ring structure has also been found in steroid hormone metabolites isolated from the urine. The metabolites identified as synthesized *in vivo* from acetate or cholesterol are: dehydroisoandrosterone, androsterone, etiocholanolone, delta^2-androstene-17-one, 11-ketoetiocholanolone, 11-OH etiocholanolone, 17-OH pregnaolone, tetrahydrocortisone, tetrahydrohydrocortisone, pregnanediol, delta^9-androstene-3 alpha-ol-17-one.

METABOLIC STUDIES OF ADMINISTERED HORMONES

The urinary steroid hormone metabolites found after administration of acetate or cholesterol have all been found after administration of the steroid hormones themselves and these studies have elucidated many of the routes of overall steroid metabolism in the body.

To date the following hormones have been administered to humans[12]: testosterone-H^2 and C^{14}; hydrocortisone-4-C^{14}; cortisone-4-C^{14}; desoxycorticosterone-21-C^{14}; corticosterone-4-C^{14}; progesterone-4-C^{14}; and estradiol-16-C^{14}. In two experiments, testosterone and hydrocortisone have been given to subjects with complete bile fistulas. In these cases, it has been demonstrated that the bile is a minor excretory route of these hormones and thus emphasizes one of the ways human steroid metabolism differs from that of the rabbit, rat and mouse. Because the hormone metabolites are present only in trace quantities in human bile, fecal excretion of these products is similarly low, since there appears to be little if any direct elimination through the intestinal wall.

When labeled testosterone is given intravenously or intramuscularly, about 60 per cent of the hormone is excreted in the urine. Most of this is ketonic and approximately 90 per cent of this fraction is present as androsterone and etiocholanolone. The ratio of these two compounds in the urine is relatively constant for each individual but varies between subjects; there are, moreover, marked variations in ratio in the same subject with time after the dose.

Progesterone appears to be unique among the hormones insofar as the route of excretion by human subjects is concerned. After administration of this substance about 30 per cent of the metabolites appear in the urine and an equal amount is present in the feces. Pregnanediol is the principal and pregnanolone the minor metabolite found in the urine. Together these products comprise about 90 per cent of the total urinary radioactivity.

The metabolism of hydrocortisone-4-C^{14} has been studied in some detail.[13] Two subjects, one a man (Experiment 1) and the other a woman (Experiment 2), each received a tracer quantity of hormone, approximately 0.25 mg, equivalent to one microcurie C^{14}; on another occasion, the same amount of labeled hydrocortisone together with 100 mg of carrier hormone was given to the same male subject (Experiment 3). This experiment permitted an evaluation of the effect of the quantity of hormone

given on its subsequent fate in the same individual. Another female subject (Experiment 4) also received 1 μc of hydrocortisone-4-C^{14} together with 100 mg of carrier hormone. Two studies were carried out on the fourth subject, a woman who was both oophorectomized and adrenalectomized. In the first of these (Experiment 5), all hormone therapy was withdrawn for 48 hours before, and 24 hours after, the intravenous administration of a tracer amount (0.25 mg) of hydrocortisone. The procedure permitted an evaluation of the metabolism of the hormone when the tissues of the recipient were depleted of this substance. A second study (Experiment 6) on this same subject was carried out while she was receiving 100 mg of hydrocortisone daily by continuous intravenous infusion. In this instance, the non-radioactive hydrocortisone infusion was interrupted for one-half hour while the tracer dose of the radioactive hormone was introduced. Infusion of the unlabeled hormone was continued for 24 hours.

A typical cumulative urinary excretion curve is shown in Fig. 8. In Experiment 1, 56 per cent was excreted in the urine at the end of 6 hours; 72 per cent was eliminated in one day and 80 per cent over three days. Virtually the same results were obtained in Experiment 2 with a tracer dose administered to the male subject. In Experiment 3, when this same subject was given 100 mg of carrier hydrocortisone together with radioactive hormone, the large additional quantity of hormone did not appreciably alter the excretory pattern. Fifty-four per cent was excreted in 6 hours, and 84 per cent in one day. The female subject who was also given 100 mg of hydrocortisone, containing 1 μc of C^{14}, showed an almost identical pattern of excretion in Experiment 4. In Experiment 5, conducted while the adrenalectomized woman was deprived of all replacement therapy, the excretory pattern was also similar and no significant changes were observed when the same subject received 100 mg of hydrocortisone daily by continuous intravenous infusion in Experiment 6.

In order to define the rate of excretion of the radioactive products derived from the administered hydrocortisone-4-C^{14}, the radioactivity in the urine was plotted semi-logarithmically against time for a typical experiment. During the first 24 hours after the dose, the data group about a straight line and suggest that the excretion approximates a first-order reaction. The rate of excretion of the products derived from the metabolism of 80 per cent of administered hydrocortisone during the first 24 hours can be characterized by an average half-life of 3.6 hours in all subjects studied.

The radioactivity found in the blood 2 hours after administration ranged from 1.2 to 3.4 $\times$ 10^{-2} μc per liter. In a blood sample obtained 15 minutes after the end of infusion, a maximum of 13 per cent of the circulating radioactivity was present as unaltered hydrocortisone as demonstrated by isotopic dilution analysis. In this short time, most of the hormone had therefore undergone metabolic transformation.

The results obtained by separating the urinary metabolites into "free" and conjugated fractions indicate that an average of 12 per cent of the radioactive hormone products excreted during a single hour 30 minutes after completion of the infusion, was present as the "free" or non-conjugated form. After the sixth hour only 1 to 7 per cent of the excreted material was still unconjugated. About 25 per cent of the urinary metabolites have been identified as pregnane derivatives in which the characteristic dihydroxyacetone side chain had been transformed to a glycerol structure. A somewhat smaller percentage may be accounted for as 11-oxygenated 17-ketosteroids and as metabolites which have the original side chain, but with the ring A reduced. In all, somewhat more than 60 per cent of the total radioactivity in the urine has been isolated and identified as pure, chemically characterized, individual compounds.

Analysis of the C^{14} content of a control respiratory carbon dioxide sample with a Libby screen wall counter gave a net activity of 15.1$\pm$0.2 dpm per gram of carbon, a value identical with that of contemporary carbon. After the administration of 0.855 μc of hydrocortisone-4-C^{14}, the pooled carbon dioxide collections contained 20.1 dpm per gram of carbon. This increase of 5 dpm per gram of carbon in the expired air represents excretion of radioactivity equivalent to 0.05 per cent of the administered hydrocortisone per day. Therefore degradation of the A ring and, by implication, the entire steroid nucleus could not have exceeded this amount and the study clearly demonstrated that about 80 per cent of the hormone administered in either trace quantities or massive doses appeared in one form or another in the urine within the course of 24 hours. Indeed, most of the end products are excreted within a much shorter period of time. Relatively little radioactivity, less than 10 per cent of the dose, was excreted into the intestinal tract. The characterization and definition of the various transformation products of the hormone has given valuable information for further studies and the dynamics of hydro-

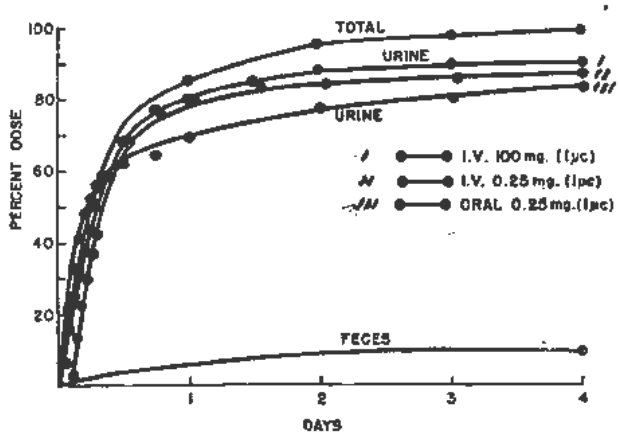

Figure 8. Cumulative excretion of radioactivity after administration of hydrocortisone-4-C^{14}

cortisone metabolism have been, in certain aspects, clarified in a manner that would have been impossible without the use of radioactive isotopes.

APPENDIX A. NOTE ON RADIOASSAY METHODS

A limiting factor in any assay procedure is the sensitivity of the detection method. Recent developments in instruments provide medical experiments with a variety of techniques for radioassay of C^{14} and tritium. These are listed in the order of their sensitivity below:

Method	*Carbon-14 sensitivity, mc/gm*
Vacuum line technique, using $C^{14}O_2$ in the vibrating reed electrometer	10^{-8}
Libby's screen wall counter, with anti-coincidence circuit	10^{-9}
Liquid scintillation coincidence counter, commercial type	5×10^{-10}
Vacuum line technique, using acetylene–C^{14} in gas counter with anticoincidence circuit	3×10^{-10}
Liquid scintillation, coincidence counter, research types	10^{-10}
Radioactivity of contemporary natural carbon, 15.2 dpm/gm	7×10^{-9}

When these values for sensitivity are considered as dilution factors at which detection is still possible in biological materials, their significance is readily apparent. As noted above, when labeled diet cholesterol is fed in milligram doses the labeled cholesterol of plasma may be diluted by a factor as great as 5×10^4. Expressed in this way it is evident that current detection methods have adequate sensitivity. Cholesterol labeled with a stable isotope would have to be fed in doses of the order of one gram or more to supply enough tracer for detection in plasma cholesterol. The best techniques for stable isotope analysis will not permit a dilution greater than 1 part in 10,000.

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Steroid Metabolism Studies with the Aid of C^{14}-Labeled Compounds

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The importance of steroidal substances in the economy of living organisms has become increasingly clear and detailed over the years. It is now recognized that in animals a significant number of steroids are present in the tissues and circulate in the blood. Two broad groups have been recognized: (*a*) the sterols, exemplified by cholesterol present in all mammalian tissues and in high concentration (100 to 300 mg %) in the blood, and which is regarded as biologically inert and (*b*) the hormonal steroids produced by certain specialized organs (the testes, the ovaries and the adrenals) which have specific mechanisms for their biosynthesis and which release these hormones into the blood where they circulate in small concentration, i.e., in μg %. The modes of biogenesis of cholesterol and the steroid hormones have been largely matters of speculation until the availability of isotopes of the atoms composing them: carbon, hydrogen and oxygen. Radioactive carbon (C^{14}) has been particularly useful in tracer studies of the simple precursors of the steroids, and C^{14}-labeled steroids have led to several notable discoveries in recent years, e.g., the establishment of cholesterol as a parent substance of hormonal steroids, of progesterone as a key precursor to adrenocortical steroids, and the delineation of biogenetic relationships between numerous hormonal steroids.

We will review certain aspects of the biogenesis of cholesterol, of adrenocortical and testis steroids, drawing primarily on the experiments performed in this laboratory.

Disturbances in the normal modes of steroid biosynthesis may be basic to important pathological consequences. Indications of such relations are presented in this paper.

CHOLESTEROL BIOGENESIS

Based on the well known work of Schoenheimer and his school,[1] later so ably continued by Rittenberg and Bloch,[2,3] we have studied the conversion of acetate-1-C^{14} into cholesterol. The important observation was made that cholesterol, when synthesized from labeled acetate either by perfusion of pig liver[4] or after injection into intact animals, is accompanied by a number of radioactive substances. These substances, designated as HCC (higher counting companions), under certain conditions, had higher specific activities than the cholesterol simultaneously formed. This suggested that at least some of these substances may be precursors of cholesterol. Some of these accompanying substances were precipitated with digitonin together with the cholesterol and it was assumed that some of the HCC is sterol in nature.

A systematic investigation of the accompanying substances was started employing column chromatography with alumina as the adsorbing material. By this means, squalene-C^{14} was isolated and identified. On feeding this substance to rats, cholesterol-C^{14} was demonstrated. These results are in accord with those previously reported by Bloch.[5] These workers had previously demonstrated that C^{14}-labeled acetate may be converted into squalene-C^{14} and that this compound administered to rats was transformed to cholesterol-C^{14}.

Two substances have been isolated from that fraction of the HCC which is precipitated with digitonin namely, 3β,5α-dihydroxy-6-ketocholestane and 7-ketocholesterol. Another substance which has been obtained in crystalline form has not yet been identified but seems to be a keto alcohol of the sterol series. The first compound, 3β,5α-dihydroxy-6-ketocholestane, is in all probability derived from 3β,5α,6β-trihydroxycholestane which apparently is formed from cholesterol since the specific activity of this material was exactly the same as that of the cholesterol itself. 7-ketocholesterol probably is also formed from cholesterol and is not a precursor.

In experiments with yeast we have found that the conversion of acetate-1-C^{14} to ergosterol-C^{14} is accompanied by a group of compounds having specific activities, greater than that found for the ergosterol. This observation parallels that of the liver experiments and cholesterol biosynthesis. The ergosterol companion substances, like the HCC from animal material, yielded squalene-C^{14} and zymosterol-C^{14}. This latter sterol proved to be a probable precursor of cholesterol since on feeding to rats cholesterol-C^{14} was formed.

* Worcester Foundation for Experimental Biology.

On theoretical grounds Ruzicka[6] has suggested that cryptosterol (lanosterol) and zymosterol should be expected to be precursors of cholesterol. Our experiments show that zymosterol is possibly a precursor of cholesterol and Bloch's group[7] has shown that lanosterol is a precursor of cholesterol. These experiments make it quite likely that the following sequence is valid for the biosynthesis of cholesterol: acetate → squalene → cryptosterol (lanosterol) → zymosterol → cholesterol. In yeast, cholesterol is replaced by ergosterol as the end product.

Experiments with blood have shown that red cells also synthesize cholesterol although on a very much smaller scale. Evidently in the living animal this part of the biosynthesis of cholesterol does not play an important role, because liver and intestine make so much larger amounts of this substance. The determination of HCC in experiments with intact animals reveals that the different tissues in different species have all very special patterns of biosynthesis of cholesterol which are characteristic for the species. We have found that the guinea pig synthesizes cholesterol in the liver and intestine on a much smaller and much slower scale than the rat. This seems to relate to the fact that the guinea pig after feeding of cholesterol becomes atherosclerotic whereas normal rats cannot be influenced in this way. It is believed that the study of the HCC under varying conditions will give interesting information on the role of cholesterol in other fields possibly also in the cancer field.

BIOGENESIS OF CORTICOIDS

Figure 1 represents a summary of the available data concerning the probable sequences involved in the biosynthesis of the adrenocortical hormones. Drawn from a variety of studies in diverse species, involving the use of C^{14}-labeled compounds, this sequence attempts to account for the production of various products in the adrenocortical secretion. Of these, corticosterone, cortisol (hydrocortisone) and aldosterone are generally recognized as the major corticoids released from the adrenal gland. Those reactions which have been definitively established are shown with solid lines; those with dotted lines are probable but have not been shown definitively.

Each of the reactions involved in the conversion of cholesterol to corticosterone and cortisol have now been demonstrated unequivocally in cell-free homogenates. Moreover, the available evidence from *in vivo* studies is basically in agreement with this phase of the postulated sequence. However, considerable uncertainty is associated with other aspects of Fig. 1. These may be summarized in the form of the following three questions:

1. Although there is evidence for an alternative pathway of corticosteroidogenesis which does not involve cholesterol as an obligatory intermediate, does such a pathway actually exist? If so, what are the intermediaries involved, and does one of these enter the "cholesterol-sequence" at the level of pregnenolone or progestrone or is another pathway operative?

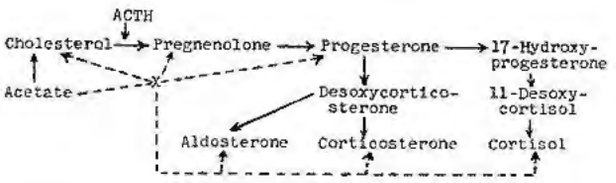

Figure 1. Biogenesis of corticoids

2. What pathways are utilized for the production of aldosterone? The discovery and elucidation of structure of this new corticoid is so recent that we do not even know whether aldosterone is derived primarily from cholesterol or the postulated alternative pathway. The limited evidence available indicates that desoxycorticosterone may be a precursor of aldosterone.[8]

3. Is the corticosteroidogenic activity of corticotrophin (ACTH) achieved as the consequence of accelerating the reaction, cholesterol to pregnenolone, which appears to be the "rate-determining step" in the sequence? If so, how does ACTH act to influence this reaction?

Our approach to the elucidation of these questions has involved search for a cell-free adrenal preparation which has the capacity of forming corticosteroids from both C^{14} cholesterol and C^{14} acetate. Given such a system, examination of the specific activities of the radioactive products formed, studied on a kinetic basis, should provide evidence concerning the postulated "non-cholesterol-pathway" and its quantitative importance in corticosteroidogenesis. Similarly, with the use of labeled precursors, the biogenesis of aldosterone could be elucidated. Finally, by suitable fractionation of the tissue preparation it might be possible to study the reaction, cholesterol to pregnenolone, upon which ACTH appears to act, and thus gain some understanding of the mechanism of this action.

Cell-free homogenates of cow adrenals were prepared and incubated with cholesterol-4-C^{14}. The major radioactive products formed which were isolated and identified are: pregnenolone (10%), progesterone (6%), corticosterone (50%) and cortisol (21%). The figures in parentheses refer to percentage of the total radioactive products formed. In addition, several zones of radioactivity were observed which represent "trace products" which could not be identified. One of these zones (about 7% of the total products) appears to contain aldosterone, (as evidenced by assay of mineralocorticoid activity) but additional unidentified products are likewise present in this zone. The question of the possible transformation of cholesterol to aldosterone cannot be answered until the aldosterone isolated in experiments of this type is chemically homogenous, at which point it should be possible to demonstrate whether or not C^{14} cholesterol is converted to aldosterone.

Having observed that adrenal homogenates of cows transform C^{14} cholesterol to products similar to those obtained following perfusion of cow glands, studies were undertaken to investigate the cholesterol catabolic activity of various fractions of the homogenates. In these studies, cow adrenals were homogenized in isotonic sucrose, and then subjected to differential centrifugation. The individual sub-cellular fractions thus obtained were incubated with C^{14}-cholesterol, using a cofactor medium containing ATP, DPN, niacinamide and Mg. The results of these studies indicated that the bulk of the cholesterol catabolic activity present in the homogenate was associated with the "mitochondrial" fraction; the "nuclear", "microsomal" and "supernate" fractions being almost completely inactive. The products obtained by incubating C^{14} cholesterol with mitochondrial fractions were analyzed by "pooling" the extracts from a large number of "small-scale" experiments involving the mitochondrial fraction from an equivalent of 40–50 gm of adrenal cortex. The major product formed by mitochondrial fractions under these conditions proved to be progesterone. Although other radioactive products were observed they could not be identified. However, the following possible transformation products which were searched for could not be detected: pregnenolone, 11β-hydroxyprogesterone, 17α-hydroxyprogesterone, desoxycorticosterone, corticosterone, and cortisol. This finding suggested that fractionation of the homogenate yielded a mitochondrial preparation wherein the later steps in the corticosteroidogenic sequence (involving 17, 21, and 11 hydroxylations) could be dissociated from the early steps involved in cholesterol catabolism.

When mitochondrial fractions were prepared on a "large-scale" (using 300 gm adrenal cortex), the products formed from cholesterol-4-C^{14} proved to be entirely dissimilar to those obtained from "small-scale" mitochondrial fractions. Using the "large-scale" conditions, the radioactive products formed proved to be progesterone, 11β-hydroxyprogesterone, corticosterone, and cortisol. Under these conditions of preparation, the mitochondrial fraction exhibits 17, 21, and 11β-hydroxylating activity. The preparation of adrenal mitochondria on a "large" and "small" scale in our laboratory are essentially similar except for one single step. In a "small-scale" experiment, the mitochondria are spun down once and then resuspended for washing. In our "large-scale" preparations, we added additional homogenate to the mitochondrial pellet of the previous spinning, and this occurred 4–6 times. It is therefore possible that under these conditions, where the mitochondria are 4–6 times concentrated, that adsorption of non-mitochondrial enzyme occurs which is not easily removed by subsequent washing with isotonic saline. While this explanation has not been tested experimentally, the differences observed between mitochondrial fractions with respect to cholesterol catabolism raises the interesting questions as to whether certain enzymes in the cell are mobile in the sense that they may shift from one site in the cell to another.

The studies completed have not answered the questions which prompted this investigation. However, the way appears to be open for a straightforward attack upon these problems, using adrenal mitochondrial preparations incubated with C^{14}-labeled substrates.

ANDROGEN BIOGENESIS

Specific steroid producing tissues appear to supply to the blood and tissues five principal androgens: dehydroepiandrosterone, Δ^4-androstene-3,17-dione, testosterone, 11β-hydroxy-Δ^4-androstene-3,17-dione, and adrenosterone. Testosterone may be a specific product of the testes or actually this androgen may be synthesized in both the ovaries and testes. Δ^4-androstene-3,17-dione is produced by the testes and probably by the ovaries as well. Evidence exists for the biosynthesis of five androgens by adrenal tissues. Four of these steroids, Δ^4-androstene-3,17-dione, adrenosterone, 11β-hydroxy-Δ^4-androstene-3,17-dione, and 11β-hydroxyepiandrosterone have been isolated from adrenal tissue and the fifth steroid, dehydroepiandrosterone, appears, on the basis of indirect evidence, to be present in the gland.

Acetate is a precursor of both testosterone and Δ^4-androstene-3,17-dione. Brady[9] demonstrated the conversion of acetate-C^{14} to testosterone when he incubated tissue slices derived from hog, rabbit and the human testis with carboxyl-labeled acetate and isolated C^{14}-labeled testosterone. In these experiments it was demonstrated that the synthesis of testosterone was significantly enhanced when chorionic gonadotropin was added to the incubation mixture. The fact that chorionic gonadotropin did not enhance the incorporation of acetate into cholesterol and that cholesterol had a lower specific activity than the formed testosterone argues for the fact that cholesterol is not a necessary intermediate between acetate and testosterone.

The results of Brady have been confirmed and extended by the perfusion studies in our laboratory. In these studies, acetate-1-C^{14} was perfused through a human testis with the result that testosterone-1-C^{14} and Δ^4-androstene-3,17-dione-C^{14} was formed. The incubation of pregnenolone with testicular interstitial cells resulted in the formation of testosterone.[10] This may represent an important pathway for the biogenesis of testicular androgens. The steps are indicated in Fig. 2.

Adrenal cancer patients, who frequently excrete enormous quantities of 17-ketosteroids, have been treated with acetate-1-C^{14}. Dehydroepiandrosterone,

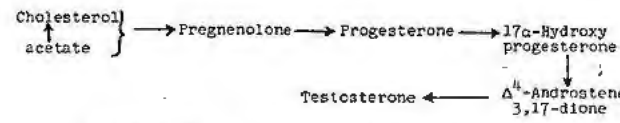

Figure 2. Possible biogenesis of testicular androgens

Δ^5-androstene-3β,17β-diol, androsterone, and etiocholanolone containing C^{14} have been isolated from the urine. The presence of the androgen, dehydroepiandrosterone, in the urine indicates that acetate is readily converted to this adrenal androgen.

Perfusion of the bovine adrenal with acetate-1-C^{14} and added ACTH resulted in the detection of C^{14}-labeled adrenosterone, 11β-hydroxy-Δ^4-androstene-3,17-dione, and Δ^4-androstene-3,17-dione.[11]

The steroid fractions isolated in the urine of an adrenal cancer patient following the oral administration of cholesterol-3-C^{14} contained C^{14} radioactivity. No positive statement can be made regarding the origin of the steroid carbon content. Although a sample of cholesterol isolated from the urine had a specific activity approximately equal to that of the blood cholesterol, androsterone and etiocholanolone isolated during this period had only $^1/_{10}$ the specific activity of the cholesterol. Thus, the 17-ketusteroids could arise from breakdown products of the administered cholesterol as well as from cholesterol directly. The possible biogenesis of adrenal androgens is represented in Fig. 3.

ADRENAL HYPERACTIVITY IN THE HUMAN

The fact that certain changes in urinary steroids are associated with adrenal hyperactivity has been known for some time.[1–3] On the basis of more recent steroid metabolism studies in humans together with new knowledge of adrenal steroid biogenesis it is now possible to suggest certain hypotheses concerning the biochemical-physiological defects associated with three types of adrenal hyperactivity.

As an aid to the understanding of adrenal hyperactivity it is proper to consider first the pituitary-adrenal system in normal individuals. This can conveniently be done with the aid of Fig. 4. We start with the anterior pituitary and the production of adrenocorticotropin (ACTH) which is known to stimulate the adrenal cortex resulting in the increased production of corticoids and androgens. This tropic hormone may be considered to have three interrelated actions: (1) the growth and maintenance of the adrenal cortex, an action which appears to take place in a matter of days, (2) the stimulation of steroid biosynthetic enzyme formation (such as 3β-dehydrogenase, 11β-hydroxylase), an action which probably takes place in hours, and finally (3) direct stimulation of the corticoid production, an action which occurs in minutes. This last action is represented by arrows between precursors such as cholesterol (directly) and acetate (probably indirectly) and the first steroid products, pregnenolone and dehydroepiandrosterone (Fig. 4).

A feature of the normal biosynthetic events in the adrenal as presented is essentially a separation of C_{19} (androgen) and C_{21} (corticoid) production. It is postulated that separate enzyme systems are required for the crucial steps and that the interconversion of C_{19} and C_{21} steroids in the adrenal gland is of minor consequence.

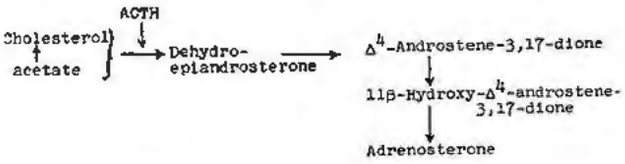

Figure 3. Biogenesis of adrenal androgens

The reactions pregnenolone to progesterone (C_{21} steroid) and dehydroepiandrosterone to Δ^4-androstene-3,17-dione (C_{19} steroids) have the common feature of the oxidation of carbon-3 so that the 3β-hydroxyl gronp is converted to the 3-ketone and the double bond is shifted from carbons 5, 6 to carbons 4,5. This reaction is catalyzed by the enzyme 3β-dehydrogenase.

Progesterone formed in the adrenal may be hydroxylated at carbon atoms 11, 17, 21. When all three hydroxylations are accomplished cortisol is formed. Six intermediate compounds are possible, all of which have been realized by isolation studies, perfusion studies, and/or in vitro studies using various adrenal preparations.[12] More recently, aldosterone, a steroid possessing high electrolyte activity and high activity with respect to life maintenance, has been isolated from adrenal tissue. The steroid may well arise from desoxycorticosterone by successive hydroxylation at carbon-18, oxidation of the 18-hydroxy group to the 18-aldehyde group followed by 11β-hydroxylation.[8]

The Δ^4-androstene-3,17-dione can be 11β-hydroxylated with the formation of 11β-hydroxy-Δ^4-androstene-3,17-dione.

A pertinent question is how does the pituitary-adrenal system continue to operate? The system can be described as a "negative feed back" which consists of the stimulation of the adrenal cortex by ACTH and the control of ACTH production and/or release by the formed corticoids. Actually, cortisol appears to be the most active substance with respect to control of ACTH concentration of the blood. Other corticoids such as corticosterone and the androgens possess so little activity that for practical

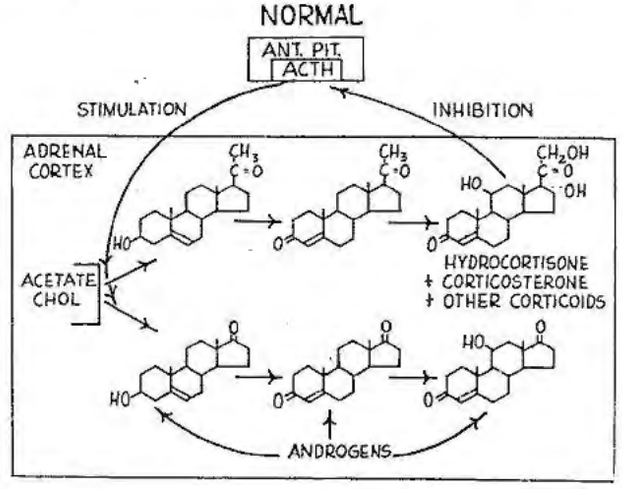

Figure 4. Pituitary-adrenal interrelationships in normal individuals

purposes it is the cortisol concentration that normally controls the blood ACTH concentration. This means that whereas the corticoid-ACTH production may be described as a self-contained and internally regulated system the production of androgens is dependent upon the ACTH-corticoid interrelationships.

Our discussions will be limited to three types of adrenal hyperactivity. Cushing's syndrome (non-malignant) will be considered as the disease attributable to increased blood concentrations of corticoids such as cortisol and cortisone and producing diabetes, hypertension, moon face and stria and the "pure form" does not produce significant masculinizing symptoms. Adrenal cancer, characterized by severe masculinization, will be the second type of adrenal hyperactivity, and the third type will be the adrenogenital syndrome characterized by excessive androgen production without adrenal malignancy and the absence of increased cortisol production. It is recognized that it is not unusual for mixed types of adrenal hyperactivity to exist, but for the sake of simplification, we shall take the liberty of dealing with the so-called pure forms.

The continuation of the Cushing syndrome requires that an excessive concentration of cortisol be maintained. It is postulated that this occurs because of three pituitary-adrenal factors: (1) An increased ACTH production by the anterior pituitary. (2) ACTH stimulation of the Cushing's adrenal causes disproportionate increases in the production of C_{21} steroids as compared to the production of C_{19} steroids. (3) The anterior pituitary is relatively refractory to the inhibitory effect of cortisol and the ACTH level in the blood remains high, and in turn the cortisol production continues at an excessive level (Fig. 5).

Adrenal cancer resulting in excessive androgen production with no Cushing's disease is consistent with the following biochemical-physiological modifications. (1) The malignant adrenal cortex produces steroids autonomously, independent of ACTH. (2) C_{19} steroid production tends to be greater than

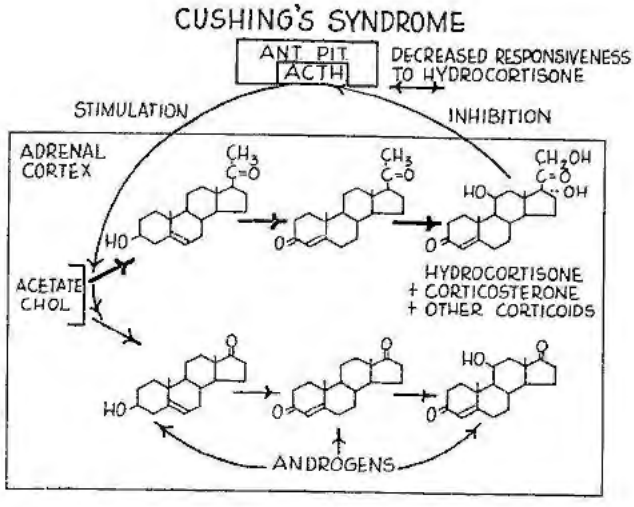

Figure 5. Pituitary-adrenal interrelationships in Cushing's syndrome

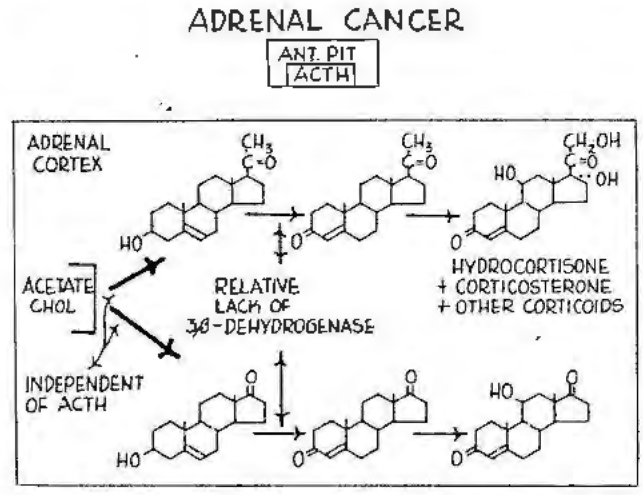

Figure 6. Pituitary-adrenal interrelationships in adrenal cancer

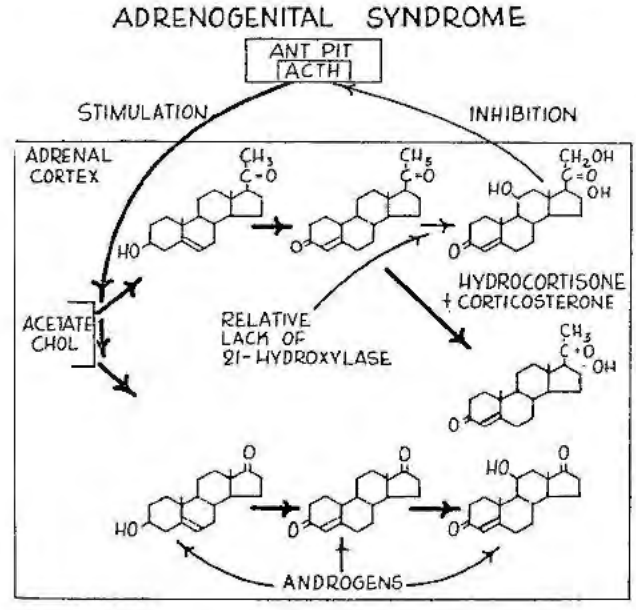

Figure 7. Pituitary-adrenal interrelationships in the adrenogenital syndrome

the C_{21} steroid production. (3) The bulk of the C_{19} and C_{21} steroids are of the Δ^5-3β-hydroxyl type due to a relative lack of the 3β-dehydrogenase system. This results in an excessive excretion of dehydroepiandrosterone (3β-17-ketosteroids) (Fig. 6).

The adrenogenital syndrome (Fig. 7) which causes masculinization of the female or precocious puberty in boys can be summarized as follows. (1) Increased concentration of ACTH produces increased quantities of both C_{19} and C_{21} steroids. (2) Although the quantity of C_{21} steroids produced by the adrenal is increased, the concentration of cortisol and corticosterone remains relatively low. Since progesterone (determined by urinary pregnane-3α, 20α-diol), 17-hydroxylated steroids (determined by pregnane-3α, 17α, 20α-triol), and 11-hydroxylated steroids (determined by 11-oxygenated androsterone derivatives) are increased whereas cortisol and corticosterone are not proportionately increased, it is suggested that

the 21-hydroxylating enzyme system (21-hydroxylase) is relatively decreased. (3) Insufficient cortisol is available to control the synthesis and/or release of ACTH leading to excessive adrenal stimulation for androgen production and in turn to the characteristic masculinizing symptoms.

The extent of the lack of the 21-hydroxylase system determines the type and extent of the clinical symptoms. If the lack of 21β-hydroxylase is relatively small, enough cortisol can be produced to maintain the individual against stress but excess androgens is evident. If 21-hydroxylation is severely impaired excessive androgens are present together with signs and symptoms of adrenal insufficiency, the latter being due to critical reduction in cortisol concentrations.

The important discovery by Wilkins and his coworkers[13] that cortisol or cortisone reverses the symptoms of the adrenogenital syndrome is a direct treatment. The administration of exogenous hormone, in effect, replaces the relative lack of cortisol (due to insufficient 21-hydroxylase) and leads to adequate suppression of the anterior pituitary with respect to production and/or release of ACTH. This in turn is reflected in a lowered androgen production and a reversal of the diseased state.

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The Metabolic Fate of "Internally" and "Externally" Labelled Protein Antigens

By Felix Haurowitz,* Wallace Friedberg,† Harry Walter* and Mutahhar Yenson,‡ USA

The metabolic fate of isotopically labelled antigens was investigated in order to get more insight into the mechanism of antibody formation. In 1930, Breinl and Haurowitz[1] advanced a theory according to which the antigen interferes with the normal process of globulin formation; the antigen, according to this view, acts as a template, and causes the formation of globulin molecules which are adjusted in space to the determinant group of the antigen molecule. Similar theories were proposed by Mudd,[2] Alexander[3] and, later, by Pauling.[4] According to these theories antibody formation can take place only in the presence of antigen. It seems that the antigen does not interfere with the first phase of protein synthesis, with the formation of the peptide chain from amino acids, but with the second phase in which the peptide chain of the globulin molecule folds to form a molecule of globular or ellipsoidal shape.[5] Burnett and Fenner[6] raised the objection that antigens, and particularly antigenic proteins, cannot persist in the animal body more than a very short period of time because they would be degraded by the ubiquitous proteolytic enzymes. These authors advance, therefore, another theory according to which the antigen molecule causes the adaptive formation of antibody producing enzymes which form antibodies after the elimination of all of the antigen from the organism.

In order to get more information on the persistence of injected antigens and, thereby, on the mechanism of antibody formation, we use three types of isotopically labelled protein antigens: (I) heavily substituted proteins which contain a radioactive isotope in their determinant group; such proteins are either iodoproteins containing about 8–10% of iodine and traces of I^{131} [7] or azoproteins produced by coupling ovalbumin or serum proteins with large amounts of diazotized S^{35}-sulfanilic acid or C^{14}-anthranilic acid;[8,9] (II) proteins labelled by traces only of I^{131} or S^{35}-sulfanilic acid;[10] (III) proteins containing S^{35}-amino acids in their molecules;[12] these "internally" labelled proteins are produced by injecting guinea pigs, rabbits, or chickens with the hydrolyzate of the protein of yeast which had been raised on S^{35}-sulfate. Some of these "internally" labelled S^{35}-proteins were also labelled "externally" by coupling them *in vitro* with traces of I^{131}. The doubly labelled protein antigens allow us to differentiate between the metabolic fates of the external label and of the protein portion of the molecule.

In our first investigations[7,9] it was found that iodoproteins and azoproteins containing more than about 20 iodinated groups or azogroups per protein molecule, disappear very rapidly from the circulation and are deposited in the organs of the reticuloendothelial system (liver, spleen, bone marrow), where the protein-bound radioactivity persists for many months. If liver or spleen are homogenized and fractionated by differential centrifugation, most of the antigen is found in the large cytoplasmic granules (mitochondrial fraction). This was confirmed[11] by autoradiography of the liver and spleen. Most of the radioactivity of injected S^{35}-sulfanil-azoprotein was found in the cytoplasm of the Kupffer cells of the liver. In agreement with similar findings of Ingraham,[8] the activity in the spleen was localized in the red pulp. Autoradiography of the pellet of large cytoplasmic liver granules showed that the radioactivity was localized in the bottom layer, close to a zone which contained flat nuclei, most probably those of the Kupffer cells; the upper portions of the "mitochondrial" pellet were free of radioactive material, in agreement with similar observations of Gitlin.[13]

In experiments with I^{131}-iodoproteins, the protein-bound radioactivity indicates the fate of the determinant iodinated tyrosine or histidine molecules of the injected antigen. When S^{35}-azoproteins were used, the possibility had to be considered that S^{35}-atoms are incorporated into cystine or methionine. Investigation of the liver proteins revealed, however, that most of the radioactivity was present as a sulfonic acid derivative and that the amino acids of the liver proteins contained only traces[14] of S^{35}. The amount of radioactivity retained in the liver and spleen proteins 5 to 7 months after injection of the antigen was small but significant. It corresponded to approximately 100–1000 antigen molecules per liver or spleen cell. It seems, therefore, that the serologically determinant groups of iodo- and azoproteins can persist in the reticuloendothelial cells for periods of many months, probably for years.

The deposition of antigen in the cytoplasmic granules of liver and spleen is not prevented by irradia-

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tion of rats with X-ray doses of 400–800 r.[15] The subcellular distribution of the antigen in liver and spleen of irradiated rats is the same as that in normal rats.

In contrast to the heavily substituted iodo- and azoproteins, proteins labelled by traces only of I^{131} or S^{35}-sulfanilic acid, were not eliminated from the circulation, but remained in the blood for 4-5 days until the onset of antibody formation. This period of circulation was followed by a period of clearance in which most of the antigen, by combination with antibody, was eliminated from the blood and was carried down in the liver, spleen, bone marrow and lung.[10] During the following period of deposition, the radioactivity was found chiefly in the large cytoplasmic granules, where it persisted for periods of several months. When beef serum gamma-globulin labelled by traces of S^{35}-sulfanilic acid was injected into the foot pad of rabbits, most of the radioactivity was found in the cytoplasm of the macrophages of the popliteal lymph nodes.[11] It is difficult, however, to interpret results obtained with trace-labelled proteins of this type which contain only 1 or 2 labelled groups per molecule. Whereas the labelled group of the heavily substituted protein antigens are at the same time the serologically determinant groups which combine with the antibody molecules, the radioactive groups of the trace-labelled proteins are serologically not determinant. The antigenic determinant part of such a molecule is its protein portion. Obviously, the metabolic fate of the protein is not necessarily the same as that of the radioactive label.

In order to get more information on the protein portion of the antigen, we prepared "internally" labelled protein antigens, containing S^{35}-cystine and S^{35}-methionine. They are differentiated from the externally labelled S^{35}-proteins, which contain diazotized S^{35}-sulfanilic acid, by the designation (S^{35})-protein, the parentheses indicating the presence of an "internal" label in the protein portion. Some of these internally labelled proteins were labelled "externally" by coupling them *in vitro* with I^{131} or with diazotized C^{14}-anthranilic acid. If such (S^{35})-I^{131}-guinea-pig serum albumin is injected into rabbits, it circulates in the blood without any change in the S^{35}/I^{131} ratio. However, if this doubly labelled protein is injected into the foot pad of rabbits, the ratio S^{35}/I^{131} in the regional lymph node, 3 days after injection, is 6 times larger than in the injected material, and 9 days after injection about 25 times larger. Similarly, the S^{35}/I^{131} ratio in the liver of rats injected intravenously with (S^{35})-I^{131}-guinea-pig serum albumin, nine days after injection, is 50-100 times larger than in the injected material at the same time.[12,16] In similar experiments in which (S^{35})-protein antigens were coupled with diazotized C^{14}-anthranilic acid, the ratio S^{35}/C^{14} changed very little. We conclude, therefore, that the large increase in the ratio S^{35}/I^{131} is due to de-iodination in liver, lymph node and probably also in other organs. If the increase in the ratio S^{35}/I^{131} were caused by incorporation of S^{35}-amino acids into the liver or lymph node proteins, one would expect a similar increase after the injection of the $(S^{35})C^{14}$-proteins. Since this is not observed, we assume that iodinated proteins are de-iodinated in the tissues and that de-iodinated (S^{35})-protein antigens are deposited in the tissues. The amount of S^{35} found in the liver proteins is very considerable. Nine days after injection, about 2-3% of the injected S^{35}, 45 days after injection about 0.6%, was found in the liver proteins.

Our experiments show clearly that protein antigens persist in rabbits and rats for very long periods of time; it is not necessary, therefore, to invoke the adaptive formation of antibody forming enzymes.[6] The persistence of antibody formation is, evidently, due to persistence of the antigen. It is not yet clear, however, why the injected antigen is not rapidly degraded by the proteolytic enzymes of the cells. It seems to penetrate very rapidly into the reticuloendothelial cells, probably by means of a process akin to phagocytosis. In the cell, the antigen seems to be present in an inactive form; for we are not able to prove the presence of nonradioactive antigens by adding radioactive antibody to liver or spleen homogenates of the injected animals.[17] It seems that the antigen is masked by combination with cellular constituents such as nucleic acids, polysaccharides, or lipids.

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Certain Chemical and Biochemical Investigations with the Help of Radioactive Isotopes at the University Institute of Chemistry, Lahore, Pakistan

By Bashir Ahmad, Pakistan

A series of investigations, started in 1949 at the University Institute of Chemistry, Lahore, has continued, as isotopes have become available, until today. The isotopes were first obtained from the Atomic Energy Commission, Oak Ridge, through the usual channels. These studies are being continued. The following is a brief account of the work so far done.

STUDIES ON METABOLISM OF IRON AND THE REGENERATION OF BLOOD IN ANAEMIC ANIMALS

A sensitive method for the estimation of iron in biological materials has been worked out and compared with other methods available for the purpose. The adsorption of radioiron, its distribution in various tissues of normal and anaemic animals and its excretion was examined. The regeneration of blood pigment in animals showing different degrees of anaemia was studied, as also the effect of feeding iron in different forms and under different conditions. The utilization of iron from biosynthesized radiohaemoglobin was also studied. While standardising the method for the estimation of radioactive iron in biological materials certain modifications were introduced which gave more accurate and reproducible results.

STUDIES ON PHYTIN

These studies were directed towards investigating the biological availability of calcium and phosphorus of phytin and the conditions under which both these minerals could be utilized and, further, to determine the biological significance of phytin itself. Radioactive phytin containing radioactive P which has a short half-life was found difficult to synthesize. Radioactive calcium phytate was synthesized containing Ca^{45} and was used in these studies. Labelled calcium phytate could best be prepared by the interaction of sodium phytate and radioactive calcium chloride. For this reaction, the best conditions of precipitation were the addition of 3.3 equivalents of sodium phytate to the solution of radioactive calcium chloride. Ca^{45} in the phytate was estimated both by precipitation as calcium oxalate and as calcium by the precipitate counting technique.

Exchange of calcium of the active phytate with other inorganic salts of calcium was studied in both *in vitro* and *in vivo* experiments. *In vitro* it was found that 29.2% of the phytate calcium is exchanged with other inorganic salts of calcium. The *in vivo* experiments were somewhat inconclusive. It was evident that appearance of active calcium in bone and other body fluids was no measure of the hydrolysis and utilization of the phytate.

INVESTIGATIONS OF IRON BLUES

A molecule of Prussian blue, which is a well known pigment used both in laundering and in the paint industry contains two atoms of iron in different valency states. The compound obtained from ferric iron and ferrocyanide is called Prussian blue while that obtained from ferrous salt and ferricyanide is called the Turnbull's blue. X-ray diffraction studies prove both of them to be identical and show that the two atoms of iron occupy equivalent positions. However, there are certain reactions where it is not clear which of the two atoms is involved. Sodium hydroxide converts Prussian blue to ferric hydroxide and sodium ferrocyanide, whereas ammonium carbonate forms ferricyanide.

To study this anomalous behaviour, Prussian and Turnbull's blue, with inner and outer iron tagged, were obtained by direct precipitation from: (*a*) active potassium ferrocyanide and inactive ferric chloride, (*b*) active ferric chloride and inactive potassium ferrocyanide, (*c*) active potassium ferricyanide and inactive ferrous chloride.

The active preparations obtained as above were decomposed with potassium hydroxide, ammonium carbonate and potassium carbonate. It was observed that whether the reaction product was in ferrous or ferric state, the outer iron was precipitated.

Further, iron exchange experiments with active preparations and solutions of inactive iron salts were carried out. The activity appeared in the solution only with those preparations of Prussian and Turnbull's blue in which the outer iron was active. Although X-ray diffraction does not differentiate between the two atoms, yet the outer one only is exchangeable.

Further studies in this field are concerned with the specific area of Prussian blues prepared under different condition of temperature and concentration. The surface area is of importance in the paint

industry as it determines the covering power of the pigment.

STUDIES ON THE METABOLISM OF SILVER AND GOLD

Both these precious metals have held a prominent place in indigenous medicine since time immemorial. Local hakims still use preparations of these metals on an extensive scale in various ailments, and as tonics. Quite appreciable amounts of these metals are also used in the form of leaf for decorating foodstuffs particularly sweets. These facts lend importance to the investigation of the metabolism of these elements.

Both metals fed to animals in the form of leaf and colloidal sols of the metal, as well as their oxides, are found to be almost wholly excreted without any absorption. The same is true of indigenous medicinal preparations known as "kushtas." The earlier studies were carried out with non-radioactive materials.

Since there is a possibility that the action of these elements is more likely to be of a catalytic nature, investigations have now been planned with radio-isotopes of Ag and Au. The results of such studies are not yet available.

BIOSYNTHESIS OF RADIOACTIVE CAROTINOIDS AND STUDY OF CAROTINOID METABOLISM

Studies have been undertaken on the biosynthesis of various carotinoids and their isomers using algae and diatoms as synthesizing organisms. The organisms were grown by the submerged culture technique, using radioactive carbon dioxide for aeration of the cultures. This was a part of the programme of the study of carotinoid metabolism which has been pursued in this laboratory for the last 10 years. So far no results of any great significance have been obtained.

STUDIES WITH TAGGED FATTY ACIDS

Work on the tagging of fatty acids with carbon is under progress. The scheme adopted for tagging of carboxylic group of linolenic acid is as follows:

Linolenic acid is obtained as hexabromide from linseed oil by crystallization from petroleum ether. The hexabromide of linolenic acid is converted into its silver salt. The reported methods for this conversion, using alcohol as the medium, failed. Therefore, pyridine solutions of the acid and the silver nitrate were mixed; which method worked nicely. The silver salt in an anhydrous state was reacted with bromine. The bromine atom replaced the carboxylic group giving a polybromide. The polybromide thus obtained could be treated with zinc dust in alcohol, which is most likely to remove the bromine atom except one at the end of the chain. The monobromide through Grignard's reagent is expected to give the linolenic acid through treatment with radioactive carbon dioxide.

Record of Proceedings of Session 16C

TUESDAY AFTERNOON, 16 AUGUST 1955

Chairman: Mr. H. H. Ussing (Denmark)
Vice-Chairman: Mr. B. Ahmad (Pakistan)
Scientific Secretaries: Messrs. E. O. Hughes and I. D. Rojanski

PROGRAMME

P/456 Some recent applications of tritium in biological research ..... R. F. Glascock

P/710 The use of radioactive isotopes in the study of functional biochemistry of the brain ..... A. V. Palladin and G. E. Vladimirov

Discussion

P/262 Use of isotopes in the study of enzyme mechanisms ..... D. E. Koshland, Jr.

P/182 The role of radioactive isotopes in immunologic investigation including recent studies on the rates of antibody synthesis ..... F. J. Dixon

Discussion

P/776 Application of C^{14}-labeled substances in the study of adipose tissue metabolism ..... B. Shapiro and G. Rose

P/377 The utilization of the sulfites by the higher animals ..... P. Fromageot and F. Chapeville

P/686 Investigation of the incorporation of amino acids into proteins *in vivo* and *in vitro* ..... V. N. Orekhovich

Discussion

P/260 Pathways of biosynthesis of nucleic acids ..... G. B. Brown

P/457 Studies on the incorporation of radioactive precursors into the nucleic acids and related compounds in living cells ..... R. M. S. Smellie and J. N. Davidson

Discussion

The Chairman: This session will conclude the series on radioactive tracers in both physiology and biochemistry.

The application of isotopic tracers in biological work which has grown out of Professor Hevesy's brilliant work has now become as natural a tool in the hands of biochemists and biologists as the microscope is in the hands of the histologist.

This attitude has been established in less than 20 years. In order to illustrate the change of attitude brought about by the use of tracers, may I mention an incident which took place in 1935. When I was a newly-hatched physiologist I became associated with Professor Hevesy and the late Professor Krogh in connection with some studies of permeability in muscles. These muscles were dried and burned and the combustion water was investigated as to the contents of heavy water, and it turned out that hydrogen had gone into the proteins. Professor Krogh became very excited about this and the implication of this observation, and he went to our greatest authority on protein chemistry, Professor S. P. L. Sorensen, and informed him that the hydrogen atoms were entering and leaving the proteins *in vivo*.

Sorensen simply refused to believe him. I am mentioning this not to deduct from the greatness of Professor Sorensen whose merits in the field of protein chemistry are beyond criticism. Actually our experiments were crude and might have been in error.

The example merely is used to exemplify the revolution in biochemical thinking which has taken place in the meantime.

Today nobody is in doubt that not only hydrogen ions but all kinds of atoms are going in and out of proteins. The afternoon session will bring a series of brilliant contributions to the already long series which was originated by the work of Professor Hevesy.

Mr. R. F. Glascock (UK) presented paper P/456.

Mr. A. V. Palladin (Ukrainian SSR) presented paper P/710.

DISCUSSION OF P/456 AND P/710

Mr. G. B. BROWN (USA): Could the asymmetry of the tritium in the oleic acid possibly be due to biological synthesis of the oleic from shorter carbon chains derived from the original labelled stearic acid?

Mr. GLASCOCK (UK): No, sir, I do not think so, because the specific activity of the short-chain acids as exemplified by those found in milk was extremely low, something of the order of 200 counts per minute per milligram, compared with 700 to 1000 counts for the long-chain milk fatty acid.

Mr. W. H. SWEET (USA): I should like not only to congratulate Mr. Palladin on this very comprehensive survey but to ask him if he could answer this question.

Are the differences in rates of renewal of proteins, ribo-nucleic acid and phospholipids due to a change in the amounts of specific enzymes present or is there some other explanations?

Mr. PALLADIN (Ukrainian SSR): It would appear from the various effects of stimulation and inhibition that we have observed—in some cases a rise in the renewal rate and in others a drop—that, in these states, conditions arise in the brain in which changes in the metabolic processes occur. It is difficult to say whether there is any change in the quantity of enzymes present. No difference was apparent in a number of cases. The reason may well vary from case to case: sometimes the activity of the enzymes changes and sometimes enzyme systems which normally play no part in the metabolism have a chance to operate.

The CHAIRMAN: We have certain requests from non-delegates, and according to rules non-delegates are unfortunately not allowed to take the floor. But perhaps, as we have a few minutes, I may be allowed to read a question:

"Dr. Glascock's evaluation of the efficiency of detection of tritium beta particles with the liquid scintillator technique is a little pessimistic. Dr. Hayes from Los Alamos has reported 25 per cent, and we achieved something like 30 per cent."

Perhaps Mr. Glascock would like to comment on that.

Mr. GLASCOCK (UK): Since preparing the script, on the basis of which that last comment was made, I have been collaborating with some friends at the Isotope Development Company in England who also report that they are now using a liquid scintillator to get much higher efficiencies than I quoted in my paper, and something of the order of that quoted by the questioner. Therefore, I feel I should perhaps apologize if the statement made in my paper was misleading.

Mr. D. E. KOSHLAND, Jr. (USA) presented paper P/262.

Mr. F. J. DIXON (USA) presented paper P/182.

DISCUSSION OF P/262 AND P/182

Mr. J. COURSAGET (France): Mr. Koshland used oxygen-18 in his work on the use of isotopes in the study of enzyme mechanisms, measuring the oxygen by mass spectrometry. I should like to ask him whether this work was not complicated by exchange phenomena and whether there were any significant memory effects.

Mr. KOSHLAND (USA): Could I ask whether you are referring to the second method or to first?

Mr. COURSAGET (France): I am referring to the first method.

Mr. KOSHLAND (USA): There was no trouble at all. What we did was distill carbon dioxide away from the water. After the equilibration it was left for two days with the carbon dioxide in water and then, by putting a dry ice trap around the system and distilling out the carbon dioxide, we had no water in the system and therefore we had no memory effects. If you get any water in the mass spectrometer, you get memory effects and these have to be recorded. There are no exchange reactions in the glass or anything else in the mass spectrometer.

Mr. COURSAGET (France): Mr. Dixon has given us an admirable account of the rate of incorporation of amino acids into antibodies and has shown that the rate of incorporation is a function of time. I should like to ask Mr. Dixon whether he has made a similar study of the rate of degradation of antibodies and whether it too varies with time.

Mr. DIXON (USA): As far as we have been able to discover the only thing that affects the degradation of antibody is the presence of antigens. In the presence of antigens most of the circulating antibodies are rapidly degraded—they are incorporated, taken out of the bloodstream and degraded. In the absence of antigens, or a significant mass of antigens, we have detected no difference in the rates of antibody catabolism.

Mr. TROITSKY (USSR): I think that both Mr. Dixon's paper and his personal observations have been a source of satisfaction to everyone.

I should like to ask whether Mr. Dixon has any information on the fate of labelled complete antigens of *Shigella* and *Salmonella* introduced into an organism. We have done a great deal of work on this question, and I would be very glad to know whether Mr. Dixon is working along these lines and, if so, what label he is using.

Mr. DIXON (USA): We have not been working with any of these fractions, and I am not acquainted with anyone who is doing work with labelled bacillary antigens other than perhaps the pneumococcal or some of the other polysaccharides derived from bacteria.

Mr. B. AHMAD, Vice Chairman, took the Chair.

Mr. E. D. BERGMAN (Israel) presented paper P/776.

Mr. P. Fromageot (France) presented paper P/377.

Mr. A. M. Kuzin (USSR) presented paper P/686.

DISCUSSION OF P/776, P/377 AND P/686

Mr. B. Hastings (USA): My first question is for Mr. Bergman. I was much interested in this fine work on the metabolism of adipose tissue. I would like to ask whether or not the authors have carried out any studies of the effect of hormones—insulin or adrenal hormones—on the metabolic reaction they have been studying, and also if they have any information, about whether changing ionic environment, particularly potassium—since that is my hobby—has any effect on the metabolism of the adipose tissues.

Mr. Bergman (Israel): As I have said, I have not carried out this work myself but am just reporting it. However, I know that no work has so far been done on the influence of hormone preparations although Mr. Shapiro is studying this aspect. Neither has anything been done in connection with Mr. Hastings' second question.

Mr. Hastings (USA): I apologize for getting up so often, but again I was very interested in Mr. Fromageot's paper and wonder if he thinks that the fixation of SO_2 is brought about by the same enzymes that fix CO_2, or are they a whole new set of different enzymes?

Mr. Fromageot (France): We have no precise experience on this point, but I feel that the analogy on which this hypothesis was based is entirely academic and merely served as a starting point for the work. I do not think, however—and various experiments bear me out in this—that these two enzymes are identical; on the contrary, they appear to be different.

Mr. M. Z. Bacq (Belgium): I should like to ask Mr. Fromageot a question. Taurin in the free state is also eliminated by the urine of mammals. The origin of the taurin in the urine is probably not the same as that of the taurin in the bile. If we could compare the specific activity of the two types of taurin—that in the urine and that in the bile—we might be able to compare the decarboxylating action of the liver and the kidneys. Do you agree?

Mr. Fromageot (France): You are quite right. I have not carried out any experiments myself to compare the two types of radioactivity, but other laboratories, such as Châtaigner's, are studying the decarboxylating action of various organs, such as the kidney or liver, of different animals with a view to finding out more about these questions of kinetics.

Mr. Koshland (USA): I would like to ask Mr. Kuzin whether Mr. Orekhovich made any studies to get a quantitative relationship of the amount of renewal as compared with the amount of over-all synthesis in metabolizing tissues? Have any experiments of this sort been carried out?

Mr. Kuzin (USSR): I understand that Mr. Orekhovich, on whose behalf I presented this paper, is developing the idea that there is no renewal in tissues where the synthesis of protein molecules is impossible. In the experiments described in his paper he is trying to show that when the possibility of synthesis is excluded no renewal of the protein molecules is apparent. Where synthesis does take place—possibly balanced by simultaneous processes of degradation in cases where both processes are permissible—what is generally known as renewal of the protein molecules can be observed.

That is the opinion of Professor Orekhovich whose report it gave me great pleasure to present.

Mr. G. B. Brown (USA) presented paper P/260.

Mr. R. M. S. Smellie (UK) presented paper P/457.

DISCUSSION OF P/260 AND P/457

Mr. Brown (USA): I would like to ask Mr. Smellie where he now stands on the long debate as to whether nuclear RNA was a regular standard precursor of cytoplasmic RNA?

Mr. Smellie (UK): There is little evidence in the work which I have just described to say that nuclear RNA is not the precursor of cytoplasmic RNA. On the other hand, there is little evidence for saying it is. However, some of the other work which we have been doing in the last year or two and which I have not reported today—work involving studies on isotope incorporation in homogenates and in isolated nuclei and in cytoplasmic material from which the nuclei had been removed—does suggest that the cytoplasm is entirely capable of synthesising RNA on its own. Moreover, the nuclei are also apparently capable of synthesising the RNA. There may be some interaction between the cell nucleus and the cell cytoplasm but my own feeling is that the two entities are themselves capable of ribo-nucleic acid synthesis.

Mr. Bergman (Israel): I have one brief comment on Mr. Brown's very interesting paper. There is one substance which is the precursor of the purines, amino-imidazole-carboxamide, which lacks only one carbon atom for the completion of the purine ring and which gets this atom from methionine. This substance can only complete the ring if it is in the form of a riboside or ribotide. Furthermore, it is known to give a transpurination reaction. Therefore, and for the reason that this completion of the ring is reversible, it might not be an uninteresting substance to consider in the scheme of biological changes in the ribo- and desoxyribo-nucleic acids and it might offer some interesting possibilities for an explanation of a number of biological features.

Mr. Brown (USA): I agree entirely that it would be very nice to have some metabolic tests made

on the rat. You mention that amino-imidazole-carboxamide undergoes a transpurination. What is the evidence you refer to for that?

Mr. BERGMAN (Israel): The carboxamide exchanges ribose with any of the purine ribosides. I don't think it has been tried with pyrimidine ribosides yet. One can transfer the ribose to the carboxamide, and probably also in the reverse direction.

Mr. BROWN (USA): I should like to see some evidence that amino-imidazole-carboxamide attached to a ribose will undergo a transpurination to a new ribose *in vivo* before I would include it in the type of transpurination I am trying to refer to here.

Mr. BERGMAN (Israel): This could only be done if you had labelled amino-imidazole-carboxamide riboside, but I think this could be made biologically from labelled carboxamide and any of the purine ribosides.

Mr. BROWN (USA): It is hard to make. I can say we have tried.

Mr. A. R. GOPAL-AYENGAR (India): I should like to ask Mr. Brown whether the very attractive scheme of the biosynthesis of nucleic acids that he has postulated for the rat could be applied with equal validity to other biological systems like the actively growing plant meristematic systems and undifferentiated tissues?

Mr. BROWN (USA): I have only tried with the rat because it is only the rat that for many years has been studied with purine, adenine and guanine. I once had a table of eleven species; they included mice, rats and hamsters. Now, we are adding man to it. The relative utilization of adenine or guanine and the relative extent of transpurination from either one into the other—guanine into adenine or adenine into guanine—varies greatly from species to species. You can go all the way from a system in which adenine goes to adenine and also to guanine, and guanine is hardly utilized, until guanine gives in and goes to adenine, and adenine, if used at all, goes only to adenine. However, I would hate to extrapolate on the whole table from the rat.

Mr. SMELLIE (UK): I wonder if I might ask whether you think there is any possibility of one of the carbon atoms of a purine ring in a polynuclear type unit undergoing an exchange, say for instance, the C_2 carbon of adenine, while still being firmly attached to the polynuclear type?

Mr. BROWN (USA): Some work with uniformly labelled biosynthetic materials has also indicated parallelism of isotopes, and that gives a little evidence for the two carbons. A couple of other laboratories—not ours—have looked for such an exchange and have not found it, but I would not exclude a small amount.

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