**The dynamic state of body constituents / by Rudolf Schoenheimer.**

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# THE DYNAMIC STATE OF BODY CONSTITUENTS

BY

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# RUDOLF SCHOENHEIMER, 1898–1941 [1]

RUDOLF SCHOENHEIMER was born in Berlin, where he received his early education and university training. After receiving the medical degree from the University of Berlin in 1922, he held for a year the position of resident pathologist in the Moabit Hospital of that city. There his interest was attracted by the problem of atherosclerosis and his first published works, dating from that period, relate to the production of this condition in experimental animals by the administration of cholesterol. Recognizing his need for a wider knowledge of biochemistry, he then studied for three years under Karl Thomas of Leipzig, from whose laboratory he published, early in 1926, an ingenious method for the preparation of peptides. During these years of supplementary training, Schoenheimer held a fellowship of the Rockefeller Foundation.

The next move was to the Pathological Institute of the University of Freiburg, where in 1926 he joined, as chemist, the staff of Ludwig Aschoff, who exerted a marked influence on his scientific development. Here, along with his regular duties in the investigation of pathological material, he again took up the biochemical study of the sterols. In 1927 he became the active, and in 1931 the titular, head of his division. During this period his researches related mainly to the metabolism of cholesterol and were continued in that field when, in 1930, he came to this country for a year as Douglas Smith Fellow in the Department of Surgery of the University of Chicago. After his return to Freiburg in 1931 his work, continued with the support of the Josiah Macy, Jr. Foundation, was rudely interrupted by political developments within Germany in the spring of 1933. The last study completed in the Freiburg laboratory was reported in the *Journal of Biological Chemistry*; it established the im-

[1] Reprinted from *Science*, 94, 553 (1941).

portant finding that in the normal mammalian organism cholesterol is continually and extensively synthesized and degraded in the tissues.

The Department of Biochemistry of Columbia University was fortunate in being able to provide facilities for Schoenheimer's subsequent researches. The first report published by him from this laboratory, recording the normal occurrence of cetyl alcohol in intestinal contents, has a peculiar significance in its bearing on his subsequent work on the intermediary metabolism of fatty acids. In collaboration with W. M. Sperry, he developed a valuable method for the precise determination of minute quantities of free and combined cholesterol and applied this technique to a comparative study of serum and plasma.

In 1934 Schoenheimer made a new contact which proved to exert a fundamental influence on the nature of his work. In order to exploit the availability of deuterium, discovered by Urey in 1932, for the development of biological research, the Rockefeller Foundation established a fund to enable chemists trained in deuterium techniques to apply their special knowledge to biochemical and allied problems. Under these auspices David Rittenberg came from Urey's group to the laboratory in which Schoenheimer had been working for a year. From their association there developed the idea of employing a stable isotope as a label in organic compounds, destined for experiments in intermediary metabolism, which should be biochemically indistinguishable from their natural analogues. Justification for this scheme was found in the established fact that the hydrogen in organic matter displays the same isotope abundance as that in common water. Exploratory experiments soon showed that the feeding of 4,5-deuteriocoprostanone led to the presence of deuteriocoprosterol in the feces and that the ingestion of fat containing combined deuteriostearic acid was, surprisingly, followed by the rapid deposition of a large proportion of it in the body fats.

A similar effect was then observed to occur in animals the

body fluids of which were enriched with heavy water; deuterio fatty acids appeared in the depot fats and reached a maximum in a strikingly short time. Conversely, with ordinary water in the body fluids, the isotopic label in the depot fats disappeared equally rapidly. This rapid interchange between components of the diet and of the tissues proved on further investigation to involve not only direct replacement of chemically identical fatty acids but rapid transformations, notably desaturation, saturation, degradation, elongation and reduction to alcohols. The only natural fatty acids which appeared not to be synthesized by the rat were the highly unsaturated acids known to be essential for health.

As soon as the stable isotope of nitrogen, $N^{15}$, became available, Schoenheimer and his colleagues applied it to an analogous study of protein metabolism. Amino acids synthesized from isotopic ammonia and added in small quantities to the diet of adult rats in nitrogen equilibrium were found to be rapidly and extensively incorporated in the tissue proteins. Like the fatty acids, they also gave evidence of chemical transformations; after the ingestion of isotopic amino acids or ammonia, heavy nitrogen was found in all amino acids isolated from the proteins, except lysine. Advantage was also taken of the possibility of labeling compounds with both isotopes, the ratio of which in the products isolated from tissue proteins indicated the extent to which the carbon chain of an amino acid had followed a different metabolic pathway from that of the nitrogen atom.

As a result of Schoenheimer's investigations, of which but a few examples have here been outlined, there has emerged a concept of metabolic "regeneration," wherein the central idea is the continual release and uptake of chemical substances by tissues to and from a circulating metabolic "pool." Coincident with these cyclic processes there occur among the components of the pool multitudinous chemical reactions, of which only relatively few are concerned with elimination of waste products.

These general interpretations were summarized by Schoenheimer in his Harvey Lecture of 1937 and his Dunham Lectures of 1941. . . .

One of Schoenheimer's most striking characteristics was his ability to correlate pertinent facts from highly diversified branches of knowledge and bring them to bear upon problems under immediate consideration. He not only sought the advice of experts in fields other than his own, but freely discussed his scientific plans with his colleagues as well as his direct collaborators. He led his research group with tact, understanding and constant stimulation.

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# PREFACE

DRAFTS of these lectures had been prepared by Dr. Schoenheimer shortly before his untimely death early in September, 1941. Their final revision, carried out by Dr. David Rittenberg, Dr. Sarah Ratner, and myself, has merely been such as he would in any case have performed in consultation with these closely associated colleagues, and involves no essential change in their form as originally planned.

HANS T. CLARKE

NEW YORK CITY
DECEMBER, 1941

# CONTENTS

# THE DYNAMIC STATE OF BODY CONSTITUENTS

# I

## THE REACTIONS OF THE BODY FATS INVESTIGATED WITH DEUTERIUM

THE general title, *The Dynamic State of Body Constituents*, designates an attempt to consider under one general concept the various topics of these three lectures, wherein there will be presented some results of modern biochemistry which suggest that all constituents of living matter, whether functional or structural, of simple or of complex constitution, are in a steady state of rapid flux.

The animal requires food, which is obviously necessary for growth. Its function in an adult animal of constant weight, however, is not so obvious, and this question of its indispensability is probably as old as human thought. Prior to the introduction of exact measurement into physiological experimentation, the general concept of the mechanism of life was mainly irrational and transcendental. During the centuries when alchemists sought the philosopher's stone, numerous attempts were made to construct perpetual motion machines which should create energy. The human body was considered an ideal "machine" of this kind and was frequently studied with the aim of disclosing its secret. Food occupied but a small place in this picture. It was frequently considered to act merely as a lubricant for the mechanical motion of muscles and joints.

These ideas were dispelled at the end of the eighteenth century, mainly by the work of Lavoisier on combustion. For some decades thereafter the working of the living organism was compared to a burning candle.

The simile was again changed by the development of thermodynamics in the middle of the last century, when Helmholtz

painted a new picture of life by comparing the animal with a combustion engine. The food represented the fuel and the organs the working parts of the engine.

Later it was realized that human and other animals are highly sensitive to the quality of the "fuel." They require not only the major energy-yielding substances like fats and carbohydrates, but also definite amounts of proteins and even some minor ingredients.

A concept of the interaction between the diet and the whole organism was advanced by the German physiologist Rubner and the American biochemist Folin, who suggested that the mechanical parts are subject to continual wear and tear and have constantly to be repaired by food materials. Some of the constituent parts of the organism which cannot be constructed within it have to be added to the system. Part of the diet is degraded for energy liberation, and a smaller fraction is employed for repair. This view has received its most direct formulation in the theory of independent exogenous and endogenous types of metabolism.

According to these successive theories, from the perpetual motion machine to the frequently repaired combustion engine, the structural elements of the body are primarily in a fixed or stable state. This hypothesis has not satisfied all investigators and has occasionally been challenged. Borsook and Keighley (1), for instance, on the basis of experiments on the rate of nitrogen and sulfur excretion, have advanced the theory of a continuous metabolism in which food and tissue take almost equal part.

The experimental investigation of the chemical reactions of the body constituents is associated with great difficulties. Food and tissues both consist of proteins, fats, and carbohydrates. When the split products liberated by digestion are absorbed, they merge with identical molecules which originate from the tissues, and the investigator loses track of them. Investigation of intermediary metabolism was therefore restricted

to exceptional or abnormal conditions that permit the accumulation of newly formed substances, as by the use of unnatural compounds, of diets enriched with or poor in test substances, of poisoned organisms, or of isolated organs, tissues, or extracts. Work has also been done with normal, growing animals, which lay down tissue material derived from food. However, methods which depend on growth rates can yield little information as to metabolic processes in adult animals of constant weight.

Animals in nutritional equilibrium may reasonably be assumed to maintain, within narrow limits, constancy not only of weight but of chemical composition.

Most of our knowledge of intermediary metabolism is the result of balance experimentation, which can give little insight into the nature and extent of chemical reactions that may conceivably take place between body constituents but do not give rise to metabolic end products. Many attempts have been made to circumvent the fundamental difficulties by introducing readily detectable chemical labels into substances under investigation. For instance, in order to follow the metabolism of a fatty acid, one or more hydrogen atoms have been replaced by halogen (2). The physical and chemical properties of the compound are, however, greatly affected by such substitution, and the highly sensitive cells or organs of the animal body cannot be expected to treat both substances alike.

In order to mark a compound for biological studies the label has to be of such nature that no change of physiological properties is effected by its introduction, but the experimenter must be able to estimate it in small amounts. Labels that satisfactorily fulfill these requirements are isotopes of elements that occur in organic matter: namely, the less abundant isotopes of carbon, hydrogen, oxygen, and nitrogen (Table 1).

The fact that the biologist is in a position to mark organic molecules with isotopes is mainly due to the work of Urey (3), who not only discovered heavy hydrogen but devised

methods for its large-scale concentration and that of the less abundant natural isotopes of carbon, nitrogen, oxygen, and sulfur.

While Urey was making available these stable, natural isotopes, radioactive elements obtained by nuclear reactions have

TABLE 1

STABLE ISOTOPES

| Element | Abundance | | Method of Concentration |
|---|---|---|---|
| | | *per cent* | |
| H | $H^1$ | 99.98 | Electrolysis of $H_2O$ |
| | $H^2$ | 0.02 | |
| C | $C^{12}$ | 98.9 | Exchange reaction |
| | $C^{13}$ | 1.1 | |
| N | $N^{14}$ | 99.62 | Exchange reaction |
| | $N^{15}$ | 0.38 | |
| O | $O^{16}$ | 99.8 | Distillation of $H_2O$ |
| | $O^{17}$ | 0.03 | |
| | $O^{18}$ | 0.16 | |
| S | $S^{32}$ | 96 | Exchange reaction |
| | $S^{33}$ | 1 | |
| | $S^{34}$ | 3 | |

been offered to the biologist. Within the period of a few years radioactive isotopes of almost all elements have been produced; indeed, some exist in several radioactive varieties.

The living organism does not discriminate between isotopes of the same element, stable or radioactive, and treats all alike. The first investigator to realize the practical value of these tools was Hevesy (4), who as early as 1923, when neither stable nor artificially radioactive isotopes were available, employed the natural radioactive isotope of lead, radium D, for the study of the course of lead in the organism. His later studies on the metabolism of inorganic ions will take their place in the history

of biochemistry as classics but will not be discussed in these lectures, which are limited to the metabolism of organic molecules.

The choice between the various types of isotope for a given investigation is at present mainly a matter of convenience. Stable and radioactive isotopes have their respective advantages and disadvantages. The analysis of the latter is generally more rapid and convenient, but the limited half-lifetime of some and the scarcity of others restrict their application to work that can be finished in a short time.

In biological work with isotopic organic compounds, the physiological substance to be investigated is synthesized in the laboratory in such a way that the isotopic composition of one or more of its constituent atoms is abnormal. If, after feeding an isotopic compound to an animal, one isolates from the organs or excreta another compound with abnormal isotope abundance, the content of the marker in the latter may be taken as indication of chemical conversion — in a broad sense — of the one into the other.

Up to the present, most of such work on intermediary metabolism has been carried out with the two stable isotopes $H^2$ (deuterium) and $N^{15}$. Deuterium, in the form of heavy water, is now readily available. Heavy nitrogen ($N^{15}$), however, is still difficult to secure. Though work with isotopic varieties of carbon is still in the first stages of development, the few papers which have been published on it have shown its fundamental value.

These lectures will include no detailed discussion of the methods employed for synthesis and isolation of the organic compounds and for the analysis of the isotope. They will be confined to the physiological application and the interpretation of the results obtained.

The living organism makes extensive use of large, complex molecules, such as proteins, fats, and polysaccharides. The amount of substances of low molecular weight, such as amino

acids, monosaccharides, or fatty acids, is comparatively small. It is a truism that the growing animal utilizes small molecules to build up larger ones, but little is known of the interaction between the large and small ones. The study of such interactions requires the use of animals of constant weight; that is, of organisms in which the relative composition remains the same. Most of the experiments here to be discussed were carried out with adult animals in nutritional equilibrium. The isotopic test substance was generally given in such small amounts that the characteristics of the stock diet were not changed.

The first biological experiments with isotopic compounds were carried out with fats. This lecture will be exclusively devoted to chemical processes in which the body fats are involved.

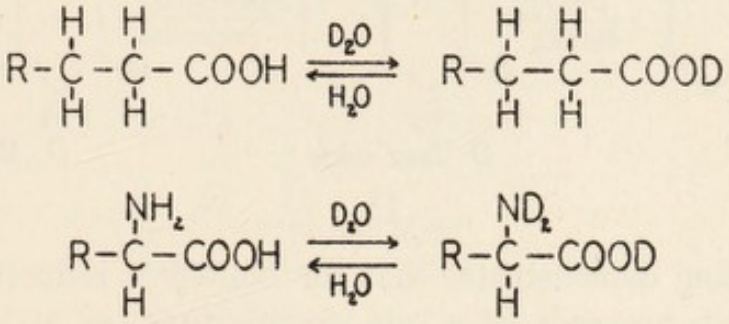
In mammals most of the fat is located under the skin, between the muscles, or around internal organs. This fat is usually called depot or storage fat and has generally been regarded as a biological energy store. According to this concept, the fat depot is outside the general metabolism and becomes active only in times of need.

Every animal has a tendency to produce a fat characteristic of its species. On the other hand, the properties of a depot fat can be changed at will by feeding large amounts of different fats; small amounts have little effect. One might therefore conclude that dietary fat is immediately burned unless given in large quantities.

The transport of fatty acids and their interconversions can be followed with isotopes. The biochemist, when following the metabolism of an organic compound, is mainly interested in the fate of the carbon and only to a lesser extent in that of other atoms. The phrase "conversion of sugar into fats," thus narrowly interpreted, designates the utilization of the carbon atoms of sugar for the biological synthesis of fatty acids. The ideal marker for fats would, then, be one of the carbon isotopes.

The syntheses of the required compounds, with carbon dioxide as a starting material, have not yet been accomplished, and deuterium is at present a more convenient substitute.

It is easy to introduce deuterium into a fatty acid by merely dissolving it in heavy water (I). The hydrogen atom of the carboxyl group, as a result of its ionization, immediately ex-

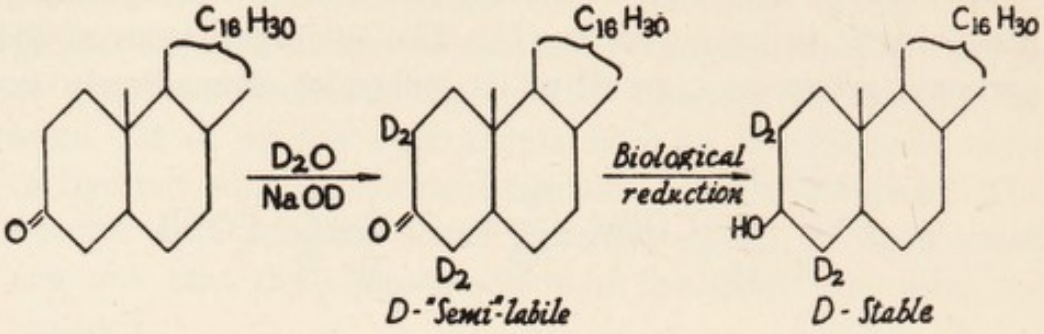


I

changes with those of the water. The same is true for the hydrogen atoms of hydroxyl-, amino-, imino-, and similar groupings in which the hydrogen is bound to atoms other than carbon. Such substances with "labile" isotopes, however, cannot be employed for biological tracer experiments, as they will lose the marker immediately after ingestion, the heavy hydrogen being replaced by the normal hydrogen of the aqueous body fluids. Hydrogen attached directly to carbon is generally stably bound and is not removed even if the isotopic compound is treated with boiling aqueous alkali or acid.

An interesting exception is hydrogen attached to a carbon atom adjacent to a carbonyl group; this can be exchanged by enolization. The occurrence of this type of exchange was first observed in the sterol field. When ordinary coprostanone was boiled with an alkaline mixture of heavy water and alcohol, the product contained appreciable amounts of deuterium. This was completely removed by the action of alkali in ordinary water (II). In exceptional cases it is possible to make use of compounds containing "semi-labile" deuterium for metabolic stud-

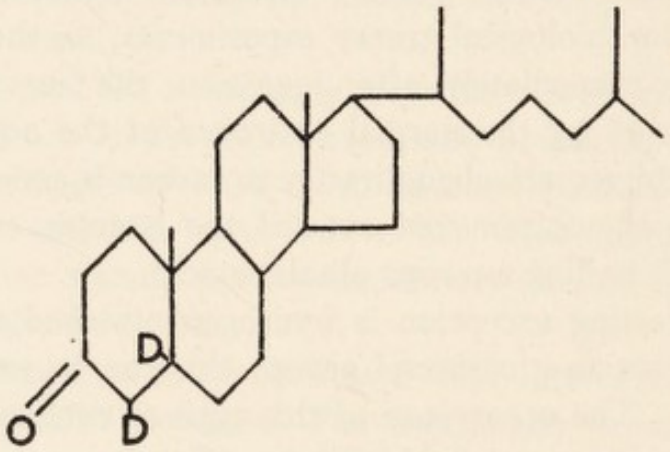
ies. When the deuterio coprostanone prepared by enolic exchange was fed to a man, the coprosterol isolated from the stools contained appreciable amounts of stably bound deuterium.




II

This finding demonstrates that the biological reduction of the keto group proceeds at a rate greater than, or at least comparable with, that of exchange due to enolization (5) in the body.

The specificity of enolic semi-lability may be illustrated by the case of the semi-labile deuterio coprostanone prepared by saturating the double bond of cholestenone (III). When this



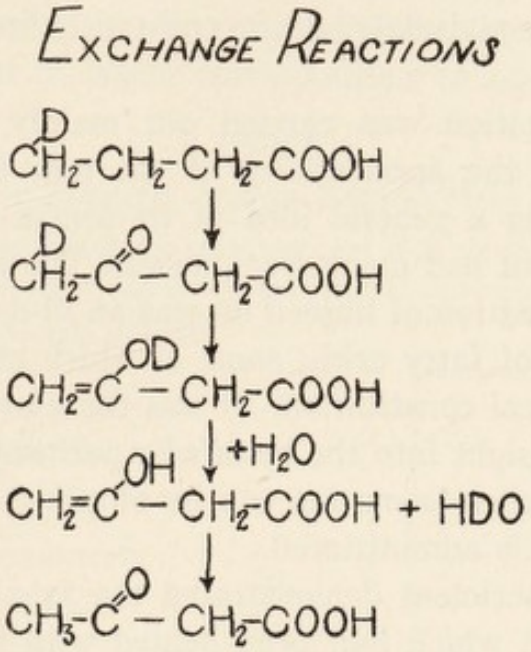

4, 5–D–COPROSTANONE

III

product was treated with a boiling solution of alkali in ordinary water, only one of the deuterium atoms was exchanged; the isotope concentration fell to one-half of its original value,

but no further. The deuterium atom in position 5 was not exchanged. In subsequent discussions, the phrase "deuterio compounds" will always imply that the isotope is stably bound.

The theoretical application of the principle of lability through enolization is illustrated by the scheme (IV) which shows the




IV

metabolic oxidation of deuterio butyric acid (6). The isotope is completely lost during the process, and merges with the hydrogen atoms of the water in the body fluids.

The first series of experiments on the metabolism of dietary fats was carried out with a deuterio fat prepared from linseed oil (7). This was partially hydrogenated with deuterium, which became attached to carbon atoms and was thus stably bound in the product. Normal adult mice were kept on a fat-low diet to which was added 2 per cent of the marked fat. At the end of four days the isotope content of the body fat indicated that at least 44 per cent of the diet fat had been deposited. This finding, later shown to be typical, must have been the result of fat replacement, as the total amount of

depot fat remained constant. An amount of depot fat corresponding to that deposited must have been degraded, or, in other words, the fats which the animals burned were not merely absorbed diet fats but consisted of a mixture of almost equal parts of food fats and depot fats. These first experiments with isotopes showed that the fats of the depots are not inert storage materials but are constantly involved in metabolic reactions.

This investigation was carried out mainly with the aim of ascertaining the applicability of the new laboratory tool and of obtaining a general idea of its sensitivity. The plan of the experiment had many weaknesses. The fat obtained by partial hydrogenation of linseed oil was an ill-defined glyceride of many kinds of fatty acids, some of which might have been of unphysiological constitution. It was therefore impossible to obtain direct insight into the metabolic pathway of any single component. Such information can be obtained only when one pure fatty acid is administered.

The next experiment demonstrated the fate in normal rats of palmitic acid which had been heated with heavy water in the presence of platinum until 22 per cent of its hydrogen atoms were in the form of heavy hydrogen almost equally distributed over the whole molecule. In the convention adopted, this compound is stated to contain 22 atom per cent deuterium. This was added to a casein-containing stock diet. In the first experiment with linseed oil the animals had been kept on a fat-low diet, and some of the fat deposition observed might have been due to the nutritional requirements of the animals for special fatty acids. In the experiment (8) here discussed the rats were supplied with 6 per cent of butter. This is a complex food fat and contains all the fatty acids which the animals require. The isotopic palmitic acid was administered as the ethyl ester at a level of 0.56 per cent of the diet.

This isotopic material must during absorption have merged with the non-isotopic palmitic acid present in the butter. As

the composition of butter is well established (9), the isotope content of the palmitic acid absorbed by our animals could be calculated to be 5.7 atom per cent deuterium. The rats were thus on a normal diet containing a large quantity and variety of physiological fatty acids, of which one was marked by the isotope tracer.

At the end of eight days the body fats were found to contain an amount of tracer corresponding to 44 per cent of that

TABLE 2

CONVERSION OF PALMITIC ACID IN NORMAL RATS ON STOCK DIET

(Values are calculated for 100 atom per cent D in compound administered.)

| Substance Isolated | Deuterium |
|---|---|
| | *atom per cent* |
| Fatty acids isolated from fats | |
| Palmitic acid | 24.2±0.3 |
| Stearic acid | 9.3 " |
| Myristic and lauric acids | 5.6 " |
| Palmitoleic acid | 6.3 " |
| Linoleic acid | 0.3 " |
| Oleic acid | 1.0 " |
| Cetyl alcohol from feces | 12.6 " |

administered. The presence of deuterium in the fat tissues might be due to one of two processes. Either the dietary fatty acid was deposited as such and had replaced palmitic acid present in the depot fat, or it was converted into other types of fatty acids, and these newly formed isotopic compounds had, in turn, replaced their analogues. For a decision it was necessary to isolate various individual fatty acids from the fat mixture and determine their isotope content.

The values in Table 2 were calculated on the basis of a deuterium content of 100 atom per cent (instead of 5.7 per cent) in the administered palmitic acid. The numbers in the table thus indicate the minimum fraction, in per cent, of each depot

fatty acid which had been derived from the dietary palmitic acid.

The palmitic acid isolated proved to have a very high isotope content. Over 24 per cent of this acid present in the depot fat had been newly introduced. Part of the palmitic acid of the food had been desaturated to palmitoleic acid, and some of this newly formed compound must have been introduced. Furthermore, palmitic acid was degraded by 2 and 4 carbon atoms to form myristic and lauric acids.

An interesting finding was the presence of considerable amounts of the isotope tracer in the stearic acid. Obviously the palmitic chain was condensed in some way with another compound so as to add 2 carbon atoms. This elongation of a fatty acid occurs so rapidly that almost 10 per cent of the stearic acid in the animals had been formed from the dietary palmitic acid. The isotopic oleic acid was probably derived by desaturation of the newly formed isotopic stearic acid.

The linoleic acid isolated was not isotopic. This finding gains significance from the observation by Burr and Burr (10) and others (11) that this compound is an indispensable food constituent which the animal cannot produce.

The results, taken together, indicate the simultaneous occurrence of many biological reactions. Part of the fatty acid of the diet was directly introduced into fats, and another large fraction was deposited after its conversion into various other compounds. This type of experimentation clearly demonstrates the conversion of one fatty acid into others. While most students of fat metabolism had suggested the occurrence of biological desaturation of fatty acids, direct proof was lacking, as it was impossible to decide whether newly appearing fatty acids had been synthesized from carbohydrates or had been derived from other fatty acids.

One of the interesting features of the experiment was the presence in the diet of butter. This contains considerable amounts of stearic, myristic, lauric, oleic, and palmitoleic acids;

that is, of all those types which the rat had formed from palmitic acid.

The animal is capable not only of producing one type of fatty acid from others but does so even if the fatty acid formed is plentifully supplied in the diet. This apparently paradoxical result was difficult to interpret. Many experiments on fats, proteins, and other body constituents had to be carried out before a logical explanation could be advanced.

All of the conversions of fatty acids seem to be "biologically reversible." Rats not only convert palmitic acid into stearic acid, as shown in the present experiment, but also degrade stearic acid into palmitic acid (12), and the process of desaturation of stearic acid into oleic acid may also be reversed.

The demonstration (13) of this last conversion required the availability of deuterio oleic acid. The product obtained by partial hydrogenation of more highly unsaturated acids, such as linoleic acid, is inhomogeneous and probably contains acids with double bonds in positions other than that in which it exists in oleic acid. Advantage was therefore taken of the ability of the living mouse to convert deuterio stearic acid into deuterio oleic acid. The unsaturated acid fraction of the fats, thus biologically prepared from deuterio stearic acid, contained deuterium. It was fed to two mice for ten days in the form of the ethyl esters admixed with ten times their weight of dry bread. Saturated and unsaturated fractions then isolated from the body fat had the isotope compositions indicated in Table 3. From calculations of the quantitative data of this experiment, it appears probable that at least two-thirds of the saturated deuterio acids originated from the ingested unsaturated acids.

The steady interconversions are not the only chemical processes in which the depot fats are involved. It is known that fats can be synthesized from other dietary material. It can be demonstrated that this fat formation also takes place continuously and rapidly in normal animals, whether fat is supplied with the diet or not.

Heavy hydrogen can be employed not only for tracing the metabolic route of organic molecules but also as a general means for determining the occurrence and rate of many biosynthetic reactions. If an animal is injected with heavy water,

TABLE 3

DEUTERIUM CONTENT OF FATTY ACIDS FROM TWO MICE FED DEUTERIO OLEIC ACID (1.06 ATOM PER CENT D) *

| Mouse | Unsaturated Acids | Saturated Acids | Body Water |
|---|---|---|---|
| | *atom per cent D* | *atom per cent D* | *atom per cent D* |
| 1 | 0.25 | 0.047 | 0.043 |
| 2 | 0.10 | 0.025 | 0.028 |

* From Rittenberg and Schoenheimer, *J. Biol. Chem.*, 117, 488 (1937).

this will soon be distributed equally over all body fluids so that the blood, lymph, urine, and cell fluids all have the same deuterium concentration (14). All chemical reactions in the animal body will then occur in a medium of heavy water. Many chemical processes carried out in an aqueous medium involve the hydrogen of the solvent so that it becomes fixed in the reaction products. One of these reactions is reduction. Whatever the intermediates in the synthesis of fats from sugar may be, reduction of hydroxymethylene groups to methylene groups takes place and should lead to an incorporation of water hydrogen in the fatty acids. If such a synthesis be carried out in heavy water, the newly formed fatty acids should be characterized by stably bound deuterium, attached to carbon.

An experiment (15) was performed to test this prediction. Adult mice on a diet of bread were injected with heavy water and then given drinking water of such isotopic composition that the deuterium content of the body fluids remained constant throughout the experimental period. Animals were killed at intervals, and the isotopic composition of the total fatty acids was determined. The solid curve of Figure 1 shows the

results, expressed as per cent synthesis, based upon the level at which the deuterium content became steady. It will be seen that the synthetic reactions had proceeded to the half way point within a few days. The biological reversibility of the process

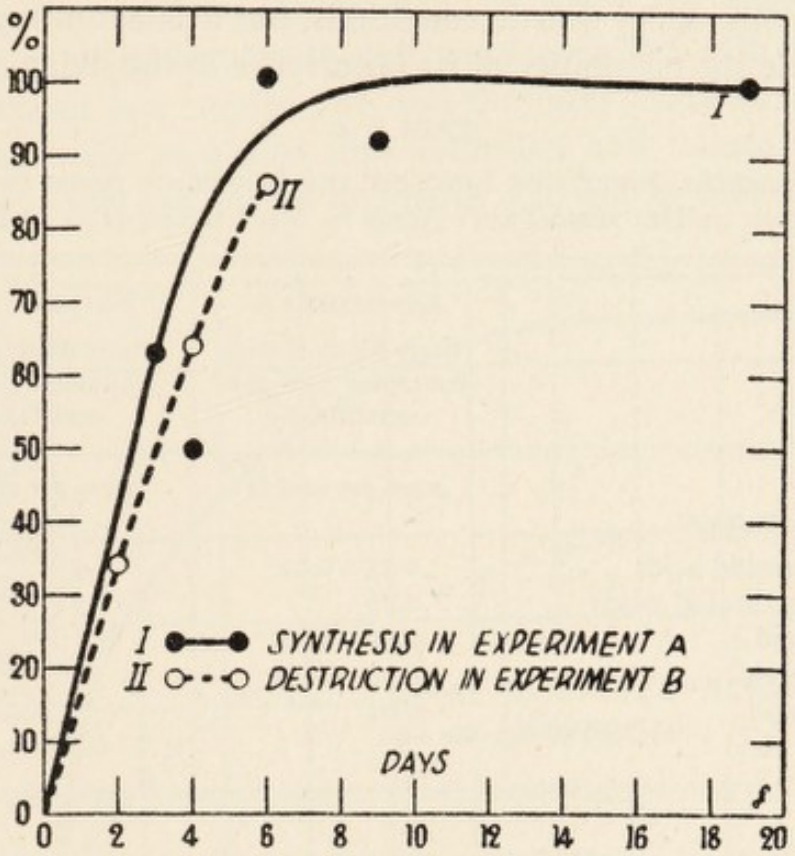



FIG. 1. — Synthesis and destruction of fatty acids in mice. (From Schoenheimer and Rittenberg, *J. Biol. Chem.*, *114*, 389, 1936.)

was shown in a separate experiment, in which mice previously fed with deuterio fat were placed on a similar diet but received ordinary water to drink. After the feeding of labeled fat had been discontinued, the isotope level in the total fatty acids of the animals fell at a rate almost identical with that found for the synthesis. The results of this reverse experiment are plotted in the broken curve of Figure 1.

In the experiment in which the body fluids were enriched

with deuterium, the interpretation is justifiable only if one can exclude the biological occurrence of other processes by which hydrogen from water could also become fixed. A possibility frequently discussed is the physical exchange of carbon-bound hydrogen with that of water. This has been observed to occur *in vitro* only under drastic conditions, but it is not always easy to exclude the possibility of its occurrence in the living animal.

TABLE 4

THE BIOLOGICAL INERTIA OF LINOLEIC AND LINOLENIC ACIDS IN MICE. FORMATION OF DEUTERIO FATTY ACIDS IN MICE GIVEN $D_2O$ TO DRINK

| | EXPERIMENT A (Body fluids of mice contained 2.96 per cent $D_2O$) | EXPERIMENT B (Body fluids of mice contained 2.25 per cent $D_2O$) |
|---|---|---|
| | *atom per cent D* | *atom per cent D* |
| Total fatty acids | .... | 0.41±0.02 |
| Total saturated acids | 0.55±0.02 | 0.58 " |
| Total unsaturated acids | 0.26 " | 0.34 " |
| Stearic acid | 0.59 " | .... |
| Palmitic acid | 0.54 " | .... |
| Linoleic acid | 0.02 " | .... |
| Linolenic acid | .... | 0.01±0.02 |

Nature has supplied us with an excellent opportunity to decide this question in at least one case. We know of two indispensable fatty acids, linoleic and linolenic acids, which have to be administered with the diet, as a rat is incapable of synthesizing them. When rats were injected with heavy water, the total depot fats isolated after some days contained deuterium. The component fatty acids were fractionated; the stearic and palmitic acids contained high concentrations of the marker (Table 4), but the highly unsaturated acids contained none (16). The presence in one and the same animal of deuterium in some fatty acids but not in others clearly demonstrates that this deuterium was introduced during fatty

acid formation and not by physical exchange in pre-existing fatty acids.

Such appearance of the isotope marker in fatty acids provides a method not only to decide which acids are formed but also how fast the reaction occurs with different types of fatty acid. A second experiment (17) in which the body fluids of mice on a carbohydrate diet were enriched with respect to deuterium was carried out, and the fatty acids secured at intervals were separated into saturated and unsaturated fractions. From the curves in Figure 2 it will be seen that, when

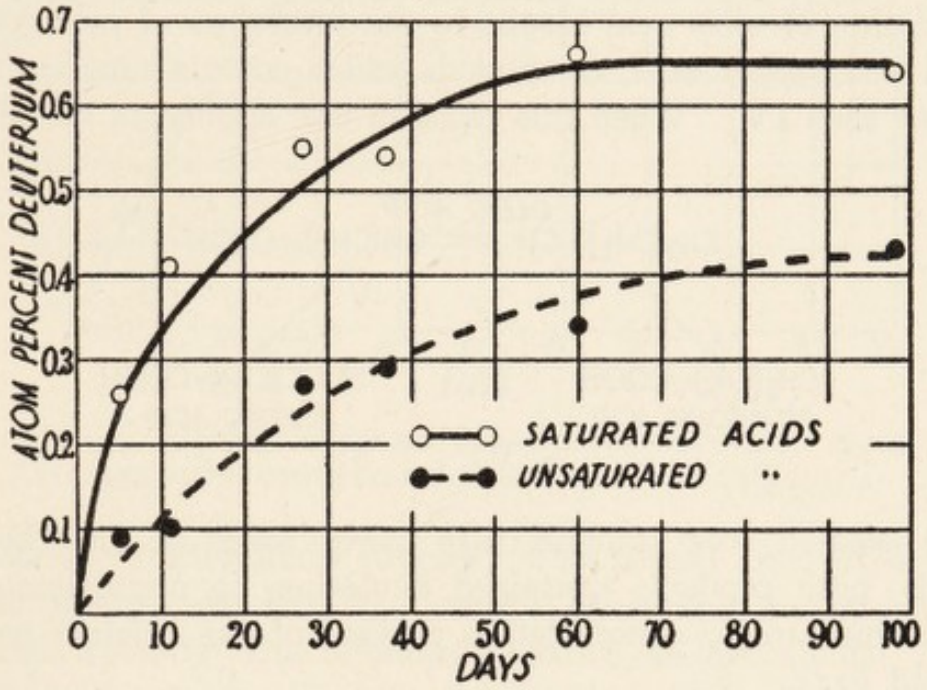


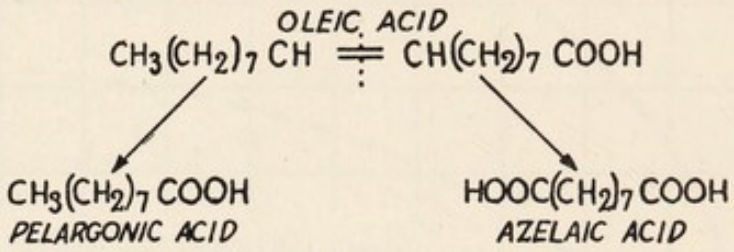

FIG. 2. — Deuterium content of saturated and unsaturated fatty acids. (From Rittenberg and Schoenheimer, *J. Biol. Chem.*, *121*, 247, 1937.)

a steady state had been reached, the deuterium content and the rate of synthesis of the saturated acids were higher than those of the unsaturated acids.

At the final level the isotope concentration of the saturated fatty acids was about half that of the water. During fatty acid formation half of the hydrogen atoms had thus been

derived from the water. This suggests that the acids were formed by the coupling of small units. The curve establishes the rate of this process. It follows an exponential course and may be characterized by its half value.

Another indication that the biological synthesis of fatty acids takes place by the condensation of relatively large numbers of small units may be found in the position in which the deuterium atoms are located within the fatty acid molecules. This field has not yet been systematically explored, but one observation points to the probability that the marked hydrogen atoms are fairly evenly distributed along the carbon chain. Oxidation of oleic acid results in the formation of pelargonic acid and azelaic acid, compounds which contain nine carbon atoms each (V). When this reaction was applied to the oleic




V

acid isolated from animals with heavy water in their body fluids, both products contained deuterium in approximately the same atomic concentration as that of the original compound (15).

As the biological synthesis of deuterio fatty acids was carried out with adult mice with constant body weight and fat content, new formation could not have occurred without simultaneous destruction. The curve thus indicates also the rate of degradation, which is likewise the rate of replacement of the body fats by those synthesized from other compounds.

Synthetic processes are not confined to the fatty acids. In the experiments just outlined, the cholesterol which accompanied the body fats was isolated and found to contain deu-

terium in concentrations which increased as the experiment proceeded (17).

The data, plotted in Figure 3, do not readily lend themselves to quantitative interpretation, but indicate that the

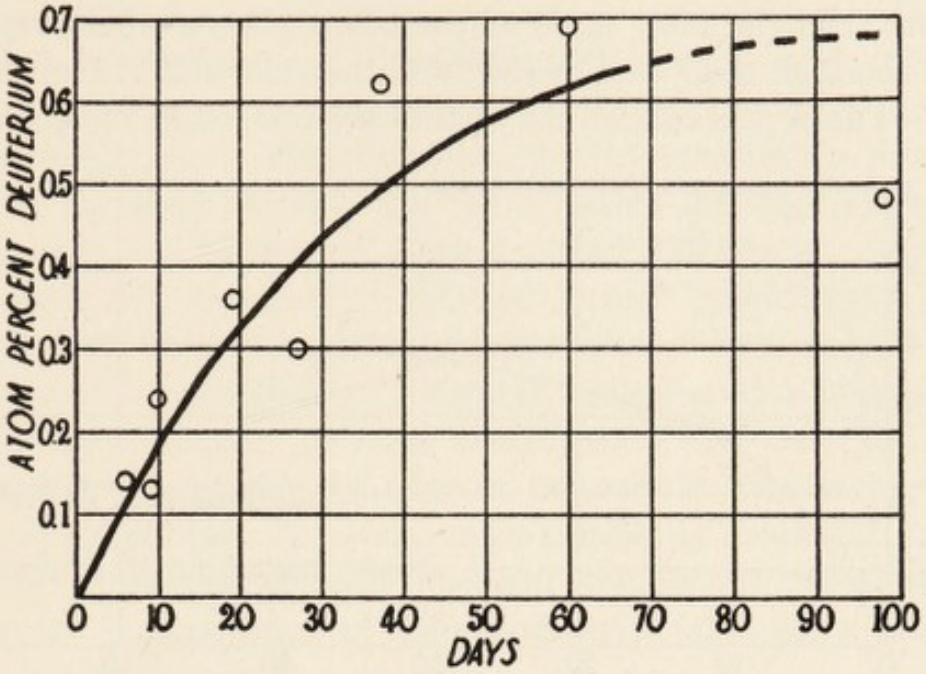



FIG. 3. — Deuterium content of cholesterol from mice. (From Rittenberg and Schoenheimer, *J. Biol. Chem.*, *121*, 248, 1937.)

synthesis of cholesterol proceeds with a half time of about three weeks, and that the isotope concentration ultimately reaches a steady value of one-half that of the body fluids. This finding suggests that in mice the synthesis of cholesterol, like that of fatty acids, involves the condensation of many small molecules.

The lipids of the fat depots are thus constantly subject to a great variety of highly complex chemical processes: synthesis, interconversion, and degradation. The sum of those biological reactions in which an organic compound is involved without ultimate change in amount or constitution may be termed "molecular regeneration." The fat of the depot and its component fatty acids are thus steadily and rapidly regenerated.

It is questionable whether the molecular regeneration occurs extensively with the fat while it is in the depots. It is probable that it takes place mainly in the internal organs, such as the liver, where the fat forms a highly disperse phase. This would connote a continuous transport of fat to and from the organs, and the fatty acids of the blood plasma would represent those on their way to and from the chief site of regeneration. The dèpots contain the bulk of the fats, while the amount

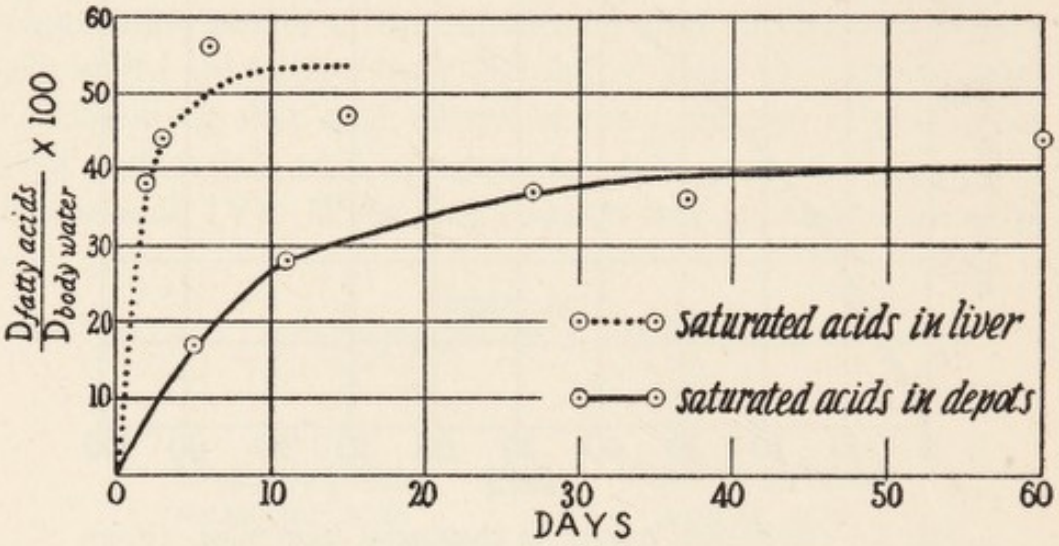



FIG. 4. — Rate of fatty acid synthesis in liver and depots.

in the organs is relatively small. Regeneration should therefore be much faster in the organs. This has been shown to be the case (Fig. 4). In the liver regeneration was so fast that it has not been found possible to determine the half time exactly; however, the trend of the curve indicates that more than half the fatty acids present in the liver of a normal animal on a fat-low diet had been synthesized within one day (18), as compared to about a week for the depot fat.

Fatty acids in the living body are not in the free state, but are bound to alcohols in ester linkage. The fact that deuterio palmitic acid supplied in the diet in the form of the ethyl ester largely and rapidly reappears as a glyceride in the depot fats indicates that ester linkages must have been broken continu-

ously and the acids at least temporarily liberated. In the free state the acids from one fat molecule must have merged with those from others.

Triglycerides are, in respect to their chemical structure, the simplest members of the lipid class. The phospholipids may be considered as triglycerides in which one fatty acid is replaced by choline- or ethanolamine-phosphoric acid. These compounds have also been found to be subject to rapid molecular regeneration. Their complex structure enables the process to be investigated with the aid of three different isotopes. Cavanagh and Raper (19) have employed deuterio fatty acids; Hevesy (20), Artom (21), Chaikoff (22), Chargaff (23), and others have used radioactive phosphates; and Stetten (24) has carried out experiments with choline and ethanolamine marked by heavy nitrogen. All these investigators have observed the respective fragment to be more or less rapidly introduced into the phospholipids. These also must constantly break into fragments which merge with identical compounds from other sources. Incorporation of dietary material takes place during the synthetic process.

The fatty acids of the depot fat are to be regarded as being constantly transported, in the form of fats or phosphatides, to and from the organs, where fátty acids are temporarily liberated by rupture of ester linkages. When fat is absorbed, the acids of dietary origin merge with those from the depot, thereby forming a mixture indistinguishable as to origin. Part of the liberated acids are converted into others, while new ones are steadily formed by condensation of small molecules derived from other substances. Some of this pool of acids is degraded, and some of it re-enters ester linkages to regenerate fat, which is transported back to the depots. All these complex reactions are so balanced that the total amount and structure of the fat mixture in depot, blood, and organs remain constant.

If one attempts to summarize the results of the isotope work on fats, one is compelled to conclude that the normal

animal's body fats, despite their qualitative and quantitative constancy, are in a state of rapid flux. It can readily be understood why the classical methods of metabolism, which were mainly limited to the measurement of changes in amount or relative composition, failed to detect this dynamic state.

# II

## THE STATE OF THE BODY PROTEINS

THE proteins are the most complex organic compounds found in nature and have molecular weights of about 35,000 up to several millions. Each of these large molecules consists of many smaller units of amino acid groups, linked together by peptide bonds in which the nitrogen of the $\alpha$-amino group is linked directly to a carbon atom of each neighboring amino acid. The replacement of one of these nitrogen atoms by another one would necessarily involve the opening of a peptide bond. It will be shown that such replacements do actually occur at rapid rates in living animals and plants.

The animal lays down proteins during growth and loses them during periods of inanition. In adult animals in nutritional equilibrium the total amount of proteins remains constant, as does, presumably, their composition. Such animals excrete an amount of nitrogen equivalent to that in the diet; they are in nitrogen equilibrium.

The isotopes, especially those of nitrogen and hydrogen, are powerful tools for the investigation of the fate of dietary amino acids and the chemical processes which the proteins in the living organism undergo. Heavy nitrogen ($N^{15}$), generously made available by Dr. Urey, has made possible a new technique for the investigation of protein metabolism. This often involves the feeding of isotopic amino acids. As the nitrogen isotope is produced in the form of ammonium salts, the amino acids had to be synthesized with ammonia as a starting material. Synthetic procedures have had to be devised to permit a quantitative recovery of the heavy nitrogen. In Table 5 are listed the isotopic nitrogenous compounds which

have been prepared. Most of these have already been tested biologically.

Amino acids may be considered to consist of two different parts, the amino group and the carbon chain, which may

TABLE 5

SYNTHETIC COMPOUNDS CONTAINING $N^{15}$

| | | |
|---|---|---|
| Urea | *dl*-Ornithine (D) | Guanidoacetic acid |
| Glycine | *l*-Leucine (D) | Betaine |
| *dl*-Alanine | *d*-Leucine (D) | Aminoethanol |
| *dl*-Tyrosine | *l*-Lysine (D) | Guanine |
| *l*-Phenylalanine | *d*-Lysine (D) | Arginine |
| *d*-Phenylalanine | *dl*-Norleucine | Hydantoic acid |
| *dl*-Glutamic acid | Choline | Methylhydantoic acid |
| *l*-Glutamic acid (D) | Creatine | *l*-α-Aminophenylbutyric acid |
| *d*-Glutamic acid (D) | Creatinine | *d*-α-Aminophenylbutyric acid |
| *dl*-Aspartic acid | Sarcosine | |
| | Thiourea | |

severally travel different metabolic pathways. For metabolic studies it is frequently desirable, and in some cases necessary, to employ independent tracers for the two parts — for example, $N^{15}$ for the amino group and deuterium for the carbon chain. Table 5 includes a number of such doubly marked amino acids in which the deuterium is bound to the carbon atom of the side chain.

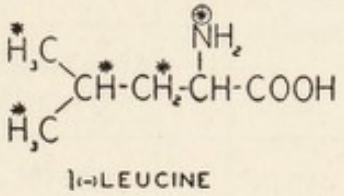
All amino acids except glycine contain an asymmetric carbon atom, and in the natural varieties only one of the optical isomerides is present. As our experiments were designed to simulate natural conditions, the synthetic amino acids were resolved, and in most cases only the natural form was fed.

If the body proteins were in a static condition, the nitrogen excreted should represent primarily that of the degraded food constituents. This concept, however, does not seem to be correct.

Two experiments will now be presented in which the path-

way of natural amino acids was investigated by adding small amounts of the isotopic analogues to the stock diet of rats. In all experiments the animals were in nitrogen equilibrium and constant in weight. The diet contained 16 per cent of casein, an amount known to be more than ample for maintenance.

In the first series *l*(−)-leucine (25), and in the second series glycine (26), were added for three days in amounts corresponding to 25 mg. (about 7 per cent of the total intake) of nitrogen per day. The leucine was doubly marked (VI).




l-(+)LEUCINE

VI

The excreta were collected, and, at the end of the period, the organs were extracted with trichloracetic acid to remove all nitrogenous compounds of low molecular weight. Samples of nitrogen were liberated from all fractions and their isotope content determined. In Table 6 are given the percentages of the labeled nitrogen distributed in the various parts of the animal. The material was well absorbed; less than 3 per cent of the heavy nitrogen appeared in the feces.

According to the concept of independent exogenous and endogenous types of metabolism, most of the dietary nitrogen should have appeared directly in the urine. This was not the case. With leucine less than one-third, with glycine less than one-half was excreted; the balance remained in the body. Of the isotopic nitrogen retained, the non-protein nitrogen fraction contained only a small amount. The proteins must, therefore, have been involved in very rapid chemical reactions resulting in the fixation of at least half of the nitrogen of the added amino acids. As the weight of the animals had remained

constant, the processes in question must have been so balanced as to avoid ultimate change in the amounts of the proteins.

The distribution of ingested nitrogen, shown in Table 6, is typical for all amino acids so far investigated. Leucine, glycine, glutamic acid, aspartic acid (66), tyrosine (27), and

TABLE 6

FATE OF AMINO NITROGEN IN NORMAL ADULT RATS

(Isotopic amino acids — corresponding to 25 mg. N per day for 3 days — were added to normal stock diet.)

| MATERIAL ANALYZED | PER CENT OF ADMINISTERED $N^{15}$ RECOVERED | |
|---|---|---|
| | After Feeding *l*(−)-Leucine | After Feeding Glycine |
| | *per cent* | *per cent* |
| Excreta | | |
| Feces | 2.2 | 2.6 |
| Urine | 27.4 | 40.8 |
| Animal Body | | |
| Non-protein N | 8.2 | 11.1 |
| Protein N | 56.5 | 44.3 |
| Total | 94.3 | 98.8 |

lysine (28) all behaved in a similar manner, and one is justified in concluding that the result indicates the general pathway of dietary nitrogen in the animal.

Different organs are not equally effective in the fixation of dietary nitrogen (Table 7). The isotope concentration in the protein nitrogen of the various organs indicates the relative activity of the respective proteins in regard to the acceptance of dietary protein nitrogen. The proteins of the internal organs, of serum, and of the intestinal tract are the most active; the proteins of muscles show less activity, but, as they constitute by far the largest part of the animal, the low concentration actually represents a high absolute amount of isotope. In fact,

two-thirds of the nitrogen deposited by the animal was recovered in the muscles, and only one-third in the combined internal organs. As might be expected, the proteins of the skin show the least activity.

The presence of isotopic nitrogen in the proteins may be

TABLE 7

$N^{15}$ CONTENT OF PROTEIN NITROGEN OBTAINED FROM DIFFERENT ORGANS AFTER FEEDING *l*(−)-LEUCINE AND GLYCINE (25 MG. N PER DAY)

(Calculated for 100 atom per cent $N^{15}$ in compound administered.)

| Organ | After Feeding *l*(−)-Leucine | After Feeding Glycine |
|---|---|---|
| Serum | 1.67 | 1.78 |
| Hemoglobin | 0.29 | 0.46 |
| Liver | 0.94 | 1.40 |
| Intestinal wall | 1.49 | 0.98 |
| Kidney | 1.38 | ... |
| Heart | 0.89 | ... |
| Spleen | 1.10 | ... |
| Testes | 0.77 | ... |
| Skin | 0.18 | ... |
| Muscle | 0.31 | 0.29 |

due to one of two processes. Either the amino acid of the diet had replaced the same species of amino acid in the proteins — leucine had replaced leucine — or the nitrogen of one amino acid was employed for the amino acid of another, which, in turn, replaced its like in the protein.

In order to investigate such reactions, one has to isolate pure amino acid samples and determine their isotope content. Some of the tissue proteins of the rats were hydrolyzed and pure samples of amino acids were secured (Table 8). The samples of leucine obtained from the bodies of the leucine-fed animals contained a very high concentration of marked nitrogen, which indicated that the dietary leucine actually had replaced leucine in the proteins. Moreover, as the isolated leucine also con-

tained deuterium, not only the amino group but the whole molecule was introduced. The results with glycine were similar; glycine replaced glycine in the proteins.

Leucine and glycine, however, were not the only isotopic amino acids isolated from the organ proteins. When leucine

TABLE 8

BIOLOGICAL NITROGEN TRANSFER IN PROTEINS: $N^{15}$ CONTENT OF AMINO ACIDS ISOLATED FROM PROTEINS OF RATS GIVEN *l*(−)-LEUCINE AND GLYCINE

(Calculated for 100 atom per cent $N^{15}$ in compound administered.)

| AMINO ACID | LIVER | | INTESTINAL WALL | | MUSCLE AND SKIN | |
|---|---|---|---|---|---|---|
| | Leucine | Glycine | Leucine | Glycine | Leucine | Glycine |
| Leucine | 7.95 | ... | 7.35 | ... | 1.90 | ... |
| Glycine | 0.74 | 8.88 | 0.63 | 4.23 | ... | 1.05 |
| Tyrosine | 0.50 | 0.46 | 0.94 | ... | 0.20 | 0.13 |
| Glutamic acid | 1.85 | 0.89 | 2.97 | ... | 0.89 | 0.27 |
| Aspartic acid | 1.16 | 0.73 | 2.30 | ... | 0.70 | 0.20 |
| Arginine | 0.89 | 0.78 | 0.43 | ... | 0.25 | ... |
| Lysine | 0.06 | ... | 0.07 | ... | ... | 0.09 |
| Amide N | 0.78 | 1.45 | 1.24 | ... | ... | 0.51 |

was fed, the isolated glycine contained the tracer, and the reverse was found when glycine was given. Leucine had yielded nitrogen to glycine and glycine had done so to leucine. Almost all other amino acids isolated contained heavy nitrogen. The results (Table 8) show that the amino acids continuously interchange nitrogen atoms.

The amino acids most active in respect to the acceptance of nitrogen from others are the two dicarboxylic acids, glutamic and aspartic acids. In all our experiments these two compounds consistently had the highest isotope content of all those which accepted nitrogen. Of the two, glutamic acid reacted somewhat more rapidly.

Among the many amino acids investigated, lysine was found

to be an exception. It has never been found to contain marked nitrogen. Its relation to these processes will be discussed later.

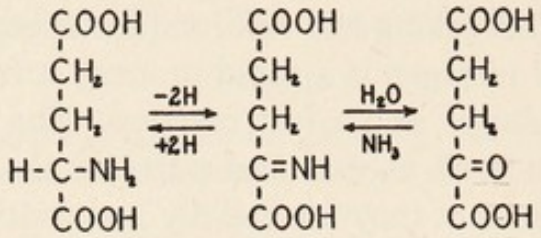
The replacement of the amino acids of tissue proteins by their dietary analogues and the transference of nitrogen must involve the rapid breaking and reformation of peptide linkages. As the uptake of nitrogen is a rapid process, it follows that the opening and closing is also a fast reaction. The peptide bonds have to be considered as essential parts of the proteins, and one may conclude that they are rapidly and continually opened and closed in the proteins of normal animals. The experiments give no direct indication as to whether the rupture is complete or partial. However, it is difficult to visualize a simultaneous attack upon all peptide linkages by enzymes.

The synthesis of amino acids took place even when they were abundantly supplied in the diet. For instance, glutamic acid, of which large amounts were newly formed, constitutes about 20 per cent of the dietary casein, and the rats were therefore certainly not in a state of nutritional glutamic acid deficiency. The synthesis of amino acids, like that of fatty acids discussed in the first lecture, proceeds even when there is no obvious need for it.

The isotope method shows the end result of a chemical process, but it does not indicate the nature of the chemical reactions involved; it does not directly reveal the mechanism of nitrogen transfer. There are, however, some results which throw some light on at least one of the links in this chain of reactions.

An important step in the degradation of amino acids is their deamination to the corresponding keto acid (VII). The amino acid is first dehydrogenated by the enzyme amino acid oxidase to the corresponding imino acid. This hydrolyzes spontaneously into the keto acid and ammonia, which is then converted into urea in the liver. These steps are formulated as reversible to indicate that the mixture of keto acid and ammonia would form the imino acid which, when hydrogenated, gives rise to

the amino acid. The formation of an amino acid by the reduction *in vitro* of a mixture of keto acid and ammonia has been effected by Knoop and Oesterlin (29), and has also been



VII

employed in the preparation of many of our isotopic amino acids. If, according to this scheme, ammonia liberated from one amino acid by dehydrogenation reacts with another keto acid, a new amino acid would be formed. Von Euler and his collaborators (30) have recently described a specific enzyme capable of carrying out the synthesis of glutamic acid from α-ketoglutaric acid and ammonia.

The hypothesis that ammonia is an intermediate in the transfer of amino groups from one amino acid to others was subjected to experimental test. If isotopic ammonia be given to rats, it must merge with the hypothetical endogenous ammonia, and in the synthesis of new amino acids, isotopic nitrogen should enter the amino group.

Rats were treated under conditions identical with those described before, except that isotopic ammonium citrate was added to the diet for nine days, instead of isotopic amino acids for three days. The amino acids (glycine, aspartic acid, glutamic acid) isolated from the proteins of these animals in fact contained the nitrogen marker (Table 9); glutamic acid had the highest isotope content of the three. As was to be expected, the nitrogen of the dietary ammonia was introduced to only a slightly greater extent in nine days than that of dietary amino acids was in three days; most of it was directly converted into urea.

Though the results strongly suggest ammonia as an intermediate, other mechanisms cannot be excluded. A reaction resulting in the transfer *in vitro* of nitrogen among amino acids without ammonia as an intermediate has recently been

TABLE 9

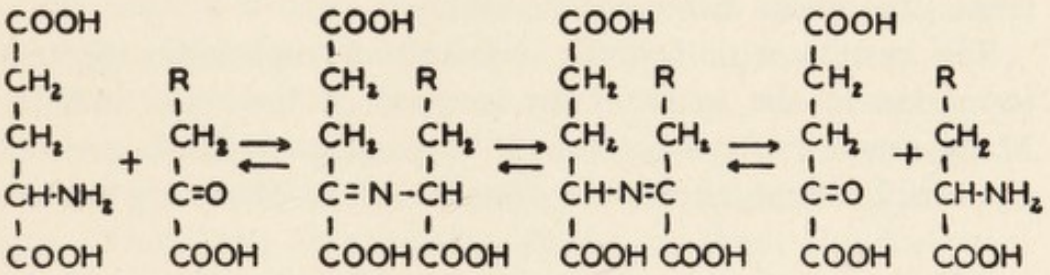
FORMATION OF ISOTOPIC AMINO ACIDS IN RATS GIVEN ISOTOPIC AMMONIA AS AMMONIUM CITRATE (25 MG. N PER RAT PER DAY FOR 9 DAYS)

(Values calculated for 100 atom per cent $N^{15}$ in administered ammonia.)

| Protein Constituent | Liver | Wall of Intestinal Tract |
|---|---|---|
| Total protein N | 1.10 | ... |
| Amide N | 1.10 | 0.95 |
| Glutamic acid | 2.30 | 1.10 |
| Aspartic acid | 1.51 | ... |
| Glycine | 0.95 | 0.55 |

described by Herbst and Engel (31). Keto acids may condense not only with ammonia to form imino acids but also with amino acids to form substituted imino acids. In these compounds shift of the double bond may occur, and hydrolysis results in the formation of two amino acids. One of these is new and contains the side chain of the keto acid employed.

Braunstein and Kritzmann (32) have provided evidence



VIII

for the biological occurrence of this general process, now called transamination (VIII), and have demonstrated the presence of a transaminating enzyme in muscle. This enzyme

is highly specific in so far as one of the pairs of the interacting compounds has to be either glutamic acid or aspartic acid, or one of the corresponding ketonic derivatives.

The two enzymatic mechanisms of von Euler and of Braunstein, both of which involve dicarboxylic acids, offered an attractive explanation for our isotope results. The ammonia liberated by deamination of amino acids was incorporated by von Euler's enzyme into glutamic acid, and the nitrogen of the latter was transferred by transamination to other amino acids. The high isotope content of glutamic acid, suggesting its central position in the metabolism, can be taken as support of this view.[1]

Isotopic ammonia can be employed for the study of the activity of body proteins of animals on a diet entirely free of proteins and amino acids. With the animals that had received amino acids and proteins, the replacement of nitrogen might have occurred before the dietary amino acids had reached the body proteins. The occurrence of amino shift without the intake of amino acids or proteins would further testify to the intense activity of the body proteins.

Immature rats were kept on a diet of cornstarch, lard, and isotopic ammonium citrate (34). On this unnatural diet the animals, of course, lost weight rapidly; but, despite the rapid disappearance of body proteins, new amino acids were introduced into the proteins (Table 10). As in previous experiments, the lysine contained no isotope.

The results with isotopic ammonia do not imply protein formation in the sense of an increase of the total amount. Many attempts have been made to employ ammonia or urea as nutritional substitutes for proteins (35). There are numer-

[1] This interpretation, however, is not entirely satisfactory. Cohen (33) has shown that the occurrence of transamination is more restricted than was originally assumed by Braunstein. Only glutamic acid, aspartic acid, and alanine, but none of the other amino acids, have been shown to take part in this reaction. The occurrence of the isotope in the other amino acids of our rat proteins can thus be attributed to neither the enzyme of von Euler nor that of Braunstein. Other mechanisms must be investigated.

ous reasons, most of which will become apparent later, why ammonia cannot fulfill that function. The formation of proteins requires, in addition to nitrogen, certain carbon chains, some of which the animal cannot form. The present experi-

TABLE 10

COMPOUNDS FROM IMMATURE RATS GIVEN LOW PROTEIN DIET AND ISOTOPIC AMMONIA

(Calculated for 100 atom per cent $N^{15}$ in the ammonia administered.)

| Compound Isolated | $N^{15}$ Excess |
|---|---|
| | *atom per cent* |
| Amide N | 94.1 ± 2.5 |
| Glycine | 41.3 " |
| Glutamic acid | 70.2 " |
| Aspartic acid | 55.3 " |
| Proline | 30.6 " |
| Histidine | 9.9 " |
| Lysine | 2.5 " |
| Arginine | 27.2 " |
| "Urea" from arginine | 57.0 " |
| Ornithine from arginine | 3.3 " |
| Creatine | 24.8 " |

ments demonstrate merely that ammonia can take part in nitrogen transfer.

The studies hitherto discussed concern the tissue proteins, which are ill-defined mixtures. It has been suggested (36) that the results with isotopes were due to the presence in tissues of limited amounts of "dispensable reserve proteins," but had not involved the "fixed" proteins with specific functions. There has been much controversy about the presence of "reserve proteins"; the most cogent evidence, advanced by Luck (37), points strongly against their existence.

This question could be tested by isolating from the experimental animals such proteins as may be considered to be specific. As such a plan is hampered by the scarcity of the

isotopic marker, recourse was had to immunological procedures. Experiments were carried out (66), in collaboration with Dr. M. Heidelberger, on the chemical reactivity of the

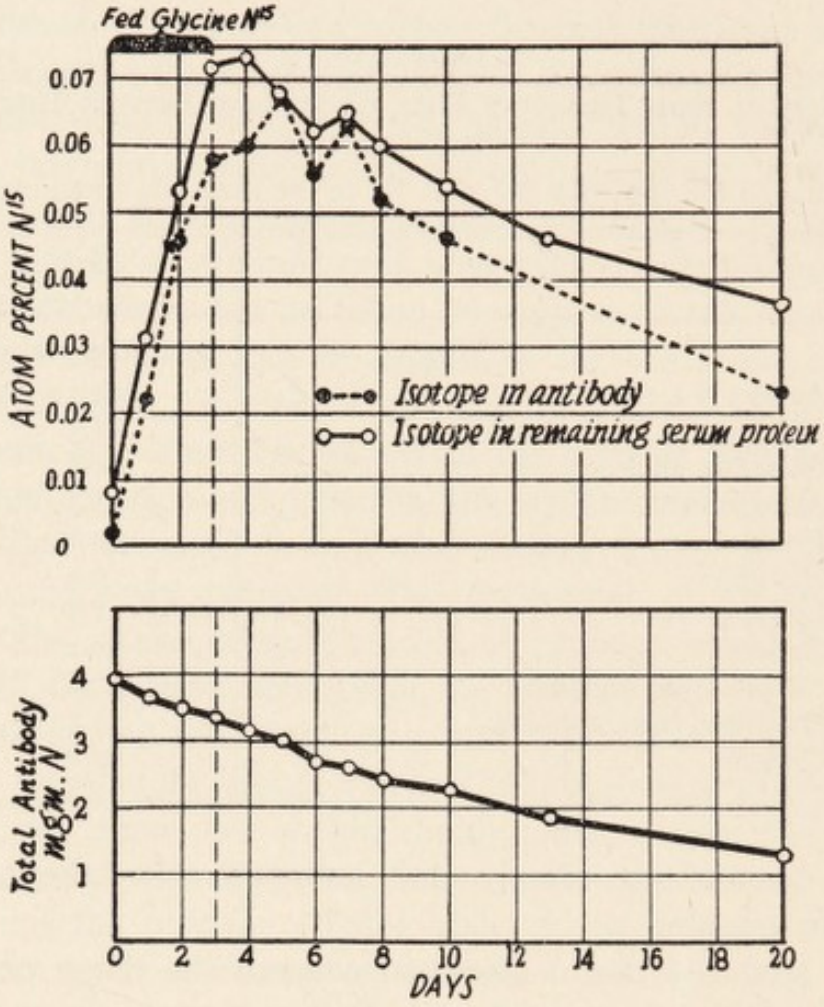



FIG. 5. — *Lower curve*, spontaneous decrease of circulating antibody in rabbit immunized with type III pneumococcus. *Upper curve*, introduction and disappearance of isotopic nitrogen in circulating antibody and serum proteins.

specific antibody protein in immunized animals. A rabbit was immunized by repeated injections of pneumococcus type III until the serum had a high antibody content. After the injections were discontinued, the antibody concentration decreased, and while it was declining, small amounts of isotopic

glycine were added to the diet for three days. Samples of serum were taken daily, antibody was precipitated (38) with the nitrogen-free type III specific polysaccharide, and its isotope concentration determined. The results are given in the dotted curve of Figure 5.

When isotopic amino acid was added, the concentration of marked nitrogen in the antibody increased immediately, but diminished steadily after the addition of glycine to the diet was discontinued. The solid line of the upper part of Figure 5 gives the isotope concentration of the other serum proteins. The two almost coincident curves indicate that the specific antibody reacts, in respect to the acceptance of dietary nitrogen, like the average serum protein.

Chemical reactions had thus occurred among the units of the antibody protein, resulting in the uptake of dietary nitrogen in the first period and its removal in the second. The formation of the isotopic proteins occurred during the period in which the antibody content of the serum was steadily decreasing. The processes in question could not have changed the specific structure of the antibody protein.

The results are similar to those observed with all the total proteins of tissues. Another rabbit was immunized and fed with isotopic glycine for three days. Total antibody and other serum proteins were hydrolyzed and several amino acids were isolated. From Table 11 it can be seen that antibodies and other plasma proteins are subject to the same type of rapid regeneration as the proteins of tissues. The high isotope content of glycine from the antibody indicates the replacement of antibody glycine by dietary glycine. The presence of isotope in the other amino acids shows nitrogen shift.

The finding of rapid molecular regeneration of serum proteins is in accord with the theory of Whipple and his collaborators (39), who consider the rapid replenishment of plasma protein lost by plasmapheresis to be due to a dynamic equilibrium between the proteins of blood and those of tissues.

These investigators summarize their findings by saying, "The evidence now appears convincing that under certain conditions (and probably more or less continuously) there is a 'give and take' between body proteins and plasma proteins." This con-

TABLE 11

$N^{15}$ CONTENT OF AMINO ACIDS FROM SERUM OF RABBITS GIVEN ISOTOPIC GLYCINE

(The values are given in atom per cent and are calculated for 100 atom per cent in the glycine administered.)

| Compound Isolated | Antibody | Globulin | Euglobulin | Pseudoglobulin | Albumin |
|---|---|---|---|---|---|
| Total protein | 0.89 | 1.9 | 2.41 | ... | 1.5 |
| Glutamic acid | 0.77 | 1.7 | 2.91 | 1.4 | 2.37 |
| Aspartic acid | 0.49 | 1.4 | ... | ... | 2.31 |
| Arginine | ... | 0.65 | 1.8 | 0.49 | 1.4 |
| Leucine fraction | 1.2 | ... | ... | ... | ... |
| Glycine | 6.92 | ... | ... | ... | ... |

The error of the analyses is ±0.06.

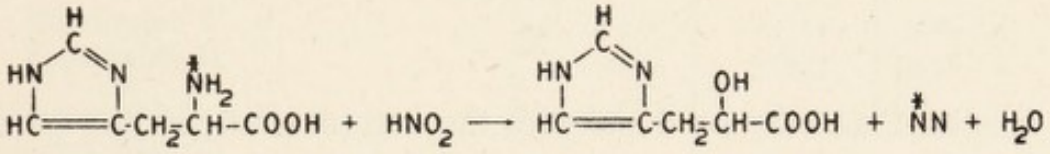
cept of a dynamic state of blood and organs, which will be discussed more fully in the last lecture, is supported by all our results.

While the rates of regeneration in the living organism may differ considerably, it is questionable whether any of its proteins are ever, even temporarily, in a static condition. Even the tendons of a rabbit, the proteins of which may be considered to have a purely structural function, revealed a slow but significant activity (66).

Nutritionally, amino acids are classified as dispensable and indispensable. Rose (40) has recently defined an indispensable dietary component as one which is not synthesized by the animal organism out of the materials ordinarily available, at a speed commensurate with normal growth. This admirable definition relates to nutritional requirements in growth but not to metabolic behavior in adult animals.

Of the ten indispensable amino acids known, six have already been investigated with the aid of isotopes: leucine, histidine, lysine, phenylalanine, methionine, and arginine. It has been shown that leucine steadily yields its amino nitrogen to, and that its keto analogue accepts amino nitrogen from, other compounds. Similarly, when isotopic tyrosine was fed, histidine isolated from the tissue proteins contained marked nitrogen. Histidine contains three nitrogen atoms, of which two are located in the imidazole ring and one in the alpha position of the side chain.

In order to locate the isotope in the molecule, the histidine was converted by means of nitrous acid into imidazole lactic acid. As this contained only normal nitrogen (41), all of the istope must have been in the $\alpha$-amino group (IX).



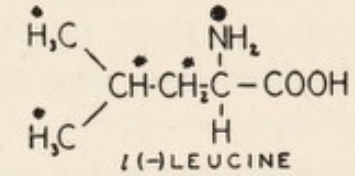

IX

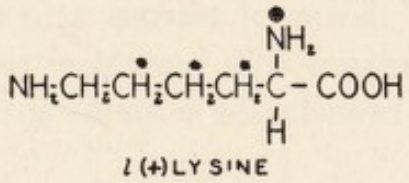
Some indispensable amino acids, including histidine, but not lysine, can be replaced in the rat's diet by some of their deamination products, such as the corresponding $\alpha$-hydroxy or $\alpha$-keto acids (42). When in need of histidine, a rat can form it by replacing the hydroxyl group of imidazole lactic acid by an amino group. As the present experiments, like all the others, were performed with full grown rats that had an ample supply of dietary histidine, the results may be taken as additional support for the contention that, if the starting materials are available, all chemical reactions which the animal is capable of performing are carried out continually.

Like leucine and histidine, which are steadily deaminated, lysine can yield its nitrogen for the formation of other amino

acids. However, the reverse of this process does not take place with lysine, which is never regenerated.

The steady process of nitrogen shift can be measured directly and accurately with the use of doubly marked amino acids. Leucine and lysine, so synthesized that the chain contained carbon-bound deuterium and the amino group $N^{15}$ (X), were




X

resolved, and the products with the natural configuration were added to the customary casein-containing stock diet. The isotopic compounds, during absorption and deposition, merged with ordinary, non-isotopic $l(-)$-leucine or $l(+)$-lysine which had originated in casein and tissues. The samples of leucine and lysine isolated from the bodies were inseparable mixtures of marked and normal leucine and lysine. If only biological mixing had occurred, the concentrations of both isotope markers would have been lowered, but their ratio would have been unchanged. If, however, $\alpha$-amino nitrogen had been removed and replaced by normal nitrogen from other sources, this ratio $D:N^{15}$ should be higher in the isolated material.

Table 12 shows the results. About one-third of the original leucine nitrogen had been replaced by other nitrogen, whereas the lysine deposited had retained all its original $\alpha$-amino nitrogen (28). Leucine was thus involved in the reversible nitrogen shift; lysine, once deaminated, was not resynthesized. The results indicate that some of the chemical reactions of in-

dispensable amino acids in normal animals are fundamentally the same as those of dispensable amino acids. The peptide linkages in the protein pattern are steadily broken, and the amino acids liberated are constantly replaced by identical acids of exogenous origin.

TABLE 12

$N^{15}$ AND DEUTERIUM CONTENT OF LYSINE AND LEUCINE FROM PROTEINS OF RATS GIVEN ISOTOPIC *l*(+)-LYSINE AND ISOTOPIC *l*(−)-LEUCINE

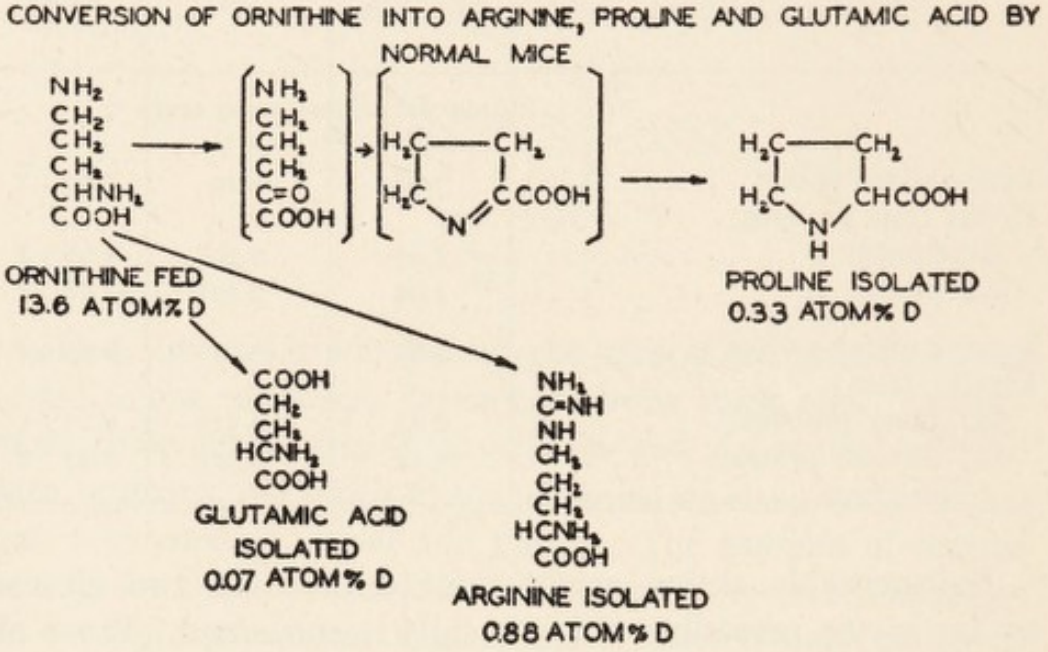
| Substance Analyzed | Deuterium | $N^{15}$ | Ratio D : $N^{15}$ |
|---|---|---|---|
| | *atom per cent excess* | *atom per cent excess* | |
| Lysine added to diet | 7.98 | 2.20 | 3.63 : 1 |
| Lysine from proteins: | | | |
| Experiment I | 1.07 | 0.294 | 3.66 : 1 |
| Experiment II | 1.04 | 0.275 | 3.78 : 1 |
| Leucine added to diet | 3.60 | 6.54 | 0.55 : 1 |
| Leucine from: | | | |
| (a) Liver proteins | 0.44 | 0.518 | 0.85 : 1 |
| (b) Carcass proteins | 0.12 | 0.124 | 0.97 : 1 |

Indispensable amino acids seem to fall into two classes as far as the reversible nitrogen shift is concerned. Those of one class, represented by leucine and histidine, continually yield and accept nitrogen. They contain two metabolically independent chemical groupings, of which one — the carbon chain — is indispensable; the other — the amino group — may be derived from some other physiological substance. The indispensability of leucine and histidine seems to inhere in the inability of the animal to build the particular carbon chain.

The other class is at present represented only by lysine. This amino acid is not reversibly involved in the nitrogen shift and may be regarded as a single indispensable chemical unit which has to be supplied as such to the animal. It cannot be replaced

by the $\alpha$-N-methyl or $\alpha$-hydroxy analogues, nor by $d(-)$-lysine (42).

The chemical reactivity of proteins in normal animals is not restricted to the amino group; the carbon chains may participate in the constant flux of transformations. An experiment has been carried out (43) on the metabolic fate of ornithine, synthesized with deuterium in the carbon chain. This was added to the stock diet of mice, and various amino acids were isolated from the body proteins. The arginine contained con-




XI

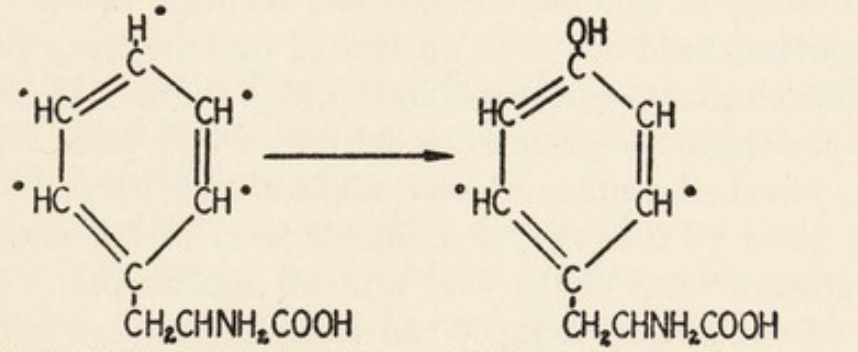
siderable amounts of deuterium; hence arginine was prepared by the animal from ornithine, and the newly formed amino acid had replaced arginine in the peptide linkage. As the proline also contained deuterium (44), it appears that some of the ornithine was cyclised; the compounds shown in parentheses are probable intermediates (XI). The newly formed proline, like arginine, also entered the proteins. In addition, the glutamic acid contained a small but significant amount of deuterium, the presence of which points to oxidative degrada-

tion of the terminal aminomethyl group, either directly or by way of proline and pyrrolidone carboxylic acid.

Another example of such conversion has been studied with phenylalanine (45), which is an indispensable amino acid, and with tyrosine, which is not. The possibility that an animal in need of tyrosine may produce it from phenylalanine has been discussed by Rose (40).

Deuterium was introduced into phenylalanine by the action of warm deuterio sulfuric acid. All the hydrogen atoms of the benzene ring then contained deuterium, which, however, hardly entered any other portion of the molecule. The tyrosine isolated from the proteins of animals that had been given this isotopic amino acid contained so high a concentration of deuterium as to indicate that at least 30 per cent of the tyrosine present in the liver proteins had been derived from dietary phenylalanine (XII). This result was calculated on the basis

CONVERSION OF PHENYLALANINE INTO TYROSINE BY RATS




PHENYLALANINE ADMINISTERED — TYROSINE ISOLATED

16.0 ATOM % DEUTERIUM — 1.25 ATOM % DEUTERIUM

XII

of a previous observation (46) that the hydrogen (or deuterium) atoms in the positions *ortho* to the hydroxyl group are labilized under the conditions of acid hydrolysis.

In all these experiments, the newly formed amino acids were already present in the casein of the diet. In order further to

test the influence of the dietary amino acids, another experiment was performed, in which rats were given a large amount of normal non-isotopic tyrosine in addition to the isotopic phenylalanine, so that the animals were undoubtedly oversupplied with tyrosine. In this case, too, deuterio tyrosine was formed in considerable quantities. This result furnishes another example of the generalization that all the reactions which the organism is capable of performing with protein constituents are carried out continually, even when the total amount and constitution of the proteins stay constant.

The rapidity with which the amino acids in the proteins of normal animals react is illustrated by the behavior of glutamic acid (66), which is not only the most reactive amino acid but also the most abundant in animal proteins. In spite of the large and continual influx of glutamic acid from the dietary casein, the metabolic reactions of this compound are so rapid that all attempts to trace it, after feeding it in isotopic form, have failed. There is no evidence as to whether the replacement of isotopic nitrogen had occurred before or after the glutamic acid had been introduced into the tissue proteins, but it is clear that all the glutamic acid of the animal, including that present in the proteins, is subject to very rapid regeneration. In experiments in which isotopic *dl*-tyrosine and *l*-leucine were given (Table 13), the isotopic level of the nitrogen of the glutamic acid of the liver proteins was almost as high as that of the urinary urea. When isotopic glycine was fed, the corresponding ratio was lower, but still strikingly high. The nitrogen of the urea is, of course, derived not from merely one amino acid, but may be regarded as a representative sample of that of all biologically reacting amino acids of exogenous and endogenous origin.

Similarly, the nitrogen introduced by amino shift into one amino acid also originates from all others. One may postulate, as an approximation, that under our experimental conditions the nitrogen mixture introduced into any amino acid had roughly

the same isotope concentration as that employed for urea formation. Thus one cannot expect to isolate a sample of glutamic acid, or of any amino acid other than that fed, with an isotope concentration higher than that of the urinary urea. If the isotope concentrations of an isolated amino acid and of the

TABLE 13

CONCENTRATION OF $N^{15}$ IN GLUTAMIC ACID OF LIVER AND URINARY UREA AFTER FEEDING ISOTOPIC AMINO ACIDS

(Calculated for 100 atom per cent in the compound fed.)

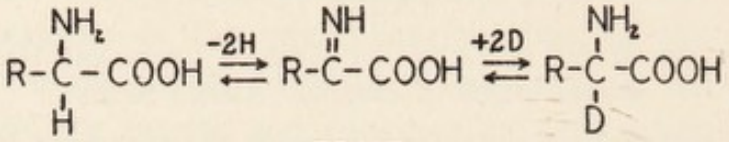
| Amino Acid Administered | Glutamic Acid from Liver | Urinary Urea |
|---|---|---|
| | *atom per cent $N^{15}$ excess* | *atom per cent $N^{15}$ excess* |
| *dl*-Tyrosine | 2.80 | 3.35 |
| *l*(−)-Leucine | 1.85 | 2.44 |
| Glycine | 0.89 | 1.88 |

urea were equal, one might assume that the nitrogen in the amino acid had been completely replaced with nitrogen from the metabolic pool. The values in Table 13 indicate that an extensive replacement had occurred within the short periods (3 days) of the experiments.

A direct method for measuring the rapidity of these reactions was provided by the use of heavy water. The reversible deamination requires not only replacement of the α-amino nitrogen but also of the hydrogen atom directly bound to the α-carbon atom. According to the scheme described earlier (VII), biological deamination requires primary dehydrogenation, and the reverse reaction, amination, is associated with hydrogenation (XIII). If this reversible reaction be carried out in a medium of heavy water, the deuterium of the solvent enters the reaction and ultimately becomes fixed in the amino acid. When all the glutamic acid molecules in the system have been subjected to this reversible reaction, all α-hydro-

gen atoms of the mixture of molecules must have the same isotope concentration as the water.

Fasting rats were injected with heavy water. After twenty-four hours all amino acids, except lysine, isolated from the proteins contained deuterium. Glutamic acid showed the



XIII

highest degree of replacement of hydrogen (66); the isotope content of a sample isolated from the liver corresponded to 80 per cent of that attained in experiments of ten and sixty days' duration (47). This suggests that at least 80 per cent of the glutamic acid of that organ had been subjected to this reversible dehydrogenation-hydrogenation reaction in the twenty-four-hour period.

The results here reported give a dim idea of the rates at which some parts of the protein molecules react in a normal animal. It would be hopeless to attempt to investigate the reactions of entire protein molecules. Too many linkages and metabolically active groups, which may react at very different rates, are involved. Even with such convenient tools as the isotopes, one cannot investigate more than a few groups at a time.

# III

## THE RÔLE OF STRUCTURAL ELEMENTS IN THE FORMATION OF EXCRETORY PRODUCTS

IN THE preceding lectures an attempt has been made to show how extensively the body constituents of living organisms are subject to regeneration. It is interesting to try to correlate processes of regeneration with those responsible for the formation of excretory products.

Most of the end products of nitrogen metabolism are removed in the urine. The quantitative and qualitative composition of the urine has been the subject of investigation since the beginning of scientific medicine. It has always been hoped that detailed knowledge of the urine constituents and their formation would add important insight into the chemical behavior of the living cell.

The main nitrogenous constituents of the urine are urea, ammonia, creatinine, and uric acid. The formation of these substances is interrelated with those processes to which the nitrogenous tissue components are subjected.

It has been pointed out that the nitrogen excreted by a normal animal in nitrogen equilibrium is a sample of the pool originating from the constant and rapid chemical interaction of food and body proteins. The formation of the excreted compounds is closely related to the chemical processes undergone by the proteins and has been found to be associated with some reactions of definite groupings of these large molecules.

From the work of Bollman and his associates (48) we know that urea formation occurs in the liver. For a long time it was assumed that urea was formed in the body from carbon dioxide and ammonia by direct dehydration of ammonium carbonate,

but in 1932 Krebs and Henseleit (49) showed by tissue slice experiments that the process is much more complicated. Urea formation was found to be accelerated by the amino acids ornithine or citrulline. These authors offered the explanation that ornithine is condensed with carbon dioxide and one mole of ammonia to form citrulline, which by reaction with a second mole of ammonia is converted into arginine. The enzyme arginase, present in the liver of mammals, in turn splits arginine into urea and ornithine. Urea is transported to the kidney for excretion, and the ornithine is again employed in a continuous cycle of urea formation.

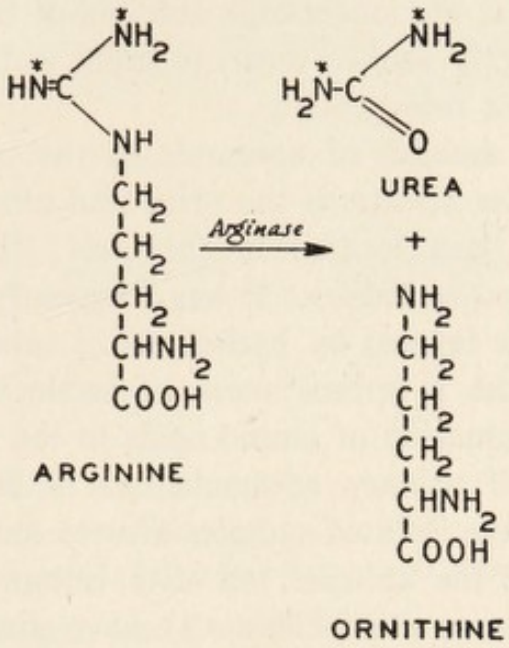
This theory has obtained ample support from isotope work. The assumption that the carbon of urea is derived directly from carbon dioxide was proved in tissue slice experiments by Evans and Slotin (50), who employed radioactive $CO_2$, and by Rittenberg and Waelsch (51), who used the stable isotope $C^{13}$.

In our experiments with living animals, the administration of isotopic ammonia or isotopic amino acids leads to the excretion of isotopic urea. Arginine isolated from the proteins of these animals also contains the nitrogen isotope. As arginine contains 4 nitrogen atoms, it is necessary to degrade it in order to locate the position of the isotope (XIV). This is readily done with the enzyme arginase, which, as mentioned, has been postulated to be involved in the urea cycle. The urea so obtained represents the nitrogen of the amidine group of the arginine. As it contained isotope, a reaction must have occurred by which the original amidine group of the arginine had been replaced by a new one, formed with nitrogen from other sources.

It appears almost certain that the arginine samples isolated had been involved in urea formation. The urea cycle postulates the intermediary action of arginase, which is known to react readily with free arginine but very slowly or not at all with arginine bound in proteins. One thus has to conclude that the

arginine isolated from the proteins had, like other amino acids, been steadily liberated and reintroduced into the proteins. While in the free state, it had been degraded by arginase to ornithine, which in turn was employed for the regeneration of arginine.

DEGRADATION OF ISOTOPIC ARGININE ISOLATED FROM PROTEINS OF RATS GIVEN ISOTOPIC AMINO ACIDS




XIV

The steady conversion of ornithine into arginine and the introduction of the latter into the body proteins has already been discussed. The replacement of the amidine group of arginine, which is one phase of the urea cycle, is extremely rapid. When arginine with an isotopic amidine group is administered, the arginine of the liver proteins has a low isotope concentration (66), and more than 60 per cent of the marker is recovered in the urinary urea.

The constant formation of urea in animals has thus been traced back to the amidine group of the proteins. Urea is formed whenever arginine, like all other amino acids, is temporarily liberated by opening of peptide links. The arginine

of the proteins has thus a double function. In addition to possessing significance as an important protein constituent, it participates in the urea cycle.

In all the experiments in which isotopic ammonia and amino acids were fed, the isotope concentration of the amidine nitrogen of the protein arginine was always less than that in the corresponding urinary urea. This observation can be explained by assuming that an appreciable fraction of the newly formed arginine is directly broken down to urea, while the remainder is reincorporated into proteins.

The relative amount of ammonia in the urine varies, and depends upon the acidity of the urine and other factors. Urea formation has been located in the liver; that of ammonia occurs largely in the kidney. It has frequently been suggested that ammonia is formed by hydrolysis of urea, but recent investigations make it appear more probable that ammonia is formed by deamination of amino acids in the kidney (52).

The source of urinary ammonia can be investigated with isotopes. We have isolated samples of urea and ammonia from the urine of all the animals fed with isotopic nitrogen compounds, and the results (Table 14) have given some insight into the biological sources of ammonia. When isotopic ammonia was fed, the isotope was recovered in the urea and practically none in the ammonia. If the ammonia were directly produced from urea, the nitrogen of both fractions should have the same isotope concentration. When proteins are digested in the intestinal tract, some ammonia is formed and absorbed. This "dietary" ammonia can be excluded as a source of urinary ammonia.

When isotopic urea was fed, the urinary urea again contained practically all the isotope and the ammonia was almost normal. Urea can thus be excluded as an important intermediate in ammonia formation.

However, when certain isotopic amino acids were fed, the urinary ammonia nitrogen had an isotope concentration con-

siderably higher than that of the urea nitrogen. This finding strongly suggests that the urinary ammonia is formed by direct deamination of amino acids without urea as an intermediate. As all amino acids so far investigated have been found to take some share in ammonia production, the nitrogen

TABLE 14

ISOTOPE CONCENTRATION IN URINARY AMMONIA AND UREA

(Calculated for 100 atom per cent $N^{15}$ excess in the substance administered.)

| Substance Administered | Urea | $NH_3$ | Ratio $\frac{NH_3-N^{15}}{Urea-N^{15}}$ |
|---|---|---|---|
| | *atom per cent $N^{15}$ excess* | *atom per cent $N^{15}$ excess* | |
| Urea | 8.09 | 0.34 | 0.043 |
| $NH_3$ | 11.7 | 4.50 | 0.384 |
| *l*(−)-Leucine | 2.43 | 3.90 | 1.60 |
| Glycine | 0.90 | 1.80 | 2.00 |
| *dl*-Tyrosine | 3.35 | 9.85 | 2.94 |
| *d*(+)-Leucine | 4.19 | 15.7 | 3.75 |

of urinary ammonia, like that of urinary urea, must be derived from the mixture of diet and tissue. The nitrogen excreted as urea has to pass through the urea cycle, but the ammonia is derived from the $\alpha$-amino groups of the amino acids liberated from proteins.

Creatinine is a more abundant constituent of normal urine than ammonia. Creatine is found in muscles, where it is reversibly phosphorylated in connection with energy production.

The daily excretion of creatinine, in contrast to that of ammonia and urea, is highly constant and is only slightly influenced by change of dietary proteins. Even after the feeding of creatine, a considerable period elapses before extra creatinine appears in the urine. This was interpreted by Benedict (53) to indicate that the conversion of creatine into creatinine is not a direct process but goes via intermediates.

The work with isotopes does not support this contention. When isotopic creatine is administered, it rapidly merges with all the creatine of the body. The isotope concentration is the same, not only in creatine isolated from the muscles and internal organs, but in the creatinine of the urine (54). This creatinine must have been formed directly from the creatine and may therefore be regarded as the anhydride of a repre-

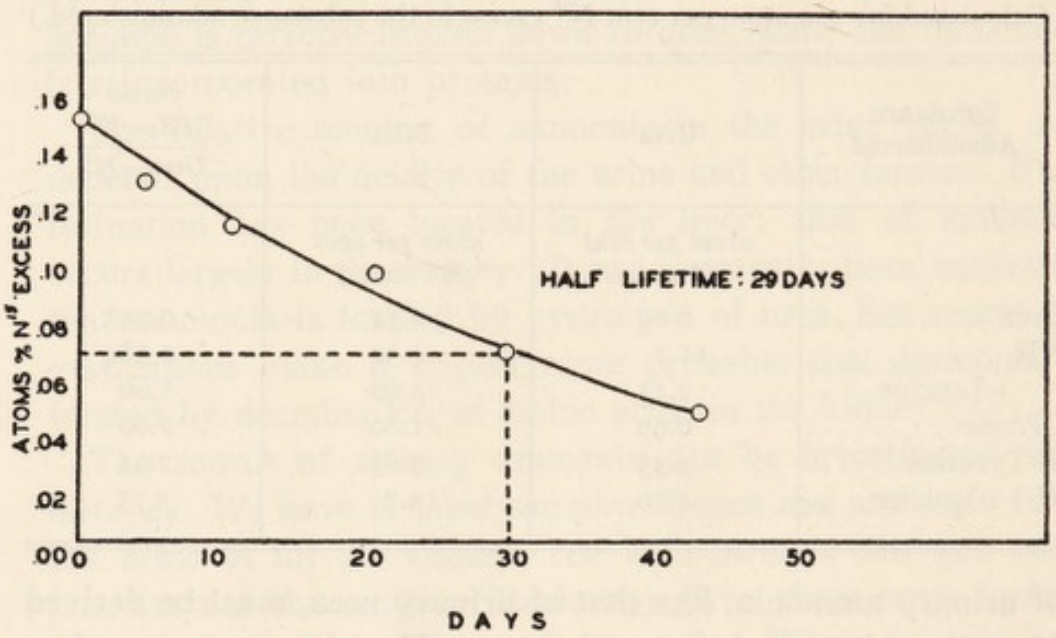



FIG. 6. — Elimination of isotopic creatinine after feeding creatine ($N^{15}$ in sarcosine). (Slightly modified from Bloch, Schoenheimer, and Rittenberg, *J. Biol. Chem.*, *138*, 156, 1941.)

sentative sample of the body creatine. Muscle creatine and urinary creatinine have thus the same origin and precursors.

It has been found possible to estimate the rate at which creatine is synthesized, by the use of isotopic creatine (55). Rats to which this compound had been administered were kept on a creatine-free diet, and the urinary creatinine subjected to isotope analysis. The rate of elimination of isotopic creatine in the form of creatinine is shown in Figure 6. The daily decrease of isotope concentration must give a measure of the amount of creatine synthesized daily, and corresponds to

about 2 per cent of the total creatine of the animal tissues. This is about the same quantity as is daily excreted as creatinine. By the thirtieth day half of the creatine molecules are replaced by newly synthesized creatine.

Creatine is not essential to the diet and must be formed from other compounds. The most probable source of its nitrogen is that of the proteins. Much work has been carried out in various laboratories with the aim of increasing creatine formation, or creatinine excretion, by feeding various proteins or amino acids to normal animals. All these attempts were fruitless; the creatine level remained practically constant. On the other hand, experiments on patients with muscular dystrophy led Brand and his associates (56) to suggest that glycine is involved in creatine formation. It is impossible here adequately to review the literature. Rose (57) has characterized the situation by stating that creatine formation cannot be increased above the physiological needs of the cell by amino acid administration. As in the study of many other biological reactions, balance studies with normal animals have thrown no light on creatine formation.

Administration of guanidoacetic acid (glycocyamine), in contrast to amino acids or proteins, increases the excretion of creatine or creatinine. Guanidoacetic acid, however, was not regarded as physiological, and little attention was given to this important fact until the substance was recently found to be a regular but minor constituent of urine and organs (58). This compound has been shown by the use of isotopes to be an intermediate in creatine formation. The feeding of isotopic guanidoacetic acid resulted in the immediate formation of isotopic creatine and creatinine (59). Creatine formation thus occurs in two steps: (1) formation of guanidoacetic acid, and (2) its methylation.

The methyl group is the simplest organic chemical grouping, and it has apparently been tacitly assumed that during biological degradation a wide variety of organic compounds

could furnish it. Recent investigations by du Vigneaud and by Borsook have shown that this is not the case.

In the mammalian body the three most abundant compounds containing methyl groups attached to a sulfur or a nitrogen atom are choline and methionine (both essential dietary constituents) and creatine (or creatinine). On a choline-free diet, rats develop a fatty liver, which may be cured by the feeding of choline (60) or of methionine (60). As shown by du Vigneaud, methionine can be replaced in the diet by its methyl-free derivative homocystine, which, however, exerts its growth-promoting effect only if choline is also present in the diet. This observation, the first experimental demonstration of a metabolic relation between two methylated compounds, suggested that the methyl groups of choline are necessary for the synthesis of methionine from homocystine (61), and was interpreted as indicating an inability of animals to form methyl groups.

The biochemical transfer of methyl groups has been directly demonstrated by Borsook and Dubnoff (62) in tissue slice experiments. Liver slices slowly form creatine from guanidoacetic acid; but when methionine is added this reaction takes place rapidly. Methionine thus constitutes a source of methyl groups for the synthesis of creatine. Unequivocal proof for the occurrence of such a shift has been furnished by du Vigneaud and his collaborators (63), who administered to rats samples of methionine and choline in which the hydrogen atoms of the methyl groups were present as deuterium. When the choline was fed, the methionine and creatine samples isolated were isotopic. After the feeding of the methionine, deuterium was found in the choline and creatine.

The results of all these experiments not only relate the metabolism of creatine to that of choline and methionine, but show the inability of the animals to form transferable methyl groups. The essential nature of choline resides only in its methyl groups, and the other part of the molecule may be derived, as shown by Stetten (24), from aminoethanol or glycine.

Methionine, on the other hand, supplies two indispensable factors, the methyl group and homocysteine, both of which have to be available from the diet. The methyl group may be administered equally well in the form of either choline or methionine. It shifts continually from one compound to another, in a manner similar to the continual transfer of amino groups among the amino acids of proteins. Those shifting methyl groups, which become incorporated in creatine, are lost to the organism by the excretion of creatinine in the urine.

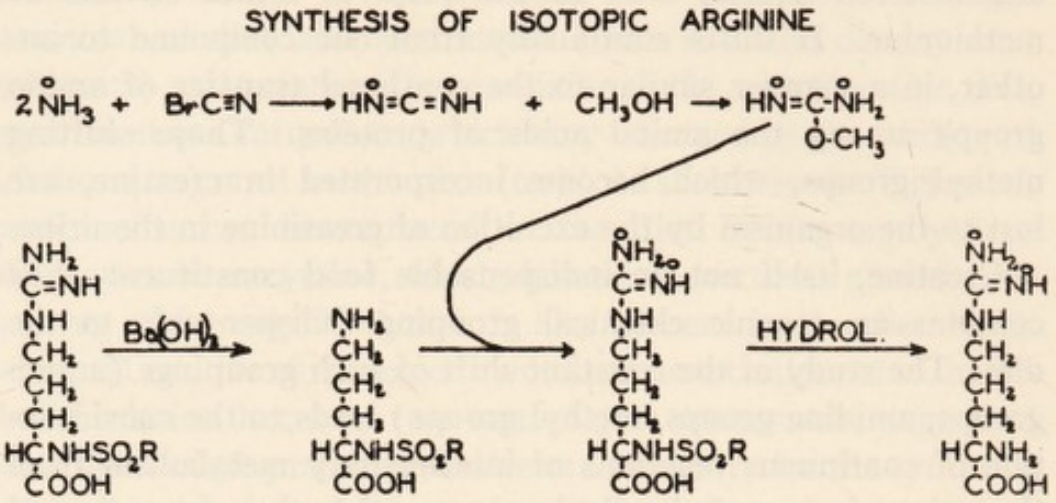
Creatine, itself not an indispensable food constituent, thus contains an organic chemical grouping indispensable to the diet. The study of the constant shift of such groupings (amino groups, amidine groups, methyl groups) leads to the consideration of continuous reactions of intermediary metabolism from the point of view of chemical groups rather than from that of the indispensability of entire molecules.

The nitrogenous part of the creatine molecule is obviously derived from proteins. Many schemes for the formation of creatine have been proposed. One of the most interesting of these is that of Fisher and Wilhelmi (64), who, on the basis of experiments with perfusion of heart muscle, concluded that arginine is a precursor.

A search for the nitrogenous precursors of creatine was undertaken by feeding a variety of synthetic isotopic compounds to normal rats, in the hope that the precursor would reveal itself by the presence of isotope in the creatine of muscles and in the creatinine of urine. Most amino acids investigated produced so little marked creatine that they could be excluded as immediate precursors. Glycine and arginine, however, were exceptions. They were so effective that they must have played an important rôle in the biological synthesis. The arginine employed for these studies was so synthesized that $N^{15}$ was only in its amidine part; that is, in the same group as is recovered as urea after administration (XV).

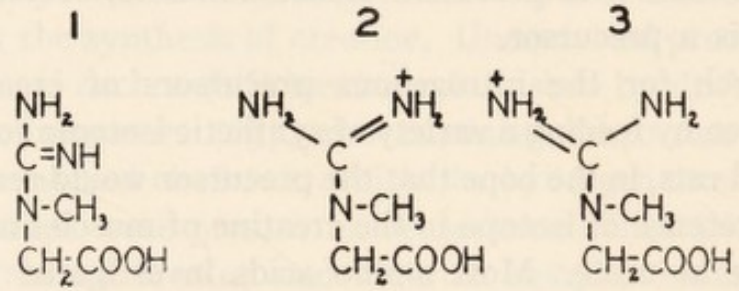
The finding by itself does not permit the formulation of the

particular steps occurring during formation of creatine. This compound has three nitrogen atoms, and it is necessary to decide which of them is related to glycine and which to argi-




XV

nine. The customary formulation (XVI, 1) of creatine suggests that each of its three nitrogen atoms differs in the way it is linked and might thus have a different chemical and metabolic function. This formula of creatine, like that of other guanido




XVI

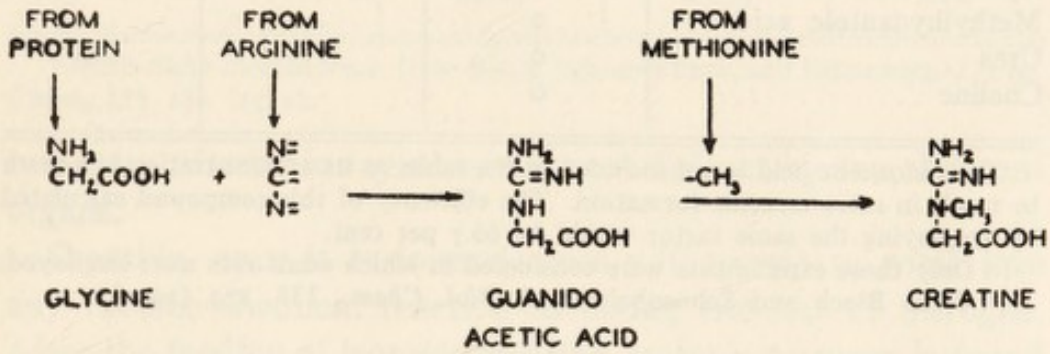
compounds, does not express all its chemical properties. The guanido group is a resonating system, and creatine is in a state which may be regarded as dynamically intermediate between the extreme forms 2 and 3. The two nitrogen atoms of the amidine groups of creatine and arginine are chemically identical and indistinguishable as to their origin. Creatine, though

it contains three nitrogen atoms, consists of only two distinct nitrogenous groupings. The amidine and sarcosine parts may be investigated separately by degradation of the creatine, and characterized by their isotope content. When treated with alkali, creatine is split into two moles of ammonia, one of carbon dioxide, and one of sarcosine. The ammonia liberated represents the nitrogen present in the amidine group of creatine.

When isotopic glycine was administered, the ammonia obtained from the creatine was normal, and the marked nitrogen was found in the sarcosine. Conversely, the feeding of the isotopic arginine gave rise to a creatine from which normal sarcosine and isotopic ammonia were obtained. It is clear that the amidine group of creatine is derived from that of arginine, and the sarcosine moiety from glycine.

The combined evidence relating to the origin of the methyl group and the nitrogenous parts of creatine demonstrates the immediate biological precursors of creatine. The amidine group of arginine condenses with glycine to form guanidoacetic acid, which, in turn, is methylated by methionine or choline (XVII).

BIOLOGICAL SYNTHESIS OF CREATINE




XVII

Quantitative calculations of the theoretical isotope content of creatine formed from the administered arginine and glycine suggested that the test substances in the diet could not have been the only precursors. A considerable number of other

biological nitrogenous compounds were tested, but all were found to be ineffective (Table 15). The most reasonable explanation for this failure is that arginine and glycine actually are the only precursors, and that not only the proteins of the

TABLE 15

FRACTION OF NEWLY FORMED CREATINE NITROGEN DERIVED FROM RESPECTIVE TEST SUBSTANCE *

| MATERIAL ADMINISTERED | FRACTION OF N DERIVED FROM TEST SUBSTANCE | | |
|---|---|---|---|
| | Of Total Creatine | Of Amidine Group | Of Sarcosine Group |
| | *per cent* | *per cent* | *per cent* |
| *dl*-Tyrosine | 0 | ... | ... |
| *l*-Leucine | 2.3 | 2.8 | ... |
| *dl*-Glutamic acid | 3.3 | ... | ... |
| Ammonia † | 2.5 | ... | ... |
| Glycine † | 11.1 | 2.2 | 25.9 |
| " | 14.4 | 3.0 | ... |
| Sarcosine | 7.7 | ... | ... |
| " | 10.2 | 3.0 | ... |
| Arginine | 22.0 | 26.4 | 0.6 |
| Hydantoic acid | 0 | ... | ... |
| Methylhydantoic acid | 0 | ... | ... |
| Urea | 0 | ... | ... |
| Choline | 0 | ... | ... |

Guanidoacetic acid is not included in the table, as its administration is known to result in extra creatine formation. The efficiency of this compound calculated by employing the same factor would be 66.7 per cent.

† Only those experiments were considered in which adult rats were employed.

* From Bloch and Schoenheimer, *J. Biol. Chem.*, 138, 179 (1941).

food but also those of the body supply these two amino acids for creatine formation. Creatine is the result of the interaction of four compounds, of which one is choline and three (arginine, glycine, and methionine) are amino acids.

Borsook and Dubnoff have been able to follow the same condensation with tissue slices (65). They have demonstrated

the synthesis of guanidoacetic acid from arginine and glycine. The methylation of guanidoacetic acid was observed only when liver slices were employed; guanidoacetic acid was synthesized by kidney slices only. These are strong indications that the

TABLE 16

BIOLOGICAL STABILITY OF THE CREATINE MOLECULE *

(The creatine administered had nitrogen isotope in the glycine part as well as in the amidine group.)

| MATERIAL ANALYZED | ISOTOPE CONTENT | | |
|---|---|---|---|
| | In Amidine Group | In Sarcosine Group | $\frac{N^{15} \text{ in Amidine}}{N^{15} \text{ in Sarcosine}}$ |
| | *atom per cent* $N^{15}$ *excess* | *atom per cent* $N^{15}$ *excess* | |
| Material administered . . . | 5.25 | 4.50 | 1.17 |
| Urinary creatinine excreted during whole feeding period . . . . . | 0.241 | 0.219 | 1.10 |
| Muscle creatine 18 days after last creatine feeding . . . . . | 0.287 | 0.259 | 1.11 |

* With slight modification, from Bloch, Schoenheimer, and Rittenberg, *J. Biol. Chem.*, 138, 160 (1941).

two steps of creatine synthesis are carried out by two different organs.

Creatine, once it is formed, does not appear to enter into any further chemical reaction involving transfer of nitrogen. After the feeding of isotopic creatine, isotopic nitrogen is found only in the creatine and creatinine. Moreover, when the creatine fed was isotopically labeled in both the amidine and sarcosine parts of the molecule, the corresponding ratio of isotope distribution in the muscle creatine and in the urinary creatinine was the same (55) as in the compound administered (Table 16). This ratio was determined by isotope analysis

of the ammonia and the sarcosine formed on alkaline hydrolysis. Ingested isotopic creatine is entirely unchanged and is excreted as such; even the urinary urea and ammonia show no isotopic nitrogen. Creatine, once formed, is not degraded.

The last nitrogenous excretion product to be discussed is the purine derivative uric acid. Mammals excrete nitrogen mainly as urea; birds excrete uric acid and are thus more suitable for the preliminary investigation of uric acid metabolism.

As might be expected, the feeding of isotopic ammonium salts to pigeons results in the excretion of isotopic uric acid, in contrast to the excretion of isotopic urea by rats. A fundamental difference in the mechanism of these types of nitrogen excretion is shown by the nature of the arginine of the proteins; that in rats given isotopic ammonium salts is marked, that in pigeons is not. Arginine, which is an intermediate in urea formation in mammals, seems to have no direct relation to uric acid production in birds. Moreover, the uric acid isolated from the excreta of pigeons given isotopic urea contained very little heavy nitrogen. These findings seem to rule out urea as one step in the production of uric acid (66).

The work with isotopes on the formation of other excretory products (urea, creatinine) indicates that small chemical groups in large tissue molecules may play a rôle as intermediates during their temporary liberation, and led to the suspicion that uric acid formation may likewise be associated with reactions of structural tissue components. A systematic search was therefore undertaken in the bodies of the pigeons which had been fed isotopic ammonia (66).

The glutamic and aspartic acids isolated from the proteins contained isotopic nitrogen at levels comparable with those observed in experiments with rats, but the arginine contained practically none. On the other hand, the amide nitrogen and the purines obtained by hydrolysis of the nucleic acids of the internal organs contained relatively high concentrations of

isotope. An even higher level was found in the excreted uric acid. Some of the nitrogen of nucleic acids must have undergone rapid replacement by nitrogen from the administrated ammonia. This suggests a common step in the syntheses which lead respectively to nucleoproteins and to uric acid. It seems possible that nucleic acids lie directly on the pathway leading from ammonia to uric acid, and may be intermediate in all uric acid production in birds. When isotopic guanine was fed to pigeons, it was rapidly eliminated as isotopic uric acid, but almost no isotopic nitrogen was found in the nucleic acids of the organs (66).

The nucleic acids of various internal organs of pigeons fed ammonia display different apparent rates of replacement; those in liver and intestinal wall are more rapidly regenerated than those in other organs (66).

Purines occur not only as nucleic acids, but are found in other metabolically active substances. Dr. Kalckar in our laboratory has isolated adenosine triphosphate from the muscles of the pigeons that had received isotopic ammonia (66). Whereas the adenine of the nucleic acid was marked, that of the triphosphate, surprisingly enough, was practically normal. This is an isolated observation and requires repetition, but suggests that the rapid transfer of nitrogen to purines may occur only while they form part of the nucleic acid molecule.

These results indicate a parallelism between the rôle in nitrogen excretion played by the arginine of rat proteins, on the one hand, and by the purines in the nucleic acid of pigeon proteins, on the other. Both nucleic acid and arginine are components of large molecules and are constantly formed from ammonia, which has been liberated from amino acids.

Urea and creatinine in mammals, and uric acid in birds, are all end products of nitrogenous metabolism, and do not enter into metabolic reactions. The immediate precursors of urea and uric acid — namely, arginine and nucleic acid — differ from

creatine, the immediate precursor of creatinine, in that they form components of large protein molecules. This difference may partially explain the fact that, in the experiments here reported, the isotope concentrations of the urea and the uric acid are higher than those of their precursors isolated from the body, whereas the isotopic composition of urinary creatinine is always the same as that of the body creatine, none of which is diverted by incorporation into protein structures.

## Summary

It may now be permissible to attempt to integrate and interpret the findings discussed in these three lectures.

The large and complex molecules and their component units, fatty acids, amino acids, and nucleic acids, are constantly involved in rapid chemical reactions. Ester, peptide, and other linkages open; the fragments thereby liberated merge with those derived from other large molecules, and with those absorbed from the intestinal tract, to form a metabolic pool of components indistinguishable as to origin. These liberated molecules are again subject to numerous processes. Fatty acids are dehydrogenated, hydrogenated, degraded or elongated, and thereby continually interconverted. While some individual molecules of these acids are completely degraded, other individuals of the same chemical species are steadily formed from entirely different substances, notably from carbohydrate. Similar reactions occur among the split products of the proteins. The free amino acids are deaminated, and the nitrogen liberated is transferred to other previously deaminated molecules to form new amino acids. Part of the pool of newly formed small molecules constantly re-enters vacant places in the large molecules to restore the fats, the proteins, and the nucleoproteins. Some of the small molecules involved in these regeneration reactions constitute intermediate steps in the formation of excretory products. Part of the nitrogen in mammals travels via the amidine groups of arginine to be either excreted as urea or

transferred to glycine to form guanidoacetic acid, creatine, and creatinine. In birds the nitrogen on its way to excretion does not take the form of amidine groups, but may, for short periods, be present in the purine groups of nucleic acids.

Components of an animal are rapidly degraded into specific molecular groupings, which may wander from one place to another. The chemical reactions must be balanced so delicately that, through regeneration, the body components remain constant in total amount and in structure. This constancy is not to be taken as an indication that the structural matter of the living organism is inactive and takes little part in metabolism.

The implications which the finding of rapid molecular regeneration seems to have on our concept of animal metabolism call for comment. Most of these reactions require the simultaneous occurrence of others. Hydrogenation of one compound must be associated with the removal of hydrogen from another, and the experiments on amination have shown directly that many different amino acids interact in the transfer of amino groups. Many of the reactions observed require the simultaneous involvement of several different compounds. Creatine formation, for instance, proceeds by condensation of three organic chemical groups from different amino acids. Two of the amino acids, arginine and methionine, after yielding the amidine or methyl groups, must then have interacted with other compounds for the replacement of the lost fragments.

Some of the molecular regenerations of amino acids can be explained by their participation in metabolic cycles. Arginine of the proteins is involved in the urea cycle; the amino dicarboxylic acids play a rôle in transamination, and the corresponding $\alpha$-keto acids concerned in this process also take part in the carbohydrate cycle.

Such metabolic cycles are, of course, part of the life process, and cannot be interrupted during life. It is therefore impossible to block the continuous new formation of metabolites by supplying with the food an excess of identical types of molecules,

and regeneration of many body components proceeds independently of the food intake. A rat forms stearic acid from palmitic acid, and tyrosine from phenylalanine, even if it be oversupplied with stearic acid or tyrosine. Specific compounds play a dual rôle: on the one hand, for the replacement of structural elements and, on the other, for the maintenance of specific chemical reactions.

All regeneration reactions must be enzymatic in nature. The large molecules, such as the fats and the proteins, are, under the influence of lytic enzymes, constantly being degraded to their constituent fragments. These changes are balanced by synthetic processes which must be coupled to other chemical reactions, such as oxidation or dephosphorylation. After death, when the oxidative systems disappear, the synthetic processes also cease, and the unbalanced degradative reactions lead to the collapse of the thermodynamically unstable structural elements. In general, every regeneration reaction involving an increase in free energy must be coupled with another process. In order to maintain structure against its tendency to collapse, work has to be done. The replacement of a brick fallen from a wall requires energy, and in the living organism energy debts are paid by chemical reactions.

For the few coupled biochemical reactions which have been carefully investigated, such as those involved in muscle contraction or respiration, it has been shown that every chemical step is specifically related to some other one. The complex organic molecules present in living matter must require for their maintenance the steady occurrence of an abundance of various reactions. The finding of the rapid molecular regeneration, involving constant transfer of specific groups, suggests that the biological system represents one great cycle of closely linked chemical reactions. This idea can scarcely be reconciled with the classical comparison of the living being to a combustion engine nor with the theory of independent exogenous and endogenous types of metabolism.

The simile of the combustion engine pictured the steady flow of fuel into a fixed system, and the conversion of this fuel into waste products. The new results imply that not only the fuel but the structural materials are in a steady state of flux. The classical picture must thus be replaced by one which takes account of the dynamic state of body structure.

A simple analogy, which may be taken as an incomplete illustration of this concept of living matter, can be drawn from a military regiment. A body of this type resembles a living adult organism in more than one respect. Its size fluctuates only within narrow limits, and it has a well-defined, highly organized structure. On the other hand, the individuals of which it is composed are continually changing. Men join up, are transferred from post to post, are promoted or broken, and ultimately leave after varying lengths of service. The incoming and outgoing streams of men are numerically equal, but they differ in composition. The recruits may be likened to the diet; the retirement and death correspond to excretion.

This analogy is necessarily imperfect as it relates to only certain aspects of the dynamic state of biological structure. While it depicts the continual replacement of structural units, it takes no account of their chemical interactions.

The question as to the forces responsible for the arrangement of atoms in biochemical molecules in living organisms is as yet beyond the scope of laboratory experimentation.

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