ISOTOPE STUDIES ON THE METABOLISM OF VALINE*

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Previous information on the catabolism of valine has been obtained by taking advantage, for the most part, of the gluconeogenic property of valine and using glucose as an indicator of its intermediary metabolism. Thus, Rose et al. (1) reported that valine and α-ketoisovaleric acid have similar gluconeogenic effects, both contributing 3 of their carbon atoms to glucose formation in phlorizinized dogs. Previously, Ringer and coworkers (2) had observed that isobutyric acid, the decarboxylation product of α-ketoisovaleric acid, also furnishes 3 carbon atoms to the formation of glucose. Studies in which isotopically labeled valine was administered to fasted rats, and the glycogen isolated and degraded, confirmed the formation of a 3-carbon intermediate, which was presumed to be propionic acid (3).

The investigation described in the present paper was undertaken in order to obtain direct evidence regarding the nature of the intermediates of valine oxidation. Using dl-valine-4, 4'-C14, we have been able to isolate and identify labeled α-ketoisovaleric, isobutyric, and propionic acids by chromatographic means. Evidence has also been obtained which indicates that carbon atoms 3, 4, and 4' of valine give rise to propionic acid and that the conversion of isobutyric acid to propionic acid proceeds by way of β-hydroxyisobutyric acid and the removal of its carboxyl group.†

EXPERIMENTAL

Incubation in Vitro—The homogenates were prepared by excising the livers of decapitated rats as quickly as possible and homogenizing them in 2 volumes of aerated, ice-cold 0.9 per cent KCl in a loose fitting Potter-Elvehjem glass homogenizer, chilled in an ice bath. The piston was allowed to work no longer than 1 minute, during which time the mixture was adjusted every few seconds with KOH to pH 7.3. The gross tissue

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1 A preliminary report of this investigation has been presented (4).
residue was removed by centrifugation at 0° in the International refrigerated centrifuge at 300 \times g for 3 minutes.

5 mg. of \textit{dl}-valine-4,4'-\textit{C}^{14}\textsuperscript{2} (6.0 \mu c. per mg.) were incubated with 5 ml. of the liver homogenate for 2 hours at 38°. The incubation mixture was then deproteinized by the addition of 0.25 volume of 5 per cent metaphosphoric acid, and, after centrifugation in the Spino preparative ultracentrifuge (at 80,700 \times g, for 15 minutes), the supernatant solution was adjusted to pH 8 with NaOH and the residual protein precipitated by the addition of 10 volumes of acetone. After evaporating the solution \textit{in vacuo} to 0.5 ml., 10 ml. of a 33 per cent (by volume) acetone-alcohol mixture were added to precipitate most of the sodium metaphosphate. One-fourth of the supernatant solution was then evaporated to dryness in a vacuum desiccator. 5 to 10 \mu moles of each of the acids under study were added, to serve as carriers in the subsequent column chromatography, and the dry residue was dissolved and brought to pH 2.5 with dilute \textit{H}_{2}\text{SO}_{4}. The aqueous solution was then absorbed on a disk punched from a Whatman ashless filter paper tablet and placed on top of the silica gel column, as described previously (5).

\textit{Experiments on Intact Animal}—10 mg. of \textit{dl}-valine-4,4'-\textit{C}^{14} (6.0 \mu c. per mg.) were dissolved in saline solution, the pH was adjusted to 7.3 with NaOH solution, the volume brought to 1 ml., and the solution injected intraperitoneally into a rat. After 10 minutes, the rat was decapitated and the liver excised as rapidly as possible and homogenized in ice-cold acetone in a Waring blender. The homogenate was transferred to a tightly stoppered flask and allowed to stand overnight in the refrigerator (6). The mixture was then centrifuged, adjusted to pH 8, and the solution evaporated to dryness. The fat was removed by repeated filtration during the evaporation process. The residue was then treated as described above, absorbed on a disk of filter paper, and applied to the top of the silica gel column.

\textit{Chromatography on Silica Gel Column}—The preparation of the silica gel column and the solvent dispenser used have been described previously (5). The effluent was collected with a Technicon fraction collector in 2.0 ml. fractions, which were received in test-tubes containing 1 ml. of water.

Two different solvent systems were used to elute the acids. The first solvent system consisted of chloroform (in the mixing vessel) and of a 1:1 (volume per volume) chloroform-\textit{tert}-amyl alcohol mixture containing 1 per cent distilled water and 0.8 per cent ethanol (in the separatory fun-

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2 \textit{dl}-Valine-4,4'-\textit{C}^{14} was synthesized by R. Ostwald and B. Tolbert through a cooperative arrangement with the Bioorganic Group of the Radiation Laboratory, University of California. The preparation was tested for purity by column chromatography and by radioautography on paper.
The second solvent system consisted of benzene (in the mixing vessel) and ether (in the separatory funnel). The chloroform, benzene, and ether were saturated with 0.05 N H$_2$SO$_4$. In the case of both solvent systems, the sequence and chromatographic positions of the acids used had previously been determined by chromatographing known acids singly, in pairs, and in larger groups and by paper chromatography and specific reactions.

**Radioactivity Measurements**—For radioactivity measurements, the aqueous phase, containing the salts of the acidic intermediates, was separated from the organic phase by means of a small separatory funnel and transferred to polyethylene planchets. 1 drop of 0.05 N NaOH solution was added to each planchet to provide an excess of base, and the solutions in the cups were evaporated to dryness under infra-red lamps.

The carboxylic acid intermediates of valine-4,4'-C$^{14}$ were identified by comparing the radioactivity curves with the titration curves obtained from the collected fractions.

**Paper Chromatography**—The isobutyric, β-hydroxyisobutyric, and propionic acid fractions isolated by silica gel chromatography were further identified by paper chromatography and radioautography with the solvent system suggested by Reid and Lederer (7). Whatman No. 1 paper was employed, which, prior to use, was washed according to the method of Kennedy and Barker (8). The chromatograms were, in most cases, developed for 48 hours, and the carboxylic acid spots were located by spraying the dried chromatograms with a 0.04 per cent alcoholic solution of bromophenol blue, the pH of which was adjusted to 5.0 with citric acid (9). To prevent the evaporation of the acids, the papers were then sprayed with 0.5 N NaOH, and radioautograms were prepared.

The α-ketoisovaleric acid isolated by silica gel chromatography was converted to its 2,4-dinitrophenylhydrazone derivative (10), and after washing it with 2 N HCl the radioactivity was measured. The dry hydrazone was then dissolved in 0.2 ml. of ethanol and neutralized with 0.3 ml. of 0.1 M phosphate buffer, pH 7.2 (11). Aliquots of this solution were used for paper chromatography (12) and radioautography. The yellow color of the 2,4-dinitrophenylhydrazone served as its own indicator.

Identification of isobutyric, β-hydroxyisobutyric, and propionic acids and of the 2,4-dinitrophenylhydrazone of α-ketoisovalerate was made by comparing the location of the color spots on the chromatograms with the radioactivity spots on the film.

**Degradation of Propionic Acid**—The propionic acid, isolated by silica gel chromatography from the incubation of DL-valine-4,4'-C$^{14}$ with rat liver homogenate, was rechromatographed and degraded stepwise by Phares' modification of the Schmidt method (13). An aliquot of the
propionic acid was totally ashed by the Van Slyke-Folch wet oxidation method (14). In all cases, the CO₂ evolved was trapped in NaOH and precipitated as BaCO₃ prior to plating.

RESULTS AND DISCUSSION

The pertinent data appear in Figs. 1 to 5 and Tables I and II. As with most other amino acids, the catabolism of valine is initiated by removal of its amino group to yield α-ketoisovaleric acid. L-Valine readily undergoes transamination (15) and is slowly attacked by L-amino acid oxidase (16), while the unnatural D-valine is readily attacked by D-amino acid oxidase (17). Administration of DL-valine to rats suffering from experimental ketosis (18) and experiments with liver slices (19) have demonstrated the antiketogenic effect of DL-valine. The gluconeogenic property of DL-valine was shown when its administration to rats resulted in deposition of hepatic glycogen (18) and its feeding to phlorizinized dogs gave rise to extra glucose in the urine equivalent to 3 of the 5 carbons of the amino acid (1). α-Ketoisovaleric acid (1) and isobutyric acid (2), its decarboxylation product, also furnish 3 of their carbon atoms to the formation of glucose. These observations led Rose and his associates (1) to suggest that valine is catabolized by way of α-ketoisovaleric acid and isobutyric acids. Propionic acid, which is glycogenic, was postulated as the 3-carbon intermediate arising from isobutyric acid, and Atchley (20) was able to demonstrate that this conversion is accomplished by the fatty acid-oxidizing system occurring in liver and kidney.

By incubating DL-valine-4,4'-Cl¹⁴ with rat liver homogenate and chromatographing the aqueous extract of the incubation mixture on a silica gel column, with a benzene-ether mixture as the solvent system, we were able to isolate and identify labeled α-ketoisovaleric, isobutyric, and propionic acids (Fig. 1). The identity of these three intermediates was confirmed when the aqueous extract of the incubation mixture was chromatographed on a silica gel column, this time with a chloroform-tert-amyl alcohol mixture as the solvent (Fig. 2). Further characterization of the chromatographically isolated isobutyric and propionic acid fractions was made by paper chromatography and radioautography (Fig. 3, A). The eluted α-ketoisovaleric acid was converted to its 2,4-dinitrophenylhydrazone derivative, which was identified by paper chromatography and radioautography (Fig. 3, B).

The formation of propionate in the intermediate metabolism of valine is in accord with the findings of Fones and coworkers (3, 21), who, after feeding labeled valine to fasted rats, determined the isotope distribution pattern in the glucose units of liver glycogen. Administration of L-valine- ³

³ Unlabeled α-ketoisovaleric acid, employed as a reference standard, was kindly supplied by Dr. Alton Meister, National Cancer Institute.
Fig. 1. Silica gel column chromatogram of the aqueous extract obtained from the incubation of DL-valine-4,4'-C\textsuperscript{14} with rat liver homogenate. A benzene-ether mixture was used as the solvent system. The five minor peaks of radioactivity were not investigated.

Fig. 2. Silica gel column chromatogram of the aqueous extract obtained from the incubation of \textit{d}-valine-4,4'-C\textsuperscript{14} with rat liver homogenate. A chloroform-\textit{tert}-amyl alcohol mixture was used as the solvent system.
Fig. 3. Paper chromatograms and radioautograms of radioactive fractions isolated by silica gel column chromatography from the incubation of DL-valine-4,4'-C14 with rat liver homogenate. The black spots indicate radioactivity, and the areas enclosed by the outlines show the location of the corresponding, unlabeled compounds, which were added for comparison. A, isobutyric acid and propionic acid. The ammonium salts of these acids were chromatographed by the descending method with an n-butanol-ammonium hydroxide solvent system. B, 2,4-dinitrophenyl-hydrazone of α-ketoisovaleric acid. The dry hydrazone was dissolved in 0.2 ml. of ethanol and neutralized with 0.3 ml. of 0.1 M phosphate buffer, pH 7.2. Aliquots of this solution were chromatographed by the descending method with a mixture of tert-amyl alcohol, ethyl alcohol, and water in the ratio 50:10:40 (by volume). C, β-hydroxyisobutyric acid. The same method was used as in A.
3-C\textsuperscript{13} gave rise to glycogen having a relatively small content of excess C\textsuperscript{13} in carbon atoms 3 and 4, but a relatively high isotope concentration in carbons 2, 5 and 1, 6 of glucose (3). The average ratio of excess C\textsuperscript{13} in the 2, 5 positions to that in the 1, 6 positions was 1.13. This is in good agreement with the corresponding ratio of 1.1 found in glycogen deriving its label from the \textalpha-carbon atom of propionate fed to fasted rats (22).

\textit{A priori}, two mechanisms for the conversion of isobutyric acid to propionic acid appear to be plausible. The first would involve the removal of one of the terminal methyl groups of isobutyric acid, and the second, proposed by Atchley (20), would occur by the decarboxylation of isobutyric acid, one of its methyl groups becoming oxidized to form the carboxyl group of propionic acid. The possibility that methylmalonic acid is an intermediate in the conversion of isobutyric acid to propionic acid was

\begin{center}
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \text{-} \text{CH}-\text{COOH} \\
\quad & \quad \text{NH}_2 \\
\quad & \quad \downarrow \\
\text{CH}_3 & \quad \text{CH} \text{-} \text{C}-\text{COOH} \\
\quad & \quad \text{O} \\
\quad & \quad \downarrow \\
\text{CH}_3 & \quad \text{CH} \text{-} \text{COOH} \\
\quad & \quad \text{CH}_3
\end{align*}
\end{center}

\textbf{Diagram 1.} Sequence of reactions in valine oxidation, illustrating the anticipated distribution of radioactive carbon in propionic acid and CO\textsubscript{2} arising from valine-4, A'-C\textsuperscript{13} and valine-2-C\textsuperscript{13} if the catabolism of the intermediate isobutyric acid occurred by the removal of either its methyl or carboxyl group.

Expected findings if methyl group of isobutyric acid is split off

\textbf{CH}_3 \text{-} \text{CH}_2 \text{-} \text{COOH} + \textbf{CO}_2

Expected findings if carboxyl group of isobutyric acid is split off

\textbf{CH}_3 \text{-} \text{CH}_2 \text{-} \text{COOH} + \textbf{CO}_2
(C\textsuperscript{14} equally distributed in carbon atoms 1 and 3)
ruled out by Atchley, since he found methylmalonic acid entirely inactive when incubated with the liver fatty acid-oxidizing system, which is able to oxidize isobutyric and propionic acids to completion.

**Table I**

*Radioactivity of Carbon Dioxide Evolved during Incubation of Labeled Valine with Fortified Triply Washed Residue of Rat Kidney Homogenate*  
Results expressed as per cent of the radioactivity of the incubated valine.

<table>
<thead>
<tr>
<th>Incubation of DL-valine-4,4'-C14</th>
<th>Incubation of DL-valine-2-C14</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>3.0</td>
</tr>
<tr>
<td>0.03</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* The triply washed residues of rat kidney homogenate were prepared as described by Green et al. (24) and used as a thick suspension in 0.9 per cent potassium chloride. The incubation medium in each Warburg flask consisted of 1.5 ml. of the washed kidney residue suspension, supplemented by 0.3 ml. of 0.125 M phosphate buffer at pH 7.3, 0.3 ml. of 0.01 M adenosinetriphosphate, 0.2 ml. of 0.02 M magnesium sulfate, 0.2 ml. of 0.0004 M cytochrome c, 0.2 ml. of pyridoxal phosphate (1 μg per ml.), 0.1 ml. of α-ketoglutarate (50 μmoles per ml.), 0.1 ml. of citrate (50 μmoles per ml.), and 0.2 ml. of DL-valine-4,4'-C14 (6.0 μc. per mg.) or DL-valine-2-C14 (3.2 μc. per mg.), each having a concentration of 50 μmoles per ml. Alkali-soaked filter paper was placed in the center well, and oxygen was used as the gas phase. The incubations were carried out at 38° for 2 hours. (Pyridoxal phosphate, as the calcium salt, was generously donated by Dr. W. W. Umbreit of the Merck Institute for Therapeutic Research. DL-Valine-2-C14 was synthesized by E. M. Gal of this laboratory and its radioactive purity established by radioautography of paper chromatograms.)

**Table II**

*Schmidt Degradation of Propionate Isolated from Incubation of DL-Valine-4,4'-C14 with Rat Liver Homogenate*  
Results expressed as per cent of total activity (obtained from total combustion of propionate).

<table>
<thead>
<tr>
<th>Degradation No.</th>
<th>Carbon 1</th>
<th>Carbon 2</th>
<th>Carbon 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48.4</td>
<td>3.6</td>
<td>42.8</td>
</tr>
<tr>
<td>2</td>
<td>48.7</td>
<td>3.5</td>
<td>42.5</td>
</tr>
</tbody>
</table>

Consideration of the isotope distribution in propionic acid arising from the incubation of valine-4,4'-C14 and of valine-2-C14, if the oxidation of isobutyric acid occurred by either mechanism, is illustrated in Diagram 1. In order to collect only the carbon dioxide formed in the oxidation of valine to the propionic acid stage, DL-valine-4,4'-C14 and DL-valine-2-C14 were incubated separately with fortified, triply washed residue of rat kidney homogenate, since the kidney does not contain the propionic acid
oxidase and, therefore, is unable to carry out the oxidation of valine beyond the propionic acid intermediate (23). The carbon dioxide evolved during the incubation of valine-2-C\textsuperscript{14} was found to be radioactive, but, with valine-4,4'-C\textsuperscript{14}, it contained no significant amount of label (Table I). The stepwise degradation of the propionic acid isolated from the incubation of D,L-valine-4,4'-C\textsuperscript{14} with rat liver homogenate showed an almost equal concentration of C\textsuperscript{14} in carbon atoms 1 and 3, while carbon 2 contained no more than a negligible amount of radioactivity (Table II). The isotope distribution in carbon dioxide and propionic acid is in agreement with the findings anticipated, if isobutyric acid is converted to propionic acid by the loss of the carboxyl group.

The formation of propionic acid by a reaction mechanism involving the decarboxylation of isobutyric acid suggests that this conversion requires the oxidation of the β-carbon atom of isobutyric acid, in accordance with the β oxidation theory. In the light of recent knowledge of fatty acid oxidation (25), isobutyric acid might be expected to be dehydrogenated to methylacrylic acid, which would be hydrated to form β-hydroxisobutyric acid. β-Hydroxyisobutyric acid, in turn, would be dehydrogenated to methylmalonic semialdehyde, which on structural grounds could decompose to carbon dioxide and propionaldehyde and then be further oxidized to propionic acid. The postulated mechanism for the oxidation of isobutyric acid to propionic acid may be summarized by the accompanying sequence of reactions (20).

Atchley (20), acting on the premise that any compound which is an intermediate between isobutyrate and propionate must, like these two compounds, be completely oxidized by the liver fatty acid-oxidizing system, showed that methylacrylic acid, β-hydroxisobutyric acid, and propionaldehyde are completely oxidized by the liver system. This observation indicates that these three compounds may be intermediates in the conversion of isobutyric acid to propionic acid. It does not, however, constitute
evidence that they actually are intermediates in this transformation, since the relatively complex liver fatty acid-oxidizing system is able to oxidize substrates other than isobutyric acid and its metabolites.

We have been able to isolate radioactive β-hydroxyisobutyric acid from the incubation of DL-valine-4,4′-C\textsuperscript{14} with rat liver homogenate and have identified it by chromatography on a silica gel column, using, in one case, a benzene-ether mixture (Fig. 1) and, in another case, a chloroform-tert-

![Silica gel column chromatogram](image)

**Fig. 4.** Silica gel column chromatogram of radioactive β-hydroxyisobutyric acid derived from DL-valine-4,4′-C\textsuperscript{14} and known, unlabeled β-hydroxybutyric acid. This chromatogram was developed with a chloroform-tert-amyl alcohol mixture and shows the differentiation of the two isomers by this method.

amylation alcohol mixture (Fig. 2) as the solvent system. The column chromatography methods employed are sensitive enough to differentiate between the β-hydroxyisobutyric and β-hydroxybutyric acid isomers, as shown by Fig. 4. The chromatographically eluted fraction was further characterized as β-hydroxyisobutyric acid by paper chromatography and radioautography (Fig. 3, C). Our findings thus indicate that the conversion of isobutyric acid to propionic acid involves the formation of the intermediate β-hydroxyisobutyric acid and the loss of its carboxyl group.

The same intermediates which have been identified from the incubation of DL-valine-4,4′-C\textsuperscript{14} with liver homogenate were isolated also from the
liver excised after the intraperitoneal injection of the methyl-labeled valine into the rat (Fig. 5). It appears, therefore, that valine catabolism follows the same pathway in vivo as is indicated by the experiments with liver homogenates in vitro.

![Silica gel column chromatogram of rat liver extract obtained after intraperitoneal injection of DL-valine-4,4'-C\textsuperscript{14}. The solvent system used was a chloroform-tert-amyl alcohol mixture.](http://www.jbc.org/)

**Fig. 5.** Silica gel column chromatogram of rat liver extract obtained after intraperitoneal injection of DL-valine-4,4'-C\textsuperscript{14}. The solvent system used was a chloroform-tert-amyl alcohol mixture.

**SUMMARY**

The catabolism of valine was studied by incubating DL-valine-4,4'-C\textsuperscript{14} with rat liver homogenate and by intraperitoneal injection of the methyl-labeled valine into the rat. In experiments both in vitro and in vivo radioactive $\alpha$-ketoisovaleric, isobutyric, $\beta$-hydroxyisobutyric, and propionic acids were isolated and identified by column and paper chromatography. Stepwise degradation of the isolated propionic acid demonstrated an almost equal concentration of C\textsuperscript{14} in carbon atoms 1 and 3, whereas carbon 2 contained only a negligible amount of radioactivity. The carbon dioxide evolved during the separate incubation of DL-valine-4,4'-C\textsuperscript{14} and DL-valine-2-C\textsuperscript{14} with fortified, triply washed residue of rat kidney homogenate, which does not contain the propionic acid oxidase, showed radioactivity only in the case of valine-2-C\textsuperscript{14}.

These findings are consistent with the hypothesis that the catabolism of
valine is initiated by transamination, resulting in the formation of α-ketoisovaleric acid, which by decarboxylation and oxidation gives rise to isobutyric acid. Isobutyric acid, in turn, undergoes β oxidation to form β-hydroxyisobutyric acid, which, by decarboxylation and oxidation, is converted to propionic acid.

BIBLIOGRAPHY

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