SCIENCE

VOL. 87 FRIDAY, MARCH 11, 1938 No. 2254

SCIENCE: A Weekly Journal devoted to the Advancement of Science, edited by J. MCKEEN CATTELL and published every Friday by

THE SCIENCE PRESS

New York City: Grand Central Terminal

Lancaster, Pa. Garrison, N. Y.

Annual Subscription, $6.00 Single Copies, 15 Cts.

SCIENCE is the official organ of the American Association for the Advancement of Science. Information regarding membership in the Association may be secured from the office of the permanent secretary, in the Smithsonian Institution Building, Washington, D. C.

THE APPLICATION OF ISOTOPES TO THE STUDY OF INTERMEDIARY METABOLISM[1]

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THE number of organic compounds involved simultaneously in the multitude of diverse chemical reactions in the living organism is exceedingly large. Substances are continually being degraded, while their split products are linked together again to form new compounds, and all these reactions and their reaction products are held in equilibrium so that the composition of the cell and the organism stays constantly within narrow limits.

By adding to this complicated system an excess of one of the components, it is in many cases possible to follow its conversions, provided one has some general idea as to its fate. Herein lies the principle of the classical balance experiments. In order to determine the fate of one of the constituents of the organism, the substance is given in large quantities and the tissues or excreta are investigated for the presence of related compounds in abnormal amounts. This method has proved to be extremely valuable, and most of our knowledge of intermediary metabolism is based on experimentation of this kind. Unfortunately this method has theoretical limitations. Many body constituents, especially the more active ones, are never produced in excess of the animal's requirements, even if the building material is available in large quantities. Another obstacle which sometimes makes the interpretation of such balance studies extremely difficult is the known fact that one substance may induce the formation of others without itself being involved in the synthesis. Thus, for instance, insulin induces glycogen formation, and fat induces an increased for-

[1] This article contains the material presented in two papers at the meeting of the American Chemical Society at Rochester, N. Y., September, 1937.

mation of cholesterol, without taking direct part in the transformations. This weakness of biological balance studies has aptly been illustrated by comparison with the working of a slot machine. A penny brings forth one package of chewing gum; two pennies bring forth two. Interpreted according to the reasoning of balance physiology, the first observation is an indication of the conversion of copper into gum; the second constitutes proof.

Several attempts have already been made to overcome the difficulties by labelling the compounds to be given with an easily detectable chemical group. A method based on this principle may give direct evidence, more convincing than the circumstantial evidence of the balance experiments. For example, hydrogen of fatty acids has been replaced by halogens and the determination of organic bound halogen used for the study of the transportation of fatty acids in the animal organism. Halogenated fatty acids, however, are so different in their physical and chemical behavior from the non-halogenated ones that the highly sensitive cell does not treat them alike. In order successfully to label an organic compound for metabolism studies, the resulting compound has to be of such a nature that it is treated by the tissues in exactly the same way as the normal analogue. On the other hand, the chemist must be able not only to detect the label, but to determine it in small quantities and in high dilutions. For obvious reasons the only type of label which will fulfil these requirements is to be found in the isotopes of the elements which constitute organic matter. Organic compounds, for instance, which have hydrogen or nitrogen atoms replaced by the corresponding isotopes have practically the same properties as the normal substances.

The separation of the isotopes of the same element is extremely difficult, and only in a few instances have such attempts been successful. Of the various elements, the concentration of the isotopes of hydrogen is the most easily accomplished in the laboratory, but the living cell is unable to perform this operation to appreciable extent. Wherever hydrogen occurs in nature, the concentration of deuterium in the total hydrogen is practically uniform (0.02 per cent.), independent of the source (sea water, lake water, biological fluids, etc.). The same concentration of deuterium as is found in water is also found in the hydrogen of organic material,[2] which is ultimately synthesized by living cells from carbon dioxide and water. The even distribution of deuterium in water and organic matter may be taken as proof that the cell does not discriminate against either form of hydrogen.

The first to realize the importance of isotopes for biological work was von Hevesy, who applied them to the investigation of the metabolism of inorganic material as early as 1926.[3] He investigated the fate of lead in the organism by adding to it a minute amount of one of its radio-active isotopes, Ra D, which can not be separated from lead by physical or chemical means. The radio-activity of the ash of organs indicates the distribution of lead in the animal after its administration. Recently he has investigated the transportation of phosphoric acid in higher plants,[4] yeast[5] and animals[6] by giving these organisms phosphoric acid containing radio-active phosphorus. His finding that ingested phosphate is distributed in animals not only throughout all the organs but also in the bones and teeth, indicates that even these structural elements are continuously and actively involved in salt metabolism. By using the same material[7] the uptake of phosphoric acid into phospholipids was studied.

The application of isotopes to the study of intermediate metabolism involves methods which are different in principle from those methods used for the study of salt metabolism. No radio-active isotopes of the elements C, H, N or O are known with a half-life long enough to permit their use for organic syntheses and metabolism experiments. One is limited to the use of stable isotopes which have been concentrated from natural mixtures. By devising methods for such fractionation, H. C. Urey has made possible a new approach to biological problems.

The study of the metabolism of organic compounds with the aid of stable isotopes involves the preparation of organic compounds in which one or more atoms are replaced by their respective isotopes. The only isotopes which have been used so far are those of hydrogen and nitrogen. As by far the most experience has been had with deuterium, we should like to discuss its application first.

In "labelling" an organic compound with deuterium, the isotope must be introduced in such a way that it will not be lost from the molecule during the course of metabolic reactions.[8] In other words, the deuter-

[2] F. Breusch and E. Hofer, *Klin. Woch.*, 13: 1815, 1934; M. Dole, *Jour. Am. Chem. Soc.*, 58: 580, 1936; M. Dole and R. B. Gibney, *Jour. Am. Chem. Soc.*, 58: 2552, 1936.

[3] G. Hevesy, *Biochem. Zeits.*, 173: 175, 1926.

[4] G. Hevesy, K. Linderstrom-Lang and C. Olsen, *Nature*, 139: 149, 1937.

[5] G. Hevesy, K. Linderstrom-Lang and N. Nielsen, *Nature*, 140: 725, 1937.

[6] O. Chievitz and G. Hevesy, *Nature*, 136: 754, 1935.

[7] G. Hevesy and E. Lundsgaard, *Nature*, 140: 275, 1937; L. Hahn and G. Hevesy, *Skand. Arch. Physiol.*, 77: 148, 1937; C. Artom, G. Sarzana, C. Perrier, M. Santangelo and E. Segre, *Nature*, 139: 836, 1937.

[8] R. Schoenheimer and D. Rittenberg, *Jour. Biol. Chem.*, 111: 163, 1935.

ium atoms should be stably bound to the carbon skeleton. From our knowledge of the reactions of organic compounds, it is in general possible to predict which of the hydrogen atoms will exchange with the hydrogen of the water in which they are dissolved. The use of deuterium has confirmed the prediction that the hydrogen atoms in carboxyl, hydroxyl, amino and other polar groups are labile and exchangeable with the hydrogen atoms of water. Since biological reactions proceed in an aqueous phase, all substances which contain deuterium in such labile positions must lose it by exchange as soon as they are dissolved in the body fluids. However, hydrogen bound directly to carbon, as in methyl or methylene groups, is, in general, stable and can not be removed by exchange with water, even under such drastic conditions as treatment with hot acid or alkali.[9] So far there has been no indication that such carbon bound hydrogen as is found to be stable *in vitro* is labile in the animal. There is good reason to believe that, if such stable deuterium is removed *in vivo* from the carbon to which it is attached, the removal is due to a biochemical reaction rather than to a simple physical exchange. This point will be discussed later.

There are many methods for the preparation of compounds containing carbon bound deuterium, the most convenient of which is the hydrogenation of double bonds with deuterium.[10] Crotonic acid when treated in this way will yield butyric acid, and oleic acid will yield stearic acid, both reaction products containing stably bound deuterium at predetermined positions in the molecule. Two other methods are based on the fact that stable hydrogen atoms of ordinary compounds may under certain exceptional conditions become labile and exchangeable with deuterium atoms of other substances. Thus many compounds, when treated with concentrated deutero-sulfuric acid (D_2SO_4) at high temperature, exchange otherwise non-exchangeable hydrogen with the deuterium of the sulfuric acid.[11] Moreover, some compounds exchange carbon bound hydrogen with the deuterium of heavy water when treated at high temperature with a suitable catalyst,[12] and fatty acids prepared by this method[13] seem to be highly valuable for metabolism studies. Another process, which will be mentioned later, is that of biological synthesis.

A prerequisite of the deuterium technique is the quantitative determination of deuterium in organic compounds. The analysis[10] is based on the fact that organic matter, when burned, yields its hydrogen in the form of water, and if deuterium is present in it this will form an equivalent of heavy water. The analysis of deuterium in organic compounds is thus in principle the determination of heavy water in the water of combustion. The methods of heavy water analysis which have been developed in different laboratories in recent years are so sensitive that a concentration as low as 0.02 per cent. can be accurately and routinely determined. Because of this the biological application of the deuterium technique is extremely sensitive. If an animal were fed a biological compound such as stearic acid in which 5 per cent. of all its hydrogen atoms are replaced by deuterium, the compound[14] could be diluted with more than 1,000 times its weight of ordinary stearic acid or an equivalent amount of other material before the analytical methods would lose track of it. Thus in general, only a small amount of a deutero-compound need be given to the animals, and even if only a fraction is converted into another substance, the analytical methods will still reveal the conversion. While this general procedure has already been applied to a number of different physiological compounds, we shall discuss, in this paper, only its application to fat metabolism.

The deuterium procedure may be employed for the study of the transport of ingested material, even if given in small quantities. On feeding deutero-fats,[15] it was found that a small part is deposited in the internal organs, such as the liver, while a very large part is deposited in the fat depots prior to its utilization. This holds true even when the amount of fat in the diet is small. Between 20 and 40 per cent. of the labelled fat was invariably recovered from the depots. Cavanagh and Raper,[16] employing the same methods, observed the transport of fatty acids through the phospholipid stage. This is in agreement with the findings of Sinclair on the rôle of phospholipins in fat metabolism.

The deposition of food fatty acids in the fat depots is well in accord with the known fact that the properties of depot fats depend on the quality of the diet.

[9] There are a number of general exceptions, such as when the hydrogen is adjacent to a carbonyl or phenol group, etc., or with some compounds of small molecular weight.

[10] D. Rittenberg and R. Schoenheimer, *Jour. Biol. Chem.*, 111: 169, 1935.

[11] C. K. Ingold, C. G. Raisin and C. L. Wilson, *Jour. Chem. Soc.*, 1643, 1936; R. Schoenheimer, D. Rittenberg and A. S. Keston, *Jour. Am. Chem. Soc.*, 59: 1765, 1937.

[12] C. Horrex and M. Polanyi, Memoirs and Proceedings of Manchester Literary and Philosophical Society, 80: 33, 1935–1936.

[13] Unpublished data of W. E. van Heyningen with the authors.

[14] On combustion, the compound will yield water containing 5 per cent. heavy water. Unless otherwise stated, the deuterium content in substances mentioned in this paper means deuterium which is stably bound in the molecule.

[15] R. Schoenheimer and D. Rittenberg, *Jour. Biol. Chem.*, 111: 175, 1935.

[16] B. Cavanagh and H. S. Raper, *Nature*, 137: 233, 1936.

These changes occur, however, to only a limited extent. All animals have a tendency to reproduce from different diets a depot fat, the composition of which is characteristic for the species. The deposition of the fats in the depots is thus followed by a change in properties. The deuterium method has enabled the physiologist to correlate this assimilation of food fat with the conversion of one kind of fatty acid to another.

The principle underlying the general experimental procedure is the following: If a deutero-compound A is given to an animal, and if subsequently a compound B, also containing deuterium, is isolated from the body, the deuterium content of B may be taken as proof that B was derived from A.

The majority of investigators have postulated the biological desaturation of fatty acids, such as the formation of oleic acid from stearic acid. This hypothesis, however, was far from being generally accepted because definite proof of the reaction was still lacking. Proof was supplied by the deuterium technique, when oleic acid with a high deuterium content was isolated from mice which had been fed with deutero-stearic acid.[17] These desaturation experiments gave no indication as to the locality of the reaction in the organism. Some investigators have suggested that the desaturation takes place in the intestinal wall during absorption of the fatty acid. Work by Dr. Smith Freeman, of Northwestern University, in collaboration with the authors[18] now shows this view to be untenable. Deutero-saturated fatty acids were fed to dogs and the absorbed material isolated from a lymph fistula directly after it had passed the intestinal wall. The deuterium analysis of the unsaturated fatty acids gave no indication of desaturation during absorption. The process must occur in other organs, most probably in the liver.

The question whether the organism is also capable of the reverse process, namely, the saturation of unsaturated fatty acids, was decided in the affirmative.[19] After feeding deutero-oleic acid to mice, deuterium was found in the saturated acids, which could therefore have had no other origin than from the administered material.[20]

Another type of biological conversion of fatty acids which would have been difficult to follow by other methods was that of the degradation of stearic acid into palmitic acid,[21] a process which involves the shortening of the fatty acid chain by two carbon atoms. These experiments, considered as a whole, clearly indicate that the three main constituents of animal fats are interconvertible and show one of the means by which animals reproduce their typical depot fat.

The typical acids of plant and animal fat contain 16 and 18 carbon atoms. As has been mentioned before, a large part of these acids is always recoverable from the fat depots after they have been fed. Eckstein[22] and others have evidence that fatty acids with short chains of 4 and 6 carbon atoms (butyric and caproic acid) are not found in the depots, even when they are fed in considerable quantities. This finding was corroborated by experiments in which the corresponding deutero-acids were fed.[23] Not only were these fatty acids not stored, but the deuterium administered in the acids was found as heavy water in the body fluids shortly (6 hours) after feeding. These results, and those of other experiments on these two acids, clearly indicate that, in contrast to the higher ones, they were rapidly and completely burned and not used for fat formation. The rapid degradation of butyric and caproic acids thus seems to resemble the labile metabolism of the carbohydrates rather than that of the higher fatty acids.[24]

While the field of application of the deuterium technique to conversion processes is very wide, the method has strict limitations. The physiologist, when studying conversions of organic compounds, is interested in the fate of the carbon rather than that of the hydrogen. The metabolic conversion of sugar into fats connotes the utilization of the carbon for the fat formation; the fate of the hydrogen is of secondary interest. The direct application of the deuterium technique is obviously excluded, when the hydrogen or deuterium becomes detached from the carbon during conversion. It is highly improbable that deuterium can be used directly for the solution of the much-labored question of the metabolic sugar formation from fatty acids. While it has been established beyond doubt that fatty acids with an odd number of carbon atoms form sugar, there is no proof that this is true for the natural fatty acids with an even number of carbon atoms.

[17] R. Schoenheimer and D. Rittenberg, *Jour. Biol. Chem.*, 113: 505, 1936.

[18] Unpublished data.

[19] D. Rittenberg and R. Schoenheimer, *Jour. Biol. Chem.*, 117: 485, 1937.

[20] At the time of the experiment no method existed for the laboratory preparation of deutero-oleic acid. This substance was, therefore, prepared biologically by feeding mice deutero-stearic acid and isolating deutero-oleic acid from the carcasses.

[21] R. Schoenheimer and D. Rittenberg, *Jour. Biol. Chem.*, 120: 155, 1937.

[22] H. C. Eckstein, *Jour. Biol. Chem.*, 81: 613, 1929; 84: 353, 1929.

[23] D. Rittenberg, R. Schoenheimer and E. A. Evans, Jr., *Jour. Biol. Chem.*, 120: 503, 1937.

[24] The interpretation given in the popular press that butter fat burns faster than other fats is incorrect. The acids investigated constitute only a small fraction of the fatty acids of butter, and corresponding experiments have not yet been carried out on the metabolism of the other fatty acids of butter. The suggestion that butter fat is not fattening lacks all foundation.

An exploratory experiment has been carried out on the applicability of the deuterium technique to the study of sugar formation.[23] Propionic acid, which contains 3 carbon atoms, is known to be a "sugar former," and when this compound is given to a diabetic animal, an equivalent amount of carbon can be recovered from the urine in the form of extra sugar. When deutero-propionic acid was given to diabetic dogs or rats, the extra sugar formation was observed just as after feeding ordinary propionic acid, yet the sugar isolated from the urine contained only an insignificant amount of deuterium. The isotope must have been lost during the conversion—most probably when intermediate compounds were formed in which the hydrogen (or deuterium) was exchangeable with the hydrogen of the body fluids.

In a number of cases where the conversion of one substance into another can not be followed on account of the labilization of the deuterium in the intermediate steps, a reverse method may be employed.[23] Instead of introducing the deuterium into the organic molecule, it is introduced as heavy water into the body fluids, or in other words, into the medium in which the conversions are to take place. If chemical reactions are carried out *in vitro* in a medium of heavy water instead of ordinary water, deuterium may enter into the reactions and subsequently be found in stable positions in the end-products. This is particularly true if reductions or condensations are involved. If the same reactions are carried out in animals whose body fluids contain heavy water, the deuterium may also be found in the products of biological synthesis. The formation of fats from carbohydrates can probably not be followed by the first method, that is, with sugars labelled with deuterium, as the isotope will be lost from the molecule during the production of intermediary compounds.[25] But, when the fat formation from sugars is studied with animals, the body fluids of which contained heavy water, the resulting fatty acids were characterized by their stably bound deuterium,[26,27] the presence of which was taken as an indication of the new formation of these fatty acids.

This conclusion, however, presupposed that the fatty acids themselves had not taken up their deuterium by simple exchange with the heavy water of the body fluids in which they were dissolved or suspended. In all biological experiments in which chemical reactions are investigated by the uptake of deuterium from the body fluids extreme care must be taken in the interpretation of the findings. Proof is essential that fatty acids, which are not undergoing chemical reactions, do not exchange hydrogen with that of the water in which they are dissolved. This proof of the stability of the hydrogen in the compounds when demonstrated in the test-tube is not sufficient indication of its stability in the living organism. Enzymes may exist which labilize otherwise stable hydrogen atoms without further changing the total molecule. In the case of most compounds the proof of the "biological hydrogen stability" can be furnished only with the greatest difficulty, but it has already been possible to supply it for a few substances. The biological stability of the hydrogen in a compound can be accepted, if this substance can be isolated in the deuterium-free state from a living organism, the body fluids of which have contained heavy water for a considerable period. Demonstration of such "biological hydrogen stability" has as yet been supplied only for the fatty acids, cholesterol and the essential amino acid lysine. Deuterium-free fatty acids[26] and cholesterol[27] were isolated from chicks which had developed in eggs, the fluids of which had been enriched with heavy water. Deuterium-free lysine was isolated from mice whose body fluids had contained heavy water for a period of more than 3 months.[28] If the hydrogen of these compounds were exchangeable under biological conditions with the hydrogen of the body fluids, the compounds isolated from these metabolically active organisms should have contained deuterium. If, under other conditions, deuterium is introduced biologically into these substances, such a finding must be taken as an indication for a chemical reaction which had occurred in the animal. The introduction of deuterium into fatty acids in mice from the body fluids demonstrates the occurrence of chemical reactions, namely, fat formation, rather than mere physical exchange.

As has just been mentioned, fatty acids do not take up deuterium during the active development of the chick embryo, but they do so in growing and adult mice, when the animal takes up food. The formation of fatty acids from carbohydrates in mice proceeds even on constant body weight. A breakdown of fatty acids concurrent with a new formation must have taken place. This turnover of fatty acids was found to be unexpectedly rapid, and is probably dependent not only on the quality of the diet but also on a great number of other physiological influences.

Cholesterol, also, takes up deuterium from the body fluids of adult animals;[27] the rate is slower than that for the fatty acids, indicating a slower synthesis of cholesterol. However, the total amount of deuterium introduced into the cholesterol during its formation is so large that it can be explained only by a synthesis

[25] R. Sonderhoff and H. Thomas (*Annalen*, 530: 195, 1937) have published an interesting paper in which they report on the conversion by yeast of tri-deutero-acetic acid into fatty acids and unsaponifiable matter.

[26] R. Schoenheimer and D. Rittenberg, *Jour. Biol. Chem.*, 114: 381, 1936.

[27] D. Rittenberg and R. Schoenheimer, *Jour. Biol. Chem.*, 121: 235, 1937.

[28] Unpublished data of G. L. Foster with the authors.

from a large number of small molecular units, such perhaps as the intermediates of fat or carbohydrate metabolism.

A highly interesting experiment in which similar methods and principles were employed is that of Ussing[29] on glycogen formation. After the body fluids of rats had been enriched with heavy water, large amounts of monohexoses were fed. The glycogen isolated from the liver contained such large concentrations of stably bound deuterium that it was suggested that glycogen formation does not proceed by simple coupling of hexose molecules, but only after their preliminary degradation to smaller molecules.

The use of deuterium as a tool for the study of intermediary metabolism is still new. While it has as yet been applied only in a limited number of investigations (experiments on sterols and bile acids),[30,31] we have no doubt that the scope of its application is much wider and that if the proper procedures are developed, it can be used for the study of almost all physiological compounds. Synthetic methods for the preparation of other deutero-compounds must be devised, and the physical and chemical properties of these new types of organic compounds must be studied more thoroughly. By far the most important task is the further investigation of the stability of carbon-bound hydrogen atoms *in vivo*.

Little has been done on the use of other isotopes for the investigation of intermediary metabolism. The oxygen isotope (O^{18}) will probably prove to be of great utility in the study of many chemical reaction mechanisms, but its application as a label for metabolic processes seems to be limited. Most cell constituents contain oxygen, but the groupings which contain this element (COOH, OH, C = O, etc.) are metabolically so active that even unimportant changes (esterification, etc.) may lead to a loss of this isotope.

The nitrogen isotope (N^{15}), on the other hand, will probably prove to be of great utility in the important field of nitrogen metabolism. The natural abundance of heavy nitrogen in all samples investigated, whether inorganic or organic, is the same, namely 0.37 atom per cent. Urey and his collaborators have succeeded in concentrating this isotope.[32] The analytical methods for its determination involve the use of the mass spectrometer. This method is sensitive enough to determine an increase of 0.003 per cent. of N^{15}. In other words, an amino acid, such as glycine, synthesized with an isotopic mixture containing 2.5 atom per cent. of N^{15} can be diluted over a hundred times with ordinary glycine and still be determined by the mass spectrometer.

The experience gained with deuterium in metabolism studies has demonstrated the importance of the stability of the isotopic tracer in the molecule. Just as stably bound hydrogen must be used for metabolic experiments, so can only such nitrogen as is stably bound and does not exchange with the nitrogen of other substances be employed for following the fate of nitrogenous compounds. A number of amino acids (glycine, alanine, phenylalanine, tyrosine, glutamic acid and norleucine) containing an increased amount of N^{15} have already been synthesized.[33] The stability of the nitrogen in amino acids has been investigated by treating a system of one isotopic and one normal compound (amino acid or ammonia) in aqueous solution at 100° C. In no case was the isotope observed to shift from one compound to another.[34] It is probable that the stability, observed *in vitro*, also exists *in vivo*; only chemical reactions (*e.g.*, deamination) will lead to a loss of the label.

So far only one series of biological experiments has been carried out with the nitrogen isotope. To test its suitability as a label for amino acids, isotopic glycine was subjected to a simple biochemical reaction, the coupling with benzoic acid to form hippuric acid. This reaction has already been extensively studied in many laboratories, and it is well known that animals when fed benzoic acid can supply more glycine for its detoxication than was originally present in the body. Animals thus have the ability to form comparatively large quantities of glycine from other nitrogenous compounds, and it was uncertain whether they can utilize preformed glycine for this reaction, or whether the benzoic acid is primarily coupled with another nitrogenous substance, which subsequently is degraded to hippuric acid. Isotopic glycine was given to rats together with benzoic acid, either *per os* or subcutaneously,[34] and the isotope content of the urinary hippuric acid showed that the administered glycine was used for the hippuric acid synthesis.

It would be premature, on the basis of these experiments, to predict the types of metabolism problems for which the nitrogen isotope can be employed. The direction in which this field will develop depends upon whether the animal can use ammonia for the preparation of amino acids, or whether this synthesis is limited to the use of carbon-bound nitrogen. While the occurrence of amino acid synthesis in animals is well established, the source of the nitrogen employed in such reactions is still obscure. These new techniques in biochemical practice may soon answer this and other fundamental questions of metabolism.

[29] H. H. Ussing, *Skand. Arch. Physiol.*, 77: 85, 1937.

[30] R. Schoenheimer, D. Rittenberg and M. Graff, *Jour. Biol. Chem.*, 111: 183, 1935.

[31] R. Schoenheimer, D. Rittenberg, B. N. Berg and L. Rousselot, *Jour. Biol. Chem.*, 115: 635, 1936.

[32] H. C. Urey, M. Fox, J. R. Huffman and H. G. Thode, *Jour. Am. Chem. Soc.*, 59: 1407, 1937; *Jour. Chemical Physics*, 5: 856, 1937.

[33] Unpublished data of S. Ratner with the authors.

[34] R. Schoenheimer, D. Rittenberg, M. Fox, A. S. Keston and S. Ratner, *Jour. Am. Chem. Soc.*, 59: 1768, 1937.