STUDIES ON THE CONVERSION OF RADIOACTIVE LEUCINE TO ACETOACETATE*

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Although it is well established that leucine gives rise to ketone bodies in the course of its metabolism in the mammalian organism, the mechanism of this transformation has not been fully elucidated. As early as 1906, Embden and his colleagues (2) presented evidence from perfusion studies that isovaleric acid is a ketogenic substance, and they proposed it as a possible intermediate in the biological degradation of leucine. Subsequently, Ringer, Frankel, and Jonas (3) reported that the administration of isovaleric acid to phlorhizinized dogs results in the excretion of extra ketone bodies. The latter investigators suggested that the compound undergoes reductive demethylation to form butyric acid, which is then oxidized directly to acetoacetic acid.

More recently, Bloch (4) has found that deuterium-labeled leucine or isovaleric acid is partially converted in the intact animal to "acetate," which can be utilized for acetylations as well as for cholesterol formation. These experiments are of particular interest in that they permit a calculation of the extent to which acetate was formed from the administered compounds. Bloch's data indicate that only half a mole of acetoacetate may be formed from a mole of leucine.

The experiments described below were undertaken in the hope that the mechanism of these reactions could be established with the aid of the C\textsuperscript{14} isotope as a tracer. For this purpose, two preparations of leucine were synthesized, with the tag in the \( \beta \) and the \( \gamma \) positions, respectively. From a study of the metabolism of the two compounds in liver slices, it was possible to demonstrate that the \( \alpha \) - and \( \beta \)-carbons of leucine furnish a 2-carbon fragment capable of condensing to form acetoacetate. The isopropyl group of the amino acid does not yield a similar 2-carbon compound to an appreciable extent.

EXPERIMENTAL

*Synthesis of \( \beta \)-C\textsuperscript{14}-Leucine—The reactions which were employed are as follows:

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RADIOACTIVE LEUCINE AND ACETOACETATE

\[(\text{CH}_3)_2\text{CHMgBr} + \text{CO}_2 \rightarrow (\text{CH}_3)_2\text{CHCOOH} \rightarrow (\text{CH}_3)_2\text{CHCH}_2\text{OH} \rightarrow (\text{CH}_3)_2\text{CHCH}_2\text{Br} \rightarrow (\text{CH}_3)_2\text{CHCH}_2\text{CHNH}_2\text{COOH}\]

Carboxyl-labeled isobutyric acid was prepared by the Grignard reaction according to the general method of Sakami, Evans, and Gurin (5). The carbon dioxide formed by the action of perchloric acid on 10 gm. of radioactive barium carbonate was allowed to react with the Grignard reagent made from 6.20 gm. of isopropyl bromide. The yield of redistilled isobutyric acid was 59 per cent of the theoretical amount. The acid was converted directly to isobutyl alcohol by the use of lithium aluminum hydride, according to the general method of Nystrom and Brown (6). Treatment of the dry alcohol with phosphorus tribromide gave isobutyl bromide in a 52 per cent over-all yield from the acid. The isobutyl bromide was then condensed with acetylamino malonic ester, according to the directions of Snyder, Shekleton, and Lewis (7), and the crude reaction product was hydrolyzed with hydrobromic acid. The free amino acid, which was obtained by the addition of ammonium hydroxide, was recrystallized from water. The yield from the isobutyl bromide was calculated as 42 per cent. After an additional recrystallization, the leucine gave a satisfactory value for total nitrogen content (micro-Kjeldahl), and it had a radioactivity of 008 counts per minute per mg. of carbon, or 3618 calculated for the $\beta$-carbon alone.

**Synthesis of $\gamma$-C$^{14}$-Leucine**—The reactions are as follows:

\[\text{CH}_3\text{MgI} + \text{CO}_2 \rightarrow \text{CH}_3\text{COOH} \rightarrow (\text{CH}_3)_2\text{CO} \rightarrow (\text{CH}_3)_2\text{CHOH} \rightarrow (\text{CH}_3)_2\text{CHBr} \rightarrow (\text{CH}_3)_2\text{CHCOOH} \rightarrow (\text{CH}_3)_2\text{CHCH}_2\text{OH} \rightarrow (\text{CH}_3)_2\text{CHCH}_2\text{Br} \rightarrow (\text{CH}_3)_2\text{CHCH}_2\text{CHNH}_2\text{COOH}\]

3 gm. of carboxyl-labeled acetic acid were prepared from radioactive barium carbonate and non-labeled methyl iodide (5). The acid was converted to the calcium salt and dried in an oven at 110°. It was then converted to acetone by heating at 430–490° in a glass tube approximately 15 cm. in length and 2 cm. in diameter. Dry, heated nitrogen gas was continuously passed through the system in order to carry the acetone vapors into a receiver cooled with liquid air. The product was washed into a reduction vessel with purified dioxane and shaken for 5 hours with Raney's nickel under hydrogen at a pressure of 40 pounds. The resulting isopropyl alcohol was separated by distillation and then treated with phosphorus tribromide to yield radioactive isopropyl bromide in an over-all yield of 50 per cent from the acetic acid. The bromide was con-
verted to α-C\textsuperscript{14}-isobutyric acid by carboxylation with non-radioactive carbon dioxide, and the acid was then transformed into γ-C\textsuperscript{14}-leucine by the reactions previously described. The radioactivity of the analytically pure amino acid was 40 counts per minute per mg. of carbon, or 240 calculated for the γ-carbon alone.

**Determination of Radioactivity**—All of the radioactivity measurements reported in this paper were performed on barium carbonate samples. The various organic compounds were submitted to the wet oxidation procedure of Van Slyke (8), and the carbon dioxide which was produced was trapped in a saturated solution of barium hydroxide. The barium carbonate was thoroughly washed with water and then dried before it was spread on plates in a manner previously reported (9). The radioactivity of the samples was determined with a bell-shaped Geiger counter having a thin mica window (2.5 mg. per sq. cm.). Under these conditions, the relationship of recorded activity in the counter to plate thickness was found to be identical with that described by Reid (10).

The counts in excess of background were in all cases corrected to activity at infinite thickness. With the exception of a few values (0.6 or less) which are of questionable significance, the radioactivities reported are subject to a standard error in counting of no greater than 5 per cent.

**Incubation of β-C\textsuperscript{14}-Leucine with Liver Slices**—The procedures described by Edson (11) and Cohen (12) for the study of leucine metabolism in liver tissue in vitro were adapted to a larger scale so as to provide sufficient material for a complete chemical degradation of the resulting acetoacetate. Adult rats which had been fasted for 24 hours were sacrificed. The livers were removed immediately, and tissue slices approximately 0.5 mm. in thickness were prepared. To 2.5 gm. of moist slices in a Warburg flask, 20 ml. of Ringer-phosphate buffer solution containing 3.0 mg. of the radioactive β-leucine were added, and the flask was shaken for 4 hours at 38° under an atmosphere of oxygen. Respiratory carbon dioxide was absorbed by a 20 per cent solution of sodium hydroxide in the center well.

Upon the completion of the incubation, the medium was decanted and the slices were washed. The combined supernatant solutions and washings from four flasks were treated with the copper-lime reagents commonly used for deproteinization. To the filtrate, containing approximately 8 mg. of acetoacetic acid produced by the tissue, 90 mg. of non-radioactive acetoacetic acid freshly prepared from the ethyl ester (13) were added.

**Degradation Procedure A**—A portion of the final solution containing carrier acetoacetate was heated under a reflux with Deniges' reagent, and the carbon dioxide which was liberated was collected as barium carbonate.
The mercury-acetone complex was separated from the solution, dissolved in 1 N hydrochloric acid, and purified by steam distillation of the acetone into fresh mercuric sulfate reagent. It is apparent from the results given in Table I that in each experiment the radioactivity was concentrated in the acetone fraction.

Degradation Procedure B—In order to establish the radioactivity in the individual methyl and methylene carbons of the acetoacetate, use was made of the degradation procedure recently reported by Weinhouse and Millington (14). A portion of the protein-free filtrate containing carrier acetoacetate was oxidized with permanganate at 0° to yield carbonic, formic, and acetic acids from the carboxyl, α-, and β- + γ-carbons, respectively, of the acetoacetic acid. To distinguish between the β- and the γ-carbons, it was necessary to degrade the acetic acid fraction further. Accordingly, the acetic acid was converted to the barium salt, which was pyrolyzed in an evacuated sealed tube to form acetone and barium carbonate. The acetone was collected in a portion of the tube which was chilled in a chloroform-dry ice mixture; it was subsequently isolated as the mercury complex and then purified in the usual manner. The crude barium carbonate, which was gray in color, was acidified, and the liberated carbon dioxide was aerated into barium hydroxide solution.

The radioactivity of the formic acid samples, as shown in Table I, indicates the presence of relatively large amounts of C14 in the methylene carbon of the acetoacetic acid. In contrast, the BaCO₃ formed by pyrolysis of the barium acetate bears only a trace of the C14 isotope in each experi-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Carbons of acetoacetic acid represented</th>
<th>Counts per minute per mg. of carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Respiratory CO₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degradation Procedure A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>COOH</td>
<td>0.6</td>
</tr>
<tr>
<td>Acetone</td>
<td>CH₂, CO₁, CH₂</td>
<td>9.3</td>
</tr>
<tr>
<td>Degradation Procedure B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
<td>CH₂</td>
<td>12.2</td>
</tr>
<tr>
<td>BaCO₃</td>
<td>CO</td>
<td>0.6</td>
</tr>
<tr>
<td>Acetone</td>
<td>CH₂, CO₁, CH₂</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>CH₂ (calculated)</td>
<td>11.6</td>
</tr>
</tbody>
</table>

* These figures cannot be compared directly with the other values in the table because the acetoacetate was diluted by carrier (see the text).
ment. The activity of the methyl carbon was calculated from that of the acetone produced by the pyrolysis of barium acetate, by multiplying by $\frac{1}{2}$.

It is apparent that in each experiment the radioactivity was concentrated almost entirely in the methyl and methylene carbons, and to approximately the same extent in each of these. It is therefore concluded that at some stage in the metabolism of leucine its $\alpha$- and $\beta$-carbons split off as a 2-carbon intermediate which is capable of condensing to form acetoacetate. The two degradative procedures which were used yielded data which are in good agreement. In Experiment 1, the radioactivity of the individual methylene and methyl carbons of the acetoacetic acid, as determined in Procedure B, accounts for 85 per cent of the activity of the acetone fraction obtained in Procedure A. In Experiment 2, a similar calculation yields a value of 91 per cent.

Administration of $\beta$-$\text{Cl}^{14}$-Leucine to Phlorhizinized Rat—A fasted, phlorhizinized rat was injected subcutaneously with 100 mg. of $\beta$-$\text{Cl}^{14}$-leucine, and the acetoacetate which appeared in the urine during the following 24 hours was degraded by standard procedures. The mercury-acetone complex was found to be radioactive, and iodoform prepared from it accounted for almost all of the isotope present. These findings are in accord with the results of the tissue slice experiments.

Unpublished data indicate that in the intact phlorhizinized rat singly labeled radioactive acetoacetate is randomized to a considerable extent prior to its appearance in the urine. Therefore, in the present experiment no attempt was made to establish the distribution of $\text{Cl}^{14}$ between the methyl and methylene carbons.

Incubation of $\gamma$-$\text{Cl}^{14}$-Leucine with Liver Slices—The results of these experiments, which were conducted in a manner similar to that already described for the $\beta$-labeled amino acid, are presented in Table II. In Experiment 1 no carrier acetoacetate was added to the protein-free filtrate, which was treated according to Procedure A. The appearance of isotope solely in the acetone fraction suggested the possibility that in the presence of the liver slices the isopropyl group of the amino acid had been directly converted to acetone. As a control experiment, a solution containing 6 mg. of $\gamma$-$\text{Cl}^{14}$-leucine and 8 mg. of non-radioactive acetoacetic acid was carried through the copper-lime treatment and the mercuric sulfate degradation. Under these conditions, the mercury-acetone complex had no measurable radioactivity. Therefore, the findings in Experiment 1 must be attributed to the action of the liver tissue on the amino acid.

In the event that the isopropyl group is converted to acetone during metabolism and that the latter compound is not further metabolized in
RADIOACTIVE LEUCINE AND ACETOACETATE

in liver tissue, one would expect to find no radioactivity in any of the volatile acids produced by the action of permanganate. Acetone, unlike acetoacetate, is not degraded under the experimental conditions of this procedure. In Experiment 2, 8 mg. of acetoacetate carrier were added to the solution before aliquots were submitted to the mercuric sulfate and the permanganate degradations. The acetone fraction exhibited significant radioactivity, whereas the carbon dioxide liberated by Denigès' reagent did not. Of the volatile acids produced in Procedure B, only the acetate exhibited significant radioactivity. This finding was confirmed in Experiment 3, where 16 mg. of carrier acetoacetate were added to the

TABLE II

Distribution of Radioactivity in Acetoacetate Formed from γ-Labeled Leucine in Liver Slices

<table>
<thead>
<tr>
<th>Compound</th>
<th>Carbons of acetoacetic acid represented</th>
<th>Counts per minute per mg. of carbon</th>
</tr>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Respiratory CO₂</td>
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<td>0.2</td>
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<tr>
<td>Degradation Procedure A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>COOH</td>
<td>0</td>
</tr>
<tr>
<td>Acetone</td>
<td>CH₃, CO, CH₃</td>
<td>5.1</td>
</tr>
<tr>
<td>Degradation Procedure B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>COOH</td>
<td>0</td>
</tr>
<tr>
<td>Formic acid</td>
<td>CH₂</td>
<td>0</td>
</tr>
<tr>
<td>Acetic &quot;</td>
<td>CH₃, CO</td>
<td>3.1</td>
</tr>
<tr>
<td>BaCO₃</td>
<td>CO</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>CH₃, CO, CH₃</td>
<td></td>
</tr>
</tbody>
</table>

combined copper-lime filtrate from eight flasks in order to provide sufficient material for degradation of the acetate fraction. The acetic acid was converted to the barium salt, which was pyrolyzed, and the products were purified. It is apparent that the radioactivity of the barium carbonate was exactly twice that of the acetate, which was therefore labeled only in its carboxyl carbon. The significance of these findings is discussed below.

DISCUSSION

In the course of their perfusion studies, Embden and his associates (2, 15) discovered that a greater amount of ketone bodies was produced from D- or DL-leucine than from the L isomer. Edson (11) confirmed this difference in behavior by his studies on the formation of acetoacetate from the L- or DL-amino acid in liver slices. Since the optical isomers
of the amino acid are known to yield the same keto acid upon oxidative
deamination (16, 17), one would not expect their carbon chains to meet
with different catabolic fates. It would appear that the rate of deamination
is the factor governing the differences observed by these workers.
Rose (18) has established the fact that D-leucine is not an efficient substi-
tute for the L isomer in stimulating growth in the rat. The rate of the
inversion is apparently inadequate to meet the demands of the organism
for this purpose. That the inversion actually can occur to a certain ex-
tent was proved by Ratner, Schoenheimer, and Rittenberg (19). They
concluded from isotopic experiments that complete deamination of the
D isomer must take place prior to asymmetrical amination. Accordingly,
the conclusions reached in the present investigation with respect to the
biological degradation of the carbon chain are believed to apply to both
enantiomorphs of leucine.

The data from the studies with the β-labeled leucine lead to the con-
clusion that during the metabolism of the amino acid its α- and β-carbons
split off as a 2-carbon intermediate which can condense to form aceto-
acetate. The experimental evidence definitely rules out the hypothesis
that 4 carbons of the amino acid might form acetoacetate directly, for in
such an event it would have been labeled in only a single position. This
deduction depends upon the well established fact that acetoacetate does
not undergo randomization in liver tissue (20).

Our findings are in agreement with the possibility that isovaleric acid
is an intermediate in the reactions under discussion. However, butyric
acid could not have been formed from isovaleric acid, as was proposed at
one time by Ringer et al. (3). Under the experimental conditions of the
present study, butyrate would have formed acetoacetate with a greater
proportion of the isotope in the methylene carbon (21). In the two ex-
periments given in Table I, the ratios of the radioactivity of the methyl-
ene carbon to that of the methyl carbon are only 1.1 and 1.2, respectively.

In the in vitro experiments with the β-C14-leucine, the respiratory car-
don dioxide had appreciable radioactivity. These figures cannot be di-
rectly compared with the other data in Table I because the acetoacetate
underwent chemical dilution with the carrier before it was degraded. Where-
as roughly 10 per cent of the administered counts appeared in the aceto-
acetic acid, less than 1 per cent was in the respiratory carbon dioxide.
It would seem likely that the latter effect is not evidence for an additional
pathway of leucine metabolism, but an indication that a small portion of
the 2-carbon fragments was oxidized via the tricarboxylic acid cycle. The
values for the radioactivity of the carboxyl and carbonyl carbons of the
acetoacetate are so low that they are of questionable significance.

The absence of significant amounts of radioactivity from the respira-
tory carbon dioxide in each experiment in Table II indicates that the isopropyl group of leucine is not completely oxidized in liver tissue. It can be concluded from the studies with the $\gamma$-labeled leucine that the isopropyl group does not furnish a 2-carbon fragment capable of giving rise to doubly labeled acetoacetate. The data support the conclusions previously reached with $\beta$-C$^{14}$-leucine as to the possible rôle of isovaleric acid in these reactions.

It remains to be established whether the isopropyl group is converted to a single compound which both forms an insoluble complex with Deniges’ reagent and is readily oxidized to acetate by the action of permanganate. The true intermediate most likely is not a glycogenic substance, because no reliable evidence exists for the metabolic conversion of leucine to extra carbohydrate.

The accompanying series of reactions, showing the proposed metabolic pathway of leucine catabolism, is in accord with the evidence which has been presented.

$$\begin{align*}
(\text{CH}_2)_2\text{CHCH}_2\text{CHNH}_2\text{COOH} & \rightarrow (\text{CH}_2)_2\text{CHCH}_2\text{COCOOH} \\
(\text{CH}_2)_2\text{CHCH}_2\text{COOH} & \rightarrow [(\text{CH}_2)_2\text{CH}-] + [-\text{CH}_2\text{COOH}] \\
(?) & \rightarrow \text{CH}_2\text{COCH}_2\text{COOH}
\end{align*}$$

We wish to express our appreciation to Dr. Sidney Weinhouse for making available to us the unpublished details of his procedure for the degradation of acetoacetate with permanganate, and to thank Dr. D. Wright Wilson for his advice and criticism.

**SUMMARY**

It has been demonstrated by the incubation of $\beta$-C$^{14}$-leucine with liver slices that the $\alpha$- and $\beta$-carbons of the amino acid split off as a 2-carbon intermediate which is capable of condensing to form acetoacetate. The findings are consistent with the hypothesis that isovaleric acid is an intermediate in leucine catabolism, but not with the assumption that isovalerate undergoes reductive demethylation to form butyrate or oxidative demethylation to form acetoacetate directly.

The administration of the $\beta$-labeled amino acid to a phlorhizinized rat, followed by degradation of the urinary acetoacetate, gave results in accord with the findings *in vitro.*

Evidence has been presented that the isopropyl group of leucine is not
completely oxidized to carbon dioxide in liver tissue, and that it does not furnish to an appreciable extent a 2-carbon fragment similar to the one arising from the $\alpha$- and $\beta$-carbons of the amino acid.

**BIBLIOGRAPHY**

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