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DEUTERIUM AS AN INDICATOR IN THE STUDY OF
INTERMEDIARY METABOLISM

XI. FURTHER STUDIES ON THE BIOLOGICAL UPTAKE OF
DEUTERIUM INTO ORGANIC SUBSTANCES, WITH
SPECIAL REFERENCE TO FAT AND
CHOLESTEROL FORMATION *

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In the application of deuterium to the problems of intermediary metabolism two different modes of attack have been developed. In the first method the metabolic route of a compound artificially labeled by deuterium atoms may be followed by deuterium analysis. Several investigations in which this method has been used to follow transportation and conversion of organic compounds in animals have been reported. If a deuterocompound is isolated from an animal which has been given another deuto compound, the result may in general be taken as proof of a conversion. This method is limited to such conversions as those in which the deuterium label is not removed by the chemical reactions involved. For this reason a negative result, namely the absence of deuterium in the predicated conversion product, cannot be taken as proof of the absence of such a conversion.

In the second method the rate of some chemical reactions in the animal organism is followed by measuring the uptake of deuterium into the organic compound from the body fluids. If organic molecules are synthesized in the laboratory in a medium of heavy water, the resulting substances will in general contain carbon-bound (non-exchangeable) deuterium. If the body fluids of an animal contain heavy water, deuterium must also enter into

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.
chemical reactions and will be found in the resulting substances (1). The rate at which deuterium appears in a compound may therefore be taken as a measure of the rate at which this was formed.

The value of this second method is limited by the fact that at the present time the reactions leading to the introduction of deuterium into the organic compounds are unknown. Only certain chemical reactions such as reduction, or the addition of water to a double bond,\(^1\) will introduce deuterium from the solvent (or body fluids) into stable positions. In these reactions deuterium is introduced at the carbon atom or atoms involved.

There is one chemically and physiologically important case in which the deuterium atoms may be introduced not only at the immediate site of reaction but also at adjacent carbon atoms. When a ketone is reduced in a medium of heavy water, deuterium atoms may be found not only at the carbon atom which was bound to the oxygen, but also in neighboring positions, since in a ketone the hydrogen of the adjacent carbon atoms may be labilized by enolization and take up deuterium by exchange. On the reduction of the carbonyl group in a medium of heavy water (conversion to \(-\text{CDOD} -\) or \(\text{CD}_2\)) the originally labile deuterium atoms can no longer exchange with the hydrogen of the solvent and thereby become stabilized.

\[
\begin{align*}
\text{O} & \quad \overset{\text{reduction}}{\longrightarrow} \\
\text{--CH}_2\text{--CH}_2\text{--C--CH}_2\text{--CH}_2\text{--D}_2\text{O} & \quad \text{\quad D}_2 \quad \text{--CH}_2\text{--CD}_2\text{--C--CD}_2\text{--CH}_2\text{--O}
\end{align*}
\]

Thus in the reduction of a ketone more deuterium may be stabilized than by the other processes mentioned. The amount of deuterium introduced by this mechanism must depend not only upon the concentration of deuterium in the solvent but also upon the relative rates of enolization and reduction.

In addition to these chemical reactions deuterium may also enter directly by exchange. Such exchanges with the hydrogen

\(^1\) There may exist other processes which have not as yet been investigated, such as the shift of a double bond, etc., which might also introduce deuterium from the medium into stable positions.
1. Rittenberg and R. Schoenheimer, of -COOH, -OH, -NH₂, etc., are extremely rapid and reversible. When the resulting compounds, -COOD, -OD, -ND₂, etc., are dissolved in ordinary water, the deuterium will immediately be removed. Such reactions have no bearing on the study of intermediary metabolism, as labile deuterium, if present, will always be removed during the course of isolation of the compounds, since they come in contact with large amounts of ordinary water or alcohol.

Besides these extremely fast exchange reactions there exist others, the rates of which cover the whole range from zero to that of the active hydrogen atoms mentioned above. In this category belongs the exchange of hydrogen attached to carbon atoms adjacent to carbonyl groups. For example, the hydrogen of acetone (2) exchanges slowly in pure water, faster in acid, and very fast in alkali, and that in the methyl group of acetic acid (3) has been reported to exchange slowly at 100°. In most other cases, however, the hydrogen of methyl and methylene groups is stable toward exchange in aqueous solutions. It is only this carbon-bound non-exchangeable hydrogen (or deuterium) which is of interest in metabolic studies. In order to remove any slowly exchangeable deuterium which may be in a compound, the substance during isolation must be treated under vigorous conditions with alkali or acid.²

In the study of the metabolism of a compound by the second method it is therefore necessary to investigate the presence of such slowly exchangeable hydrogen in the product under consideration.

In this paper, which is concerned with the in vivo synthesis of fatty acids and cholesterol, we present additional data on the stability toward exchange of the C—H linkage in these compounds. When treated with heavy water in alkali or acid and recrystallized from aqueous acetone or alcohol, they do not contain deuterium.

The stability of the C—H linkage in methyl or methylene

² In the case of a few amino acids the occurrence of such slow exchanges (semilabile deuterium) complicates the experimental procedure. When such compounds are isolated from an animal whose body fluids contain heavy water, they must be treated in a manner known to remove all such semilabile deuterium.
groups is probably only relative, and appropriate conditions may exist under which even these hydrogen atoms exchange. If in the course of a metabolic experiment a deuterio compound is isolated from an animal, the body fluids of which are enriched in heavy water, the stable deuterium in the compound may have entered by means of two different reactions. It may arise either from the occurrence of chemical reactions in the body or from an exchange brought about by conditions which we are not able to reproduce in vitro. It is conceivable that enzymes exist which labilize the hydrogen of certain C—H linkages, thus making the hydrogen exchangeable with that of the solvent. While there as yet does not exist any evidence for such an exchange in vivo which cannot be duplicated in vitro, the occurrence of such a biological reaction would seriously interfere with the interpretation of the data obtained by the second method. While the determination of stability in vitro is relatively easy, the exclusion of an in vivo exchange is generally more difficult. If there is isolated from an animal a deuterio compound with deuterium stable in vitro, i.e. deuterium which cannot be removed by treating the compound with boiling water, alkali, or acid, it is not possible to exclude directly the possibility that the deuterium had entered into the substance in vivo by an exchange reaction. The isolation of a deuterium-free substance, however, excludes the in vivo exchange of the carbon-bound hydrogen in this compound.

The occurrence of chemical reactions in the living organism is so widespread that most organic constituents of an animal take up deuterium under our experimental conditions. In the course of our work with deuterium we have already isolated fourteen different compounds from mice whose body fluids were enriched in heavy water for at least 10 days. All these compounds with the exception of lysine contain deuterium. We can thus definitely state that there is no in vivo exchange of the hydrogen

3 Ingold, Raisin, and Wilson (4) have demonstrated an exchange of the hydrogen of some aliphatic hydrocarbons with the hydrogen of concentrated sulfuric acid (77 moles per cent). Dilute sulfuric acid (below 50 moles per cent) does not cause this exchange.

4 The compounds are cholesterol, stearic acid, palmitic acid, oleic acid, tyrosine, cystine, arginine, histidine, lysine, glutamic acid, aspartic acid, leucine, proline, and glycine. The results on the amino acids will be reported with Dr. G. L. Foster in a subsequent publication.
of lysine which does not occur \textit{in vitro}. The unique absence of deuterium in lysine is probably associated with the fact that this is an indispensable amino acid which cannot be synthesized by the animal. It therefore seems probable that just as the hydrogen of the four methylene groups of lysine does not exchange \textit{in vivo}, so the methylene groups of other compounds should not exchange either.

In a previous paper we have described the uptake of deuterium by the fatty acids of mice, the body fluids of which contained deuterium (1). Similar findings have been reported by Smith, Trace, and Barbour (5) and by Krogh and Ussing (6). In our publication we presented proof for the non-existence of an \textit{in vivo} exchange of the hydrogen of the fatty acids. The fatty acids of chicks which had developed in a medium of heavy water contained no deuterium. From the absence of deuterium in the acids of the chick the absence of an \textit{in vivo} exchange in the mouse was inferred.

In continuation of our previous work we are reporting in this paper experiments of long duration on the incorporation of deuterium into fatty acids and cholesterol.

The heavy water content of the body fluids of mice was raised to about 1.5 per cent by injection of D\textsubscript{2}O, and kept there by giving 2.35 per cent D\textsubscript{2}O to drink. As in the previous experiments the mice received whole wheat bread \textit{ad libitum}. The experiment was continued for 98 days. At various periods groups of mice were killed and the cholesterol and fatty acids of the animals isolated. The fatty acids were separated into saturated and unsaturated fractions. Stearic acid and palmitic acid were isolated by fractional distillation of the methyl esters. Considerable amounts of deuterium were found in the cholesterol, as well as in the fatty acids.

As mentioned before, we have not been able to introduce deuterium into cholesterol \textit{in vitro} by exchange from heavy water even under drastic conditions. In accordance with the above considerations, investigations as to the occurrence of an \textit{in vivo} exchange were carried out, since such exchange might have been responsible for the presence of the deuterium in the mouse cholesterol. The same procedure was followed as for the fatty acids. Cholesterol isolated from chicks which had developed in a medium
of heavy water contained no deuterium. We therefore feel justified in concluding that the deuterium of the cholesterol which was isolated from the mice is the result of chemical reactions.

The fact that cholesterol is synthesized in mammals is well known. Synthesis of cholesterol in mice has recently been followed quantitatively by balance experiments on the same bread diet and a synthesis of about 1.8 mg. per day was found (7). It is therefore not surprising to find deuterium in the cholesterol of mice, as during the process of cholesterol synthesis deuterium must be incorporated into the molecule. We are inclined to believe that the reaction which we are measuring is the synthesis of cholesterol from other compounds.

In all biological balance experiments on sterols, these substances have to be determined by means of their insoluble addition compounds with digitonin. If, in the course of metabolism, any substance becomes precipitable by digitonin, the reaction would be called a sterol synthesis, while, conversely, any reaction which eliminates the precipitability by digitonin would be termed a destruction. The reaction with digitonin, however, is not confined to cholesterol or other typical sterols, but is a group reaction for all substances chemically related to sterols, in which the steric configuration of the carbinol at the carbon atom 3 corresponds to that of cholesterol (8). Numerous substances of animal and plant origin are known which share with the sterols the cyclopentenophenanthrene skeleton (steroids) but are not precipitable with digitonin (bile acids, saponins, heart poisons, sex hormones, etc.).

It is therefore impossible to decide on the basis of any balance experiments whether the process called biological sterol synthesis in animals actually involves the new formation of the ring skeleton or represents merely minor changes by which a non-precipitable steroid of the diet may be rendered precipitable. Furthermore, balance experiments cannot give the actual rate of synthesis, but only the difference between the rates of synthesis and degradation.

*Balance experiments in which cholesterol is determined colorimetrically by the Liebermann-Burchard or Salkowski reactions are open to criticism. The plant sterols of the diet and the fecal sterols give different color reactions, some of them giving no color at all.
The deuterium content in the cholesterol isolated from our mice after 2 months of heavy water feeding was quite high. Extreme care must be taken at the present time in drawing conclusions from the amount of deuterium in the synthesized compound as to the mechanism responsible for its introduction. As stated above, the amount of stable deuterium in the organic compound is dependent not only upon the deuterium content of the medium but also upon the rate of enolization of ketones which may have occurred as intermediates.

Irrespective of the specific reaction mechanism, the deuterium content of an organic compound obtained by our experimental procedure will indicate the minimum number of carbon atoms which must have been involved in the synthesis. Reduction of a --CH₂COCH₂ -- group may introduce a maximum of 6 deuterium atoms. The other elementary reactions introduce no more than 2 stable deuterium atoms.

The cholesterol isolated from the mice after 60 days of heavy water feeding contained 0.78 atom per cent deuterium, while the body fluids had 1.7 atom per cent. The concentration of deuterium in the hydrogen of the cholesterol was thus about half of that of the water in which the cholesterol was formed. On the assumption that deuterium and hydrogen have been treated alike in this process, this finding indicates that during the various stages of the process of cholesterol formation about half of its stable hydrogen atoms were exchangeable with those of the body fluids. Chemical reactions must therefore have occurred at a great number of carbon atoms.

The possibility that the cholesterol was derived from a dietary component of similar structure, such as a non-precipitable steroid, is therefore excluded. A conversion of this kind could not have led to the introduction of so much stable deuterium. The findings indicate rather that formation of cholesterol in the animal body is a more fundamental process and involves the coupling of a large number of small molecules.

Most investigators of the problem of cholesterol metabolism in the developing hen's egg agree that the total amount of cholesterol does not change during the entire period of development (9). This constancy, however, does not necessarily indicate that cholesterol is not formed during this period. A balance
whereby synthesis is accompanied by the degradation of an equivalent amount of cholesterol would lead to the same experimental result.

As stated before, the cholesterol of chicks which had developed in a medium of heavy water contained no deuterium. This result excludes the occurrence of cholesterol synthesis during development. As the total amount of cholesterol remains constant, the occurrence of cholesterol destruction in eggs can be excluded with the same certainty.

The metabolism of cholesterol in eggs thus differs from that of the fatty acids. A large part of the fatty acids is burned for energy requirements, while the cholesterol molecule is not attacked at all. However, during development neither fatty acids nor cholesterol is synthesized. The same fatty acid and cholesterol molecules which are found in the hatched chick were already present in the new laid egg. Although the cholesterol metabolism rests during development, it becomes active again immediately after hatching, as Dam (10) has demonstrated a destruction of cholesterol in chicks.

**EXPERIMENTAL.**

*Exchange Reactions*

*Palmitic Acid*

Experiment A—0.5 gm. of palmitic acid, 10 cc. of 27 per cent D$_2$O, and an excess of KOH were kept in a sealed tube at 100° for 120 hours. The acid was isolated and recrystallized from aqueous acetone. It contained 0.00 atom per cent deuterium.

Experiment B—1.0 gm. of palmitic acid was refluxed for 48 hours in a solution of 5 cc. of 55.1 per cent D$_2$O, 15 cc. of absolute alcohol, and 0.5 cc. of concentrated sulfuric acid. The ester was saponified and the acid recrystallized from aqueous acetone. It contained 0.00 atom per cent deuterium.

*Cholesterol*

Experiment A—0.5 gm. of cholesterol and 0.3 cc. of 98 per cent D$_2$O were sealed in a tube in vacuo and heated for 8 hours at 230°. After recrystallization from aqueous acetone, the product, m.p. 146°, contained 0.00 atom per cent deuterium.
Experiment B—1.0 gm. of cholesterol was refluxed for 24 hours with 4.8 cc. of 55.1 per cent D₂O + 7.2 cc. of ethyl alcohol + 2 drops of concentrated HCl. The cholesterol was isolated, refluxed with acetic anhydride, and the acetate recrystallized from alcohol, m.p. 113–114°. The cholesterol acetate contained 0.00 atom per cent deuterium.

Isolation of Cholesterol from Mice

The method of keeping the deuterium content of the body fluids constant was the same as described in the study of fatty acids (1). At the beginning of the experiments the mice were injected with enough concentrated heavy water to raise the deuterium content to approximately 1.5 atom per cent. To keep the concentration at this level the animals were supplied with 2.35 per cent heavy water instead of ordinary drinking water. The heavy water content of the body fluids was relatively constant during the 3 months of the experiment. From the moment of heavy water injection chemical reactions in the animal occurred in a medium of about 1.5 per cent D₂O. As in our previous experiments, the mice received whole wheat bread ad libitum.

Groups of mice were killed at intervals and for each analysis the carcasses of a number of animals were pooled. From these, water was distilled off in vacuo to determine the heavy water content of the body fluids (11). The residue was refluxed for 2 hours with 7 per cent alcoholic potassium hydroxide. The bones were removed by filtration through glass wool, and most of the alcohol was distilled off. The residue was diluted with water and repeatedly extracted with ether. The ether was washed with water until neutral and dried over sodium sulfate.

The isolation of cholesterol from these ethereal solutions was carried out by three different methods.

Experiments 4, 5, 6, 7, and 11—The cholesterol was precipitated with digitonin from 80 per cent ethanol. The digitonide was extracted for 2 hours in a Soxhlet extractor with low boiling petroleum ether and dried in vacuo at 100° for 3 hours. The digitonides were burned for deuterium analysis. For the calculation of the deuterium in the cholesterol the formula C₅₆H₉₂O₂₂ + C₂₇H₄₆O for cholesterol-digitonide was used.

Experiments 9 and 10—The digitonides were refluxed for 30
minutes with acetic anhydride and the cholesterol acetate precipitated with 50 per cent ethyl alcohol. The preparations melted at 112°. Before combustion the samples were dried in vacuo at 80° for 6 hours. From the value obtained the amount of deuterium in the cholesterol was calculated by multiplying by the

![Table 1](http://www.jbc.org/)  

**Deuterium in Cholesterol and Fatty Acids from Mice**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Duration of experiment</th>
<th>No. of animals</th>
<th>Average weight of animals at end of experiment*</th>
<th>Deuterium in</th>
<th>Body fluids</th>
<th>Total fatty acids</th>
<th>Saturated fatty acids</th>
<th>Unsaturated fatty acids</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days</td>
<td>gm.</td>
<td></td>
<td>atom per cent</td>
<td>atom per cent</td>
<td>atom per cent</td>
<td>atom per cent</td>
<td>atom per cent</td>
<td>atom per cent</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>17</td>
<td>1.21</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>4</td>
<td>18</td>
<td>1.09</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>18</td>
<td>1.70</td>
<td>0.18</td>
<td>0.29</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
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<td>17</td>
<td>1.43</td>
<td>0.21</td>
<td>0.41</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>18</td>
<td>1.50</td>
<td>0.20</td>
<td>0.41</td>
<td>0.13</td>
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<tr>
<td>6‡</td>
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<td>1.48</td>
<td>0.15</td>
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<td>0.24</td>
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<td>7</td>
<td>19</td>
<td>18</td>
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<tr>
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<td>0.58</td>
<td>0.31</td>
<td>0.67</td>
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<td>0.31</td>
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<tr>
<td>10</td>
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<td>28</td>
<td>1.70</td>
<td>(0.50)†§</td>
<td>0.75</td>
<td>0.38</td>
<td>0.78</td>
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</tr>
<tr>
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<td>0.59</td>
<td>0.43</td>
<td>0.47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The average weight of mice at the beginning was 18 gm. except in Experiment 6 in which it was 25 gm.
† For these analyses the fatty acids of Experiments 4, 5, and 7 were pooled.
‡ In Experiment 6 the animals were not injected with concentrated heavy water at the beginning but only given dilute heavy water to drink.
§ These values were not obtained by analysis but were calculated on the assumption that one-third of the total fatty acids was saturated.

ratio of the number of hydrogen atoms in the cholesterol acetate to that in cholesterol (48/46).

*Experiment 8*—The cholesterol was not precipitated by digitonin but was purified by twice recrystallizing the ether residue from methanol. It then melted at 147.5°.

**Isolation of Fatty Acids from Mice**

The fatty acids were obtained from the alkaline layer remaining after the ether extraction. The isolation and separation into
saturated and unsaturated fractions were carried out in the manner previously described (12).

The results of the deuterium analysis of the cholesterol and of the fatty acids are given in Table I. In this table we have included some results on fatty acids which have already been published (1) (Experiments 1, 2, 4, 5, and 7), since both sets of data are intimately connected. The cholesterol analysis of our earlier mouse series, Experiments 4, 5, and 7, have not been published before.

**Separation of Palmitic and Stearic Acids from Mice**

**Experiment A—3.90 gm. of saturated acids from Experiment 3 containing 0.29 ± 0.02 atom per cent deuterium were esterified with methyl alcohol and fractionated by distillation (13). Thirteen fractions were obtained. Fraction 3, weighing 452 mg., had the correct melting point for methyl palmitate (28.8–29.2°). The palmitic acid contained 0.30 ± 0.03 atom per cent deuterium. Fractions 10 and 11, weighing 95 mg. and 114 mg. respectively (mp. 33.8–34.2° and 35.2–35.9°), were combined and saponified. The free acid had a melting point of 65.7° and a mean molecular weight of 282, indicating a mixture of about 90 per cent stearic acid and 10 per cent palmitic acid. It contained 0.27 ± 0.05 atom per cent deuterium.

**Experiment B—2.456 gm. of the saturated fatty acids of Experiment 9, containing 0.58 ± 0.02 atom per cent deuterium, were esterified, and the methyl esters separated into ten fractions. Fractions 3, 4, 5, and 6, weighing 1.095 gm. and melting between 29.0–30.0°, were combined. The fractions were saponified. The acid, after recrystallization, melted at 61.5–62.3°. It contained 0.51 ± 0.02 atom per cent deuterium. The last two fractions, Nos. 9 and 10, were analyzed separately. Fraction 9, weighing 221 mg. and melting at 33.4–34.0°, was saponified. The acid melted at 62.5–64.0°. The mean molecular weight was 279, indicating a mixture of about 80 per cent stearic and 20 per cent palmitic acids. It contained 0.63 ± 0.05 atom per cent deuterium.

Fraction 10, the residue, weighing 292 mg. and melting at 33.3–33.5°, yielded an acid melting at 59.5–60.8° with a molecular weight of 296. It contained 0.72 ± 0.05 atom per cent deuterium.

In both experiments, one of which was of 5 days and the other
of 37 days duration, the palmitic and stearic acids had approximately the same deuterium content. Fraction 10 of Experiment B contained fatty acids of molecular weight greater than that of stearic acid. The deuterium content was higher than that of the palmitic and stearic acid fractions.

Cholesterol from Chick Embryos Developed in a Medium of Heavy Water

The cholesterol was isolated from the same eggs as were used for the investigations of fatty acids (1). Five fertilized eggs were injected on the 1st day of development with 0.35 cc. of a solution of 0.9 per cent sodium chloride in 98 per cent heavy water. Another group of five eggs received 0.5 cc. of the same solution. Eight of these eggs developed normally. The remaining two were probably not fertilized. No infection occurred. Two eggs were worked up after 9 days of development and the rest at the 20th day; i.e., immediately before hatching. The cell water of the eggs injected with 0.35 cc. contained 0.47 to 0.65 atom per cent deuterium, and the water of the eggs injected with 0.5 cc. contained 0.81 to 0.98 atom per cent. Each egg was worked up separately.

The cholesterol was isolated from the unsaponifiable matter by recrystallization from methanol until the melting point was 147°. In none of the samples was the deuterium concentration of the water obtained by combustion higher than 0.02 atom per cent.

DISCUSSION

Barbour and Trace (14) have found that high concentrations of deuterium (20 per cent) in the body fluids of mice affect the metabolic rate and give rise to toxic symptoms. In order to avoid these complications the deuterium content of the body fluids of our mice was kept low. In the range in which all our experiments have been carried out (1.5 per cent D₂O) no such effects are to be expected nor were they ever found.

The data given in Table I show that the deuterium content of the fatty acids and of the cholesterol depends on the duration of the experiment.
**Results on Fatty Acids**

In Figs. 1 and 2 we have plotted the deuterium content of the total fatty acids and of the saturated and unsaturated acids *versus* the time of the experiments. In Experiment 6 the mice were not injected with D$_2$O at the start of the experiment but only given dilute heavy water to drink. We have therefore plotted in Fig. 1 and Fig. 3 the values for total fat and cholesterol from Experiment 6 as equivalent to a 10 day experiment. To put
all experiments on a comparable basis it was necessary to correct for the variations in the deuterium content of the body fluids in the different experiments. We have recalculated the values for a deuterium concentration in the body fluids of 1.5 per cent. If the heavy water concentration in the body fluids was higher than 1.50 per cent, the value for the fatty acids was decreased proportionately and vice versa. We are not certain that this correction is justified, since the deuterium content of the body fluids was determined only at the time the mice were killed. The fluctuations in heavy water concentration, however, could not have been very great.

![Graph](http://www.jbc.org/)

**Fig. 3. Deuterium content of cholesterol from mice**

Drawing quantitative conclusions from biological data of such nature is rather difficult. The elimination of the biological fluctuations by the use of still larger groups of mice was not practicable owing to the expense of the heavy water.

Our mice gained weight in the course of the experiments, especially near the end. All experiments were begun with adult male mice weighing 17 to 19 gm. After 60 and 98 days they averaged 28 and 25 gm. respectively. This gain must have been accompanied by an increase of fat. It is difficult at the present time to evaluate the effect this would have on the deuterium content of the fatty acids.

\[\text{For each mouse an average of about 3 cc. of water per day was required.}\]
With these influences in mind, it is nevertheless apparent that the deuterium content of the total fatty acids rises and becomes constant after about 30 to 40 days. In the previous paper (1) in which we reported the first part of this curve (Experiments 1, 2, 4, 5, and 7) we had tentatively concluded that the turnover of the fatty acids on our bread diet was complete in about 6 to 8 days. Since our values must follow an exponential curve, it is more convenient to use the concept of the "half-lifetime." In our case it is defined as the time necessary for the deuterium content of the fatty acids to reach half of its maximum value.

We had concluded that the half-lifetime was about 3 days. The additional data obtained from the long feeding experiments, together with those previously secured, indicate that the turnover is somewhat slower, with a half-lifetime of about 5 to 9 days. The difference between the old and the new value of the half-lifetime is due to the fact that in our earlier experiments the values obtained after 6, 9, and 19 days were the same. It is now apparent that this was fortuitous. It is to be expected that a great many factors such as diet, temperature, metabolic rate, etc., may influence this value. It may, furthermore, depend upon the amount of carbohydrate (or protein) absorbed in excess of that which can be immediately burned or deposited as glycogen.

In Fig. 2 are plotted the deuterium contents of the saturated and unsaturated acids in the same manner as the total fatty acids in Fig. 1. The values for the combined fatty acids of Experiments 4, 5, and 7 have been plotted as if they corresponded to an 11 day experiment. The two fractions take up deuterium at somewhat different rates. The deuterium content of the saturated acids is always higher than that of the unsaturated ones. This is the same as we had found in our short time experiments. It is difficult to tell from the data whether the deuterium content of the unsaturated acids has reached a maximum or whether it is still slowly increasing after 98 days. If the latter

7 The half-lifetime was also determined as before by investigating the rate at which the deuterium-containing fatty acids, deposited in the fat tissues by feeding, disappeared on our bread diet. In an experiment with thirty mice we have followed the deuterium content for 16 days. Half of the deuterium was gone after about 8 days. This value is in better agreement with the one obtained from the complete heavy water experiment than with the one previously suggested.
were true, it would seem that there were two processes operating: a fast one which accounts for the rapid initial uptake and a slow one which is still going on, even after this long period. If, however, equilibrium were attained, it would be very difficult to interpret the results. One would expect that when equilibrium is attained, after prolonged feeding with heavy water, the deuterium content of both fractions would approach the same value, since the mechanisms of formation are, in principle, probably the same. The experimental diet contained a small amount of fat (3 per cent). If this fat were responsible for the difference, one would have to assume that the unsaturated acids of the diet were selectively deposited, while the saturated acids were burned. This explanation does not appear to be plausible. Experiments on a completely fat-free diet may throw light on this mechanism. The almost equal concentration of deuterium in the palmitic and stearic acids suggests a close metabolic relationship between them.

*Results on Cholesterol*

The uptake of deuterium by the cholesterol is interpreted as discussed above to indicate a synthesis of cholesterol from other compounds. This process is slower than that of the fatty acids, a finding well in accord with other studies on fat and cholesterol metabolism. The half-lifetime, assuming we have reached the maximum value (see Fig. 3), seems to be about 15 to 25 days. Some of the difficulties of the fat experiment do not appear in the cholesterol experiment. The sterol content of our diet is only 0.03 per cent and consists primarily of plant sterols which are not absorbable. Also, the cholesterol content of mice, in contrast to that of the fatty acids, is remarkably constant from mouse to mouse (0.28 to 0.31 per cent) (7).

The results of the exchange experiments as well as those with the hen's eggs show that the deuterium could not have entered by exchange but must have been introduced during chemical reactions, *i.e.* formation of cholesterol.

While it is not possible, from the quantitative data, to draw definite conclusions as to sources used for the cholesterol formation, some mechanisms frequently discussed can be excluded. As mentioned in the introduction, the new findings definitely rule
out the formation of cholesterol from a steroid present in the food. A mechanism must be postulated whereby about half of the hydrogen atoms of the cholesterol were at some stage in exchange with the hydrogen of the body fluids.

As cholesterol is always associated in the organism with other lipids, its direct formation from fatty acids has frequently been discussed and several schemes for the conversion of fatty acids into cholesterol have been proposed. All these schemes involve the utilization of the fatty acids without previous degradation. None of the known biological findings, however, can be taken as proof for the formation of sterols from fatty acids. Miniović (15), in a series of experiments on cockroaches, dogs, and mice, found feeding of oleic acid to lead to an extraordinary increase of cholesterol synthesis. His findings on mice are in disagreement with the results of Schoenheimer and Breusch (7), who fed large amounts of lard (containing 50 to 60 per cent oleic acid (16)) to mice without observing a significant increase of cholesterol synthesis compared with controls on a low fat diet. Miniović determined the sterols in his balance experiments by the Windaus digitonin method, as well as by colorimetric methods, and found in almost all analyses an agreement within a few per cent. This agreement is difficult to understand, as the sterols which he analyzed were mixtures of cholesterol, plant sterols, fecal sterols, etc., which all precipitate quantitatively with digitonin but give widely different color intensities.

Recently, Eckstein and Treadwell (17), in well controlled experiments, have found that rats fed large amounts of soy bean oil show a small but significant increase of their cholesterol synthesis. The authors discuss the possibility of a conversion of fat into cholesterol. While these experiments can be taken as an indication that a high fat diet may increase the cholesterol synthesis to a small extent, they do not offer a proof for the conversion of fatty acids into cholesterol. The increase of cholesterol is minute, compared with the large amounts of fatty acids utilized by the animal, and probably does not exceed a fraction of a per cent. The results could equally well be explained by the frequently discussed theory (see (9)) that cholesterol plays a rôle in the transport of fatty acids in the organism. The handling of a larger amount of fatty acids may require the presence of a
larger amount of cholesterol, the latter being formed according to the need. The amount of cholesterol might thus have been increased according to the requirements, but not because the starting material for the synthesis was available in large amounts.

We interpret our results as ruling out a process by which cholesterol in the animal is formed by cyclization of fatty acids, and suggest instead that it is formed by the coupling of smaller molecules, possibly those which have been postulated to be intermediates in the fat and carbohydrate metabolism.

SUMMARY

1. The reactions are discussed by which deuterium may enter from the water of the body fluids of animals into organic compounds.

2. In vitro experiments on the exchange of the hydrogen of palmitic acid and cholesterol with that of heavy water were carried out. Neither of the two compounds contains any slowly exchangeable (semilabile) hydrogen.

3. The heavy water content of mice was raised by injection of D₂O to 1.5 per cent and kept there for periods up to 98 days. Groups of mice were killed at intervals and fatty acids and cholesterol isolated. The fatty acids were separated into the unsaturated and saturated ones and from the latter palmitic and stearic acids were isolated.

4. As in previous experiments the deuterium content of the total fatty acids increased with time and reached a constant value. The complete data indicate a half-lifetime of about 5 to 9 days for the fatty acids.

5. The deuterium content of the saturated acids in all samples was higher than that of the unsaturated; stearic and palmitic acids had about the same deuterium content.

6. The deuterium content of the cholesterol rose to one-half that of the body fluids. The rate was slower than that of the fatty acids. The half-lifetime is about 15 to 25 days.

7. Cholesterol was isolated from chick embryos which had developed in eggs containing heavy water. It did not contain deuterium. This proves the absence of an in vivo exchange which is not reproducible in vitro.
8. During the developmental period of hen's eggs there occurs neither synthesis nor degradation of cholesterol.

9. The high deuterium content of the cholesterol of mice suggests that in mammals it is prepared by the coupling of a large number of small molecules.

BIBLIOGRAPHY
