Cholesterol, which is formed by biological synthesis, contains angular methyl groups and branched side chains. A few other tissue constituents exist which contain branched chains, notably valine and leucine, but these are all essential dietary constituents. It has been shown (1) that acetic acid participates in the synthesis of the entire structure of cholesterol, but no obvious mechanism is known by which acetic acid could react to form branched carbon chains. Our attempts to find specific cholesterol precursors other than acetic acid have been unsuccessful; in all instances in which the administration of a labeled test substance led to the formation of deuterio cholesterol, the effect has been attributable to the intermediary formation of deuterio acetic acid (2).

Experiments were carried out to test the effect of deuterio valine and deuterio leucine on the biological formation of cholesterol. Cholesterol isolated from animals which had received deuterio leucine contained significant concentrations of isotope, but only normal cholesterol was formed when deuterio valine was the test substance. Of the two branched chain fatty acids which are likely to be degradation products of leucine and valine respectively, deuterio isovaleric acid gave an effect similar to that of leucine, whereas deuterio isobutyric acid, like valine, failed to produce isotopic cholesterol.

The appearance of deuterium in cholesterol following administration of deuterio leucine is evidence of the ability of leucine to supply carbon atoms for sterol synthesis, but provides no proof of a specific utilization of the isopropyl groups. Leucine is a ketogenic substance; i.e., a source of acetoacetic acid. Though it is recognized that in ketosis the acetone bodies can arise either as primary oxidation products or by recondensation of acetic acid, little is known as to the metabolism of acetoacetate or other ketogenic substances under normal conditions. In the preceding paper evidence is presented that extensive hydrolysis of acetoacetate to acetate can occur in normal animals. Intermediary formation of acetic acid may be detected by the appearance of deuterio acetyl groups in acetylamino acids. This

* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation and from the Nutrition Foundation, Inc.
method has now been employed to investigate the degradation of leucine, valine, isovaleric acid, and isobutyric acid. The acetyl groups of the excreted acetylphenylaminobutyric acid contained deuterium when appropriately labeled leucine or isovaleric acid was fed, but contained no excess of isotope after administration of deuterio valine or deuterio isobutyric acid. Thus, only the two substances which are known to be ketogenic produce acetic acid. Since deuterio cholesterol is formed from the same two substances, their effect in cholesterol synthesis may well be the result of intermediary formation of acetic acid, which in turn is utilized for cholesterol synthesis.

Acetic acid supplies carbon atoms for the nucleus as well as for the side chain of the cholesterol molecule (1). The cholesterol isolated after deuterio leucine feeding was degraded into the iso-octane-iso-octene mixture corresponding to the cholesterol side chain and the polynuclear hydrocarbon \( C_{18}H_{30} \). The isotope concentrations in the two fragments were almost equal, as was the case with cholesterol resulting from deuterio acetate feeding. This finding strengthens the view that a similar mechanism is responsible for the appearance of deuterium in cholesterol, and that leucine is probably not a specific cholesterol precursor.

**EXPERIMENTAL**

*Preparation of Deuterio dl-Leucine. Isocaproic Acid*—Since pure isocaproic acid can be separated only with difficulty from commercial mixtures of the isomeric acids, it was prepared by a modification of a method described by Braun (3). Isobutyraldehyde\(^1\) was condensed with malonic acid and the resulting isopropylacrylic was hydrogenated. To 136 ml. of freshly distilled isobutyraldehyde were added 104 gm. (1 mole) of powdered malonic acid and 100 ml. of dry pyridine. The mixture was kept at room temperature for 12 hours, and was then heated under a reflux for 6 hours. The excess aldehyde was distilled off and the mixture refluxed again for 1 hour. The cooled reaction mixture was diluted with 3 volumes of water, made acid to Congo red with sulfuric acid, and extracted with ether. The solvent was removed from the dried ether solution and the residue was distilled \textit{in vacuo}. The fraction distilling at 112–114° at 20 mm. of Hg was collected. 53 gm. of isopropylacrylic acid were obtained (47 per cent of theory). 35 gm. of the unsaturated acid were dissolved in 350 ml. of absolute ethanol and hydrogenated in the presence of active platinum. Distillation of the reduction product yielded 32 gm. of isocaproic acid, b.p. 103–104° at 13 mm. of Hg.

*Deuterio Isocaproic Acid*—The isocaproic acid was converted into the sodium salt and exchanged with D\(_2\)O in the presence of activated platinum.

\(^1\) We are indebted to Dr. Roland Kapp of the National Oil Products Company for supplying us with the isobutyraldehyde.
in a sealed flask. The contents were shaken at 130° for 14 days. The isocaproic acid recovered contained 47.9 atom per cent excess of carbon-bound deuterium.

Deuterio dl-leucine was prepared by bromination of the isotopic isocaproic acid and treatment of the α-bromoisocaproic acid with ammonia (4). An over-all yield of 53 per cent, calculated for isocaproic acid, was obtained. N, Kjeldahl, found, 10.5 per cent; calculated for C₉H₁₆D₄NO₂, 10.3 per cent. The compound contained 37.0 atom per cent excess deuterium. Of the 13 hydrogen atoms of leucine, the 2 of the amino group and that of the carboxyl group must be non-isotopic. The remaining 10

<table>
<thead>
<tr>
<th>Compound fed</th>
<th>Duration of experiment</th>
<th>Body water</th>
<th>Acetyl group of acetyl-phenylalanine-butyric acid</th>
<th>Coefficient* of utilization</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium deuterio acetate</td>
<td>1.55</td>
<td>27.6</td>
<td>8</td>
<td>0.07</td>
<td>2.00</td>
</tr>
<tr>
<td>Deuterio dl-leucine...</td>
<td>1.13</td>
<td>48.1</td>
<td>16</td>
<td>0.15</td>
<td>2.50</td>
</tr>
<tr>
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<td>23.5</td>
<td>8</td>
<td>0.10</td>
<td>1.19</td>
</tr>
<tr>
<td>Deuterio dl-valine...</td>
<td>1.10</td>
<td>34.0</td>
<td>8</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>Sodium deuterio isobutyrate...</td>
<td>1.10</td>
<td>24.0</td>
<td>8</td>
<td>0.03</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* Coefficient of utilization = D₀ₑ/nₑ × mM × Dᵣ, where D₀ₑ = per cent deuterium in the acetyl group, nₑ = total number of hydrogen atoms in the compound fed, Dᵣ = per cent deuterium in the compound fed.

hydrogen atoms will, therefore, contain 37.0 × 13/10 = 48.1 atom per cent excess deuterium. As this value is almost identical with that found for the carbon-bound hydrogen of isocaproic acid, the hydrogen lost from the α-carbon atom of isocaproic acid by bromination must have had the same isotope concentration as the remainder of the carbon-bound hydrogen atoms. The catalytic exchange of isocaproic acid must, therefore, have led to fairly uniform distribution of deuterium along the carbon chain.

In Table I, the isotope concentration of dl-leucine given is not that actually found, i.e. 37 per cent, but 48.1 per cent, i.e. the deuterium concentration of the 10 carbon-bound hydrogen atoms. This is justified because in acetate formation only the carbon-bound hydrogen atoms need be considered.
Deuterio dl-Valine. Isovaleric Acid—The compound was prepared from isopropyl bromide and diethyl malonate according to Marvel and du Vigneaud (5). For introduction of deuterium, sodium isovalerate was exchanged with D₂O in the presence of activated platinum. The reaction mixture was shaken at 130° in a sealed flask for 12 days. The isovaleric acid recovered contained 34.8 atom per cent excess deuterium. The deuterio isovaleric acid was brominated and the bromo acid allowed to react with ammonia (6). N, Kjeldahl, found, 11.7 per cent; calculated for C₆H₇D₃NO₂, 11.7 per cent. The amino acid contained 24.7 atom per cent excess deuterium. As in the case of deuterio leucine, the 3 hydrogen atoms in the amino group and the carboxyl group must be non-isotopic. The carbon-bound hydrogen atoms will, therefore, contain 24.7 × 11/8 = 34.0 atom per cent excess deuterium. This compares with a deuterium concentration of 34.8 per cent found for isovalerate; the rate of exchange must, therefore, have been roughly the same for all carbon-bound hydrogen atoms of isovaleric acid. The isotope concentration for valine given in Table I is again that of the carbon-bound hydrogen atoms; i.e., 34.0 atom per cent.

Deuterio Isovalerate and Deuterio Isobutyrate—The isotopic isovaleric acid employed in the feeding experiment was prepared in the same manner as that used for valine synthesis. It was fed in the form of the sodium salt which contained 23.5 atom per cent excess deuterium. Isotopic isobutyrate was prepared from commercial isobutyric acid by exchange of the sodium salt with D₂O in the presence of activated platinum. The mixture was shaken at 127° for 16 days and the sodium isobutyrate recovered contained 24.0 atom per cent excess deuterium.

Feeding of dl-Deuterio Leucine to Mice—Twenty adult mice were placed in three groups on a stock diet containing 77 per cent starch, 6 per cent casein, 5 per cent yeast, 6 per cent Wesson oil, 2 per cent cod liver oil, 2 per cent salt mixture (7). Each animal consumed about 3 gm. daily of this diet. In addition, each mouse received per day 40 mg. of dl-leucine containing 37.0 atom per cent excess deuterium and 75 mg. of sodium chloride, mixed with the stock diet. The sodium chloride was added in order to increase consumption of drinking water and thereby maintain the deuterium concentration of the body fluids at a low level. After 8 days ten mice of Group I were killed. A sample of body water was distilled from the tissues and cholesterol isolated from the pooled carcasses in the usual fashion. The body water contained 0.055 and the cholesterol 0.136 atom per cent excess deuterium.

The four mice of Group II were kept on the deuterio leucine-containing diet for 15 days, when they were killed. The body water contained 0.051 atom per cent excess deuterium. Cholesterol was isolated from the pooled animal carcasses as the digitonide. The cholesterol regenerated from the digitonide contained 0.232 atom per cent excess deuterium.
The remaining six animals (Group III) were killed after they had received the deuterio leucine-containing stock diet for a period of 22 days. The body water of the pooled tissues contained 0.049 per cent D₂. Cholesterol from the combined carcasses was isolated as the digitonide. The cholesterol digitonide contained 0.112 atom per cent deuterium excess, whereas the cholesterol contained 0.336 per cent. The finding that during the experimental period of 22 days the isotope concentration of cholesterol increases almost linearly reflects the slow metabolic turnover of cholesterol, in agreement with earlier results obtained in this laboratory (8).

Feeding of dl-Deuterio Leucine to Rats—Three adult rats having an average weight of 300 gm. were kept on the same low protein (6 per cent casein) diet as described above. In addition, each rat received per day 0.443 gm. of dl-deuterio leucine (37.0 atom per cent excess deuterium) and 0.300 gm. of sodium chloride, mixed with the stock diet. The animals were kept on this diet for a total period of 16 days. From the 12th to the 15th day one of the rats was given daily, in addition, 0.300 gm. of dl-phenylaminobutyric acid. The urine of this animal was collected and the excreted acetyl-l-phenylaminobutyric acid was isolated as described in the foregoing paper (2). It contained 0.50 atom per cent deuterium; i.e., 2.50 per cent in the acetyl group. At the end of the feeding period the three rats were killed; a sample of body water was secured and cholesterol isolated from the pooled carcasses as the digitonide. After decomposition of the digitonide by pyridine ether (9) 1.1 gm. of cholesterol were obtained. This cholesterol was converted into cholesteryl chloride. The latter contained 0.56 atom per cent deuterium excess. Cholesteryl chloride was degraded into the isooctane-isooctene mixture, as described earlier (1). The hydrocarbon mixture representing the cholesterol side chain contained 0.50 atom per cent deuterium excess and the nuclear hydrocarbon C₁₉H₃₉, 0.54 per cent. The average deuterium content of a compound composed of the fragments C₉H₁₈ and C₁₉H₃₀, as calculated from their isotope content, would be \((18 \times 0.50 + 30 \times 0.54)/48 = 0.53\) per cent. This agrees well with that of the cholesteryl chloride.

Feeding of Deuterio Valine—Two rats weighing about 180 gm. each were kept on the same stock diet which was employed in the leucine experiment. In addition, each rat received daily 0.230 gm. of dl-deuterio valine containing 24.7 atom per cent excess deuterium for a period of 8 days. One of the rats was given 0.20 gm. of dl-phenylaminobutyric acid per day. Cholesterol was isolated from the carcasses and acetyl-l-phenylaminobutyric acid from the urine. The isotope concentrations in the isolated compounds are listed in Table I.

Feeding of Deuterio Isovaleric and Deuterio Isobutyric Acids—The isotopic fatty acids were administered to rats as the sodium salt added to the usual stock diet. The diet further contained 0.100 gm. of dl-phenylamino-
butyric acid per 100 gm. of rat weight. The deuterium concentrations of the isolated cholesterol and acetylphenylaminobutyric acids are listed in Table I.

**DISCUSSION**

In Table I are listed the isotope concentrations in cholesterol and in the acetyl groups of acetylphenylaminobutyric acid excreted after the administration of the labeled test substances. It has been pointed out (1) that the presence in cholesterol of isotope in concentrations greater than half of that of the body fluids constitutes evidence for the utilization of the test substance in cholesterol synthesis. After the administration of deuterio leucine the concentration of isotope in the cholesterol was almost 4 times that in the body fluids; when deuterio isovalerate was fed, the corresponding ratio was nearly 2. The values resulting from the feeding of labeled valine or isobutyric acid are too low to be considered significant. Since leucine was given over twice as long a period as acetate or isovalerate, the relative efficiencies of the various compounds cannot be compared directly. It is permissible, however, to assume that after a period of 8 days the cholesterol in the leucine experiment contained roughly half of the isotope concentration which was found after 16 days.\(^2\) Intermediary acetate formation from leucine and isovaleric acid, which is evident from the high deuterium concentrations in the acetyl groups, can account satisfactorily for the effect given by these compounds in cholesterol synthesis. This relation is illustrated by the ratio of isotope concentrations in the acetyl group to that in cholesterol. The quantities of deuterio acetate formed from leucine and isovaleric acid seem adequate to produce cholesterol with the observed concentrations.

As in experiments in which acetate was fed, the isotope in cholesterol following administration of deuterio leucine is distributed fairly evenly over the side chain and the nucleus. With regard to the possibility that leucine, aside from being a source of acetate, also supplies the isopropyl group of the cholesterol side chain or the angular methyl groups, it must be pointed out that, as the five methyl groups contain only 15 out of a total of 46 hydrogen atoms in the cholesterol molecule, such an effect might not be detectable in our data.

The equivalence of leucine and isovaleric acid with respect to acetate formation supports the hypothesis that degradation to isovaleric acid is a step in the normal metabolism of leucine. The present findings demonstrate the formation of acetic acid as a normal breakdown product of leucine and of isovaleric acid but do not indicate whether acetoacetate is an intermediate. In order to evaluate the analytical data, it will be useful

\(^2\) This is the case in the experiment in which mice were used.
to consider the coefficient of utilization defined in the foregoing paper (2). This coefficient, which is a measure of efficiency of acetyl formation, when calculated for the 9 carbon-bound hydrogen atoms of isovaleric acid, is 5.1 and has an identical value for the corresponding 9 hydrogen atoms of leucine. Since the coefficient has a value of 15.6 for acetic acid itself, a value of 5.1 for leucine and isovaleric acid is taken to mean that of the 9 hydrogen atoms under consideration 3 appear as acetic acid. These facts, which indicate that 1 mole of acetic acid is formed from leucine, are compatible with the view that isovaleric acid is an intermediate in its degradation. As leucine was administered as the racemic compound, this hypothesis is valid for both isomers. The inversion of d-leucine in vivo is well established (10). The formation of acetic acid from leucine could conceivably result from either of two processes shown in the accompanying diagram. The steps involved in the degradation of isovaleric acid to acetic acid must in both cases lead to a loss of carbon-bound deuterium. If demethylation of the isopropyl group takes place and acetoacetate is an intermediate (Pathway A), then isovaleric acid would be expected to yield acetate to the same extent as butyric acid, and it should have a coefficient of utilization intermediate between those found for \( \alpha,\beta \)- and \( \beta,\gamma \)-dideuterio butyric acids (2). In this case, 5 of the 9 hydrogen atoms, namely those at the \( \alpha \)- and \( \gamma \)-carbon atoms, will remain for acetate formation, and on
this basis the coefficient of utilization becomes \( \frac{9}{5} \times 5.1 = 9.18 \). If acetoacetate were the intermediate, the coefficient should fall between the values 3.8 and 7.6 found in Experiments 4 and 5 of the paper just cited.

The second process (Pathway B) postulates the formation of 1 mole each of acetone and acetic acid. According to this scheme the acetoacetic acid arising from isovaleric acid in ketosis would have to be formed by the well recognized (11) condensation of 2 moles of acetate. Only the 2 hydrogen atoms in the \( \alpha \) position of isovaleric acid could appear in the acetic acid, and the coefficient of utilization would be \( \frac{9}{21} \times 5.1 = 22.9 \), which is greater than that (15.6) calculated for acetic acid itself. Neither one of the two schemes explains satisfactorily the large production of acetate from isovaleric acid. Pathway B would conform with experimental data only if the acetone were to break down \textit{in vivo} to acetic acid. In the absence of such evidence no decision can be made as to whether one of the two pathways mentioned, or a different one, is involved.

The failure of valine and isobutyric acid to yield acetyl as well as cholesterol is consistent with the known glycogenic action of these compounds. According to Rose \textit{et al.} (12), 3 out of the 5 carbon atoms of valine are converted to glycogen. Formation of pyruvate as an intermediary step in this conversion is improbable, since pyruvate is at least partly converted to acetyl. Propionic acid is a possible intermediate in isobutyric degradation, since it is incapable of producing acetic acid (2).

**SUMMARY**

1. The preparation of \( dl \)-deuterio leucine, \( dl \)-deuterio valine, deuterio isovaleric acid, and deuterio isobutyric acid is described.

2. Cholesterol isolated from rats which had received labeled leucine or isovaleric acid contained appreciable concentrations of deuterium, but only normal hydrogen when labeled valine or isobutyric acid was fed.

3. When phenylaminobutyric acid was administered simultaneously with labeled leucine or isovaleric acid, the excreted acetyl derivative of the amino acid contained a high isotope concentration, demonstrating that these two compounds were degraded to acetic acid. Labeled valine and isobutyric acid do not form deuterio acetyl groups. It is suggested that the effect of leucine and isovaleric acid shown in cholesterol synthesis is not specific but due to intermediary acetate formation.

4. From quantitative data it is concluded that isovaleric acid is an intermediate in the oxidative breakdown of leucine.

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K. BLOCH

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