STUDIES ON THE UTILIZATION OF ISOVALERIC ACID IN
CHOLESTEROL SYNTHESIS*

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Previous studies in this laboratory with isovaleric acid-4,4'-C13,1-C14
have led to the conclusion that this branched chain fatty acid can be cleaved
at the tertiary carbon atom to yield a 3-carbon unit and a C2 fragment as
intermediary products (1). While the C2 unit was found to resemble ace-
tate in its metabolic reactions, the isopropyl portion of isovalerate proved
to be a much more efficient carbon source for cholesterol than any sub-
stance previously tested. It was, however, not clear from these experi-
ments whether isovalerate served as a specific precursor for the sterol
molecule.

In the present investigation the incorporation of carbon from isovalerate-
4,4'-C13,1-C14 into cholesterol and some of its degradation products has
been studied. In addition, isovaleric acid-4,4'-C13,3-C14 has been pre-
pared and tested as a carbon source for acetyl groups, fatty acids, and
cholesterol. Evidence for the fixation of carbon dioxide in vivo in the
course of isovalerate metabolism has been obtained from an experiment in
which non-isotopic isovalerate and CaC14O3 were fed to rats.

EXPERIMENTAL

Isotopic Compounds—The synthesis of isovaleric acid-4,4'-C13,1-C14 has
been described previously (1). Isovalerate-4,4'-C13,3-C14 was prepared by
mixing isovalerate-4,4'-C13 and isovalerate-3-C14. The latter compound
was obtained from the condensation product of acetone-2-C14 and ethyl
cyanoacetate. The isotope concentrations in the test substances are given
in Tables I and II.

CaC14O3—C14O2 was released from BaC14O3 by addition of HCl and was
trapped in a slight excess of sodium hydroxide. Somewhat more than the
calculated amount of calcium chloride was added and the precipitate of
CaC14O3 was repeatedly washed with water.

Feeding Experiments—In the experiment in Table I, rats of the Sprague-

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Dawley strain weighing about 200 gm. received isovaleric acid-4',4'-C13, 1- C13, containing 18.9 atom per cent excess C13, specific activity $1.7 \times 10^5$ c.p.m. of C13, mixed with 12 gm. of a fat-free diet (1). Rat 1 was given 2 mm of the labeled compound for 1 day; Rat 2, 1 mm per day for 2 days; and Rat 3, 0.25 mm per day for 8 days. The heads were discarded, and the carcasses of all three rats and the internal organs (except for the livers) of Rats 1 and 2 were pooled for isolation of cholesterol.

In the experiment in Table II, two rats of the same strain and weight were fed the same stock diet containing in addition 0.5 mm of isovalerate- 4',4'-C13, 3-C14 per 100 gm. of rat weight per day, and 100 mg. of L-γ-phenyl-α-aminobutyric acid per day for 3 days. For the isolation of the various compounds the organs and urines of the two animals were pooled.

In the experiment in Table III, each of two 200 gm. male Sprague-Dawley rats was given 50 mg. of L-γ-phenyl-α-aminobutyric acid and 100 mg. of CaC14O3 mixed with 12 gm. of stock diet, per day for a period of 2 days. The diets of one of these rats contained in addition 1 mm of non-isotopic sodium isovalerate.

**Isolation and Degradation Procedures**—Acetyl-L-γ-phenyl-α-aminobutyric acid, cholesterol, and fatty acids were isolated as described (2, 3). Cholesteryl chloride, prepared from 0.56 gm. of cholesterol isolated in Experiment 1, was degraded by thermolysis to the nuclear hydrocarbon C19H30 and a mixture of isoctane and isoctene (4). The residue which remained after thermolysis was distilled at 1 mm. of Hg. The fraction distilling at 230–240° represents the hydrocarbon C19H30. A portion of this hydrocarbon was oxidized with chromic acid (5) and the resulting acetic acid was isolated as the silver salt. This acetic acid is derived from the two angular methyl carbons and from the ring carbons 10 and 17 of cholesterol.

**Isotope Analyses**—All isolated compounds were converted to CO2 in a micro combustion apparatus at 900°. The isotope analyses were carried out as previously described (6). The C14 values are expressed as counts per minute of infinitely thick samples of BaCO3 of constant area.

**RESULTS AND DISCUSSION**

The terminal methyl carbons of isovalerate which are labeled by C13 are incorporated into the nuclear hydrocarbon and into the isooyctyl side chain of cholesterol to nearly the same extent, a result similar to that obtained with methyl-labeled acetate (5) (Table I). If the isopropyl group had served as a specific carbon source for the branched portions of the sterol, the C14 concentrations should have been higher in the isooyctyl moiety than in the nucleus, because 3 out of 8 carbon atoms in the side chain, but only 2 out of 19 carbon atoms in the polynuclear moiety, are branched carbon atoms. The acetic acid which obtains on oxidative degradation of the
hydrocarbon C_{19}H_{30} and which is derived from the angular methyl carbon atoms C_{18} and C_{19} and ring carbons C_{18} and C_{17} also contained C^{13} at a level not exceeding that of the total molecule or of the two products of thermolysis. On the same basis, the C^{13} concentrations of these angular methyl groups should also have been considerably higher. The results actually obtained offer no evidence to indicate that the isopropyl carbons of isovalerate are used as precursors for specific positions or portions of the sterol skeleton, but rather suggest that the isotope distribution is uniform as in cholesterol formed from labeled acetic acid.

In cholesterol derived from isovalerate-1-C_{14}, the relative isotope distribution in the degradation products closely resembles that observed in cholesterol formed from acetate-1-C_{14}. It is reasonable to assume that the

| Table I |

Incorporation of Labeled Isovaleric and Acetic Acids into Cholesterol and Degradation Products

Data for C^{13} expressed as atom per cent excess C^{13}; for C^{14} as counts per minute.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Cholesterol</th>
<th>Nucleus C_{19}H_{19}</th>
<th>Isooctyl side chain</th>
<th>Acetic acid by oxidation of nucleus*</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isovaleric acid-4,4'-C^{13}</td>
<td>0.136</td>
<td>0.124</td>
<td>0.136</td>
<td>0.141</td>
<td>0.91</td>
<td>1.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid-2-C^{14}\dagger</td>
<td>71</td>
<td>76</td>
<td>74</td>
<td>76</td>
<td>1.03</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isovaleric acid-1-C^{14}</td>
<td>320</td>
<td>350</td>
<td>270</td>
<td>165</td>
<td>1.30</td>
<td>0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid-1-C^{14}\dagger</td>
<td>93</td>
<td>98</td>
<td>72</td>
<td>64</td>
<td>1.35</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Carbon atoms 10, 18, 17, and 19 of cholesterol.
\dagger Data taken from Little and Bloch (5).

fragment derived from the carboxyl group of isovalerate enters into cholesterol synthesis by way of acetate or a metabolically active C_{2} unit.

The utilization of the isopropyl group of isovalerate cannot be explained on the basis of a preliminary conversion to acetate because this grouping, when tested under comparable conditions, is several times as efficient a carbon source for cholesterol as is acetate or the carboxyl fragment of isovalerate (1). A more direct conversion of the isopropyl moiety is therefore indicated. On the other hand, the evidence that cholesterol can be derived from acetate as the sole carbon source (5) makes it unlikely that isovalerate is an independent precursor. These findings are not contradictory if it is assumed that the pathways from acetate and isovalerate to cholesterol lead to a common intermediate, and that in the formation of this intermediate labeled carbon from isovalerate is not as extensively diluted by endogenous metabolites as is labeled acetate.

When isovalerate-4,4'-C^{13},3-C^{14} was fed to rats (Table II), the acetyl
groups of the excreted acetylaminoo acid contained C^{13} and C^{14} in a ratio of
about 2:1. This result shows that the acetyl precursor was derived from
an intermediate which contained 2 methyl carbons (C-4 and C-4') for
every tertiary carbon atom (C-3) of isovalerate. A direct conversion of
the isopropyl group to a C_2 unit would yield a C^{13}:C^{14} ratio of 1 and must
therefore be excluded. On the other hand the observed ratio strongly

**Table II**

*Isotope Concentrations in N-Acetyl, Cholesterol, and Fatty Acids of Rats
Fed Isovalerate*

Isovalerate contains 54.5 atom per cent excess C^{13} in C-4 and C-4' and 10.2 X 10^4
c.p.m. of C^{14} in C-3. Rats fed 0.5 mm per 100 gm. of rat weight per day of labeled
compound for 3 days.

<table>
<thead>
<tr>
<th>Compound isolated</th>
<th>Found</th>
<th>Relative isotope concentration*</th>
<th>Found</th>
<th>Relative isotope concentration*</th>
<th>Ratio, C^{13}:C^{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m.</td>
<td>atom per cent excess</td>
<td>c.p.m.</td>
<td>atom per cent excess</td>
<td></td>
</tr>
<tr>
<td>Acetyl group†</td>
<td>765</td>
<td>0.75</td>
<td>0.79</td>
<td>1.45</td>
<td>1.98</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>240</td>
<td>0.24</td>
<td>0.22</td>
<td>0.40</td>
<td>1.67</td>
</tr>
<tr>
<td>Small intestine</td>
<td>565</td>
<td>0.55</td>
<td>0.55</td>
<td>1.01</td>
<td>1.84</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>135</td>
<td>0.13</td>
<td>0.19</td>
<td>0.35</td>
<td>2.69</td>
</tr>
<tr>
<td>Small intestine</td>
<td>205</td>
<td>0.20</td>
<td>0.24</td>
<td>0.44</td>
<td>2.20</td>
</tr>
<tr>
<td>Unsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>80</td>
<td>0.08</td>
<td>0.10</td>
<td>0.18</td>
<td>2.25</td>
</tr>
<tr>
<td>Small intestine</td>
<td>120</td>
<td>0.12</td>
<td>0.14</td>
<td>0.26</td>
<td>2.17</td>
</tr>
</tbody>
</table>

* C.p.m. or atom % excess in compound isolated

C.p.m. or atom % excess in labeled atoms of compound fed X 100.

† Acetylphenylamino butyric acid was isolated and analyzed. The values found
were multiplied by 6.

suggests that a C_4 compound is formed by addition of a C_1 unit to the
isopropyl group and that subsequent splitting of this intermediate yields
2 C_2 units for acetylation. In studying the formation of ketone bodies in
isolated liver, Coon has obtained evidence for the condensation of carbon
dioxide with a 3-carbon intermediate derived from leucine and isovalerate
to yield acetoacetate (7). The present findings provide independent sup-
port for the view expressed by both laboratories that the breakdown of
isovalerate involves the formation of a 3-carbon intermediate and of a C_2
unit which can be independently transformed into acetoacetate (6, 7).
The results also substantiate the earlier conclusion that a demethylation of isovalerate to a 4-carbon compound is not a major pathway.

The two carbon isotopes from isovalerate-4,4'-C\textsubscript{13},3-C\textsubscript{14} are incorporated into cholesterol and into fatty acids in a ratio which is close to 2, and therefore in the synthesis of these lipides as well the intact isopropyl group appears to be utilized. The C\textsubscript{14}:C\textsubscript{13} ratio is slightly greater than 2 in the fatty acids and somewhat smaller than 2 in cholesterol. Since only a small number of samples were analyzed, it is not certain that these differences are significant. As in previous experiments with isovalerate (1), the relative isotope concentrations were appreciably higher in cholesterol than in the fatty acids, a relation which is the reverse of that found with either

**TABLE III**

Isotope Concentrations in N-Acetyl, Liver Cholesterol, and Liver Saturated Fatty Acids of Rats Fed Calcium Carbonate with and without Sodium Isovalerate

<table>
<thead>
<tr>
<th>Compound isolated</th>
<th>No isovalerate</th>
<th>1 mm isovalerate per day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>Acetyl groups*</td>
<td>615</td>
<td>880</td>
</tr>
<tr>
<td>Liver cholesterol</td>
<td>59</td>
<td>292</td>
</tr>
<tr>
<td>&quot; saturated fatty acids</td>
<td>125</td>
<td>295</td>
</tr>
</tbody>
</table>

* Acetylphenylaminobutyric acid was isolated and analyzed. The value found was multiplied by 6.

acetate or isovalerate-1-C\textsubscript{14}. This finding again points to a more direct utilization of the 3-carbon split-product in sterol synthesis.

The observation of Coon which indicates CO\textsubscript{2} fixation in the course of ketone body formation from isovalerate *in vitro* (7) raised the possibility that carbon dioxide could be incorporated into various cell constituents by a mechanism different from that involving intermediates of the citric acid cycle. The effect of isovaleric acid on CO\textsubscript{2} fixation *in vivo* was therefore tested. Rats were given CaC\textsubscript{14}O\textsubscript{3} in their diets, and one of the animals received in addition non-isotopic sodium isovalerate. Carbon from C\textsubscript{14}-carbonate is incorporated to some extent into acetyl groups, cholesterol, and fatty acids (Table III). In the presence of isovalerate the quantities fixed are markedly increased. The increase is about 40 per cent in the acetyl groups, 2.5-fold in the fatty acids, and 5-fold in the cholesterol. These results are entirely consistent with the assumption that isovaleric acid and CO\textsubscript{2} interact to form a 4-carbon compound, which is subsequently utilized in the formation of acetyl groups and in lipide synthesis. Plaut and Lardy (8) have pointed out that the carboxylation of acetone provides
a mechanism for the conversion of CO₂ to the carboxyl group of acetate and that this reaction would explain the appearance of isotopic carbon in the carboxyl group of higher fatty acids in rats fed C⁴-carbonate (9). The present data provide more direct evidence that in the intact animal one of the mechanisms for CO₂ fixation involves a C₂ + C₁ condensation, which takes place in the course of the breakdown of leucine and isovaleric acid. Previous evidence obtained in this laboratory (1) suggests that the 3-carbon intermediate is not identical with acetone.

The metabolism of isovaleric acid and its relation to lipide synthesis, as it appears on the basis of available information, are illustrated in the accompanying diagram.

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

According to this scheme, the isopropyl group of isovaleric acid enters into cholesterol synthesis by way of acetoacetate. The keto acid can be utilized directly, and therefore labeled carbon from the isopropyl group will be less diluted than acetate or substrates which enter sterol synthesis after mixing with the acetate "pool." The postulation of acetoacetate as an intermediate provides for the fact that isopropyl-labeled isovalerate affords the same distribution of isotopic carbon in cholesterol as labeled acetate, even though isovalerate carbon is incorporated at a much higher level. On the other hand the isotope concentrations found in the higher fatty acids after isovalerate feeding do not exceed the values observed after administration of acetate and it appears therefore that acetoacetate is split prior to the recondensation of C₂ units. Both the present results with isovaleric acid and those with butyric acid reported in the preceding paper (10) are in accord with the conclusion that acetoacetate is an intermediate in cholesterol synthesis but is not an obligatory stage in fatty acid formation.

**SUMMARY**

1. Cholesterol isolated after the feeding of isovalerate-4',4'-C¹³, 1-C¹⁴ to rats was found to have the same isotope distribution in the products of partial degradation as cholesterol derived from acetate.
2. All carbon atoms of the isopropyl group of isovalerate were found to be utilized in the formation of acetyl groups, fatty acids, and cholesterol.

3. The incorporation of carbon into acetyl groups, fatty acids, and particularly cholesterol after the feeding of C\textsuperscript{14}-calcium carbonate is greatly increased in the presence of isovalerate.

4. It is suggested that acetoacetate or a similar 4-carbon compound is formed \textit{in vivo} from the isopropyl group of isovaleric acid by a C\textsubscript{2} + C\textsubscript{1} condensation and that this 4-carbon compound is the intermediate in the synthesis of cholesterol from isovalerate.

BIBLIOGRAPHY
