Acetoacetate and Acetate, Intermediates in Glutarate Catabolism*

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The labeling patterns found in tissue glutamate, aspartate, and alanine after administration of glutaric acid-3-C¹⁴ to male rats (1) indicated that the major catabolic route of glutarate is via acetate (2) and not via a direct conversion to the carbon chain of α-ketoglutarate (3-5). In further studies on the metabolism of glutarate-3-C¹⁴ in rats, we have observed the formation of urinary acetyl groups labeled in the carboxyl position and of urinaly acetocetate labeled chiefly in the carbonyl carbon. Also, rat liver mitochondria have been found to form acetoacetate, labeled predominately in the carbonyl carbon, from glutarate-3-C¹⁴. To date, our efforts to implicate acetonedicarboxylic acid or β-hydroxyglutaric acid as intermediates in glutarate catabolism have been unsuccessful.

EXPERIMENTAL PROCEDURE

Preparation of Compounds Used—The synthesis of glutarate-3-C¹⁴ has been described (1). The two preparations used had specific activities of 330 and 368 μc per mmole.

Acetonedicarboxylic acid was prepared from citric acid (6) and recrystallized from ethyl acetate.

β-Hydroxyglutaric acid was prepared in 70% yield by the reduction of acetonedicarboxylic acid (sodium salt) in aqueous solution with 2 moles of NaBH₄ (7), acidified to pH 2 with H₂SO₄, concentrated to dryness under vacuum, and the residue extracted with boiling ethyl acetate. Most of the boric acid which dissolved in ethyl acetate precipitated when the solution was concentrated and cooled. Concentration of the remaining solution to dryness under vacuum followed by trituration of the residue with cold ether yielded a white product which gave a single spot on paper chromatography in butanol-acetic acid-water, 9:1:2.5, and a single peak on Celite chromatography (8). This crystalline product melted at 87° to 91°, a range which is considerably lower than the reported value of 95°. In some preparations a product melting at 92 to 93° was obtained. It is felt that these low melting points may have been due to traces of boric acid. Oxidation with dichromate in the presence of HgSO₄ gave the mercury-acetone complex (9).

Sodium acetoacetate was prepared by hydrolysis (10) of acetoacetic acid at room temperature with 0.5 N NaOH followed by lyophilization of the solution to remove ethanol.

Cyclohexyl-β-alanine was prepared by reduction of L-tyrosine (11) and was acetylated by conventional methods.

Degradation Procedures—Acetoacetic acid and acetonedicarboxylic acid were decomposed by heat, and the liberated CO₂ was trapped in alkali. Before heat decomposition, preformed CO₂ was removed by sweeping the cold solutions, at pH 3, with CO₂-free air. In some instances the acetone was swept out and trapped in cold H₂O; in others the mercury-acetone complex was prepared (9).

The middle three carbons of β-hydroxyglutaric acid were converted to the mercury complex of acetone by dichromate oxidation (9). Acetone was oxidized to iodoform and acetate with NaIO₄. After filtration of the iodoform precipitate, acetate was removed from the acidified filtrate by steam distillation and, in some instances, was purified by chromatography on Celite (8). The iodoform was recrystallized from ethanol-water. The dinitrophenyldiazonium of acetone was prepared by conventional methods. Acetone was recovered from its mercury complex or from its dinitrophenyldiazonium by steam distillation from 2 N HCl.

Acetylcyclohexylalanine (approximately 1 mmole) was hydrolyzed by heating under reflux with 10 ml of 10% H₂SO₄ for 1 hour. Acetic acid was recovered by steam distillation and purified by Celite chromatography (8).

Acetate was decarboxylated by the Schmidt reaction (12). Some of the methyl amines produced during the degradation of acetate were trapped in 1 N HCl and the resulting hydrochloride was oxidized to CO₂ by the method of Van Slyke et al. (13, 14).

Animal Experiments—Male albino rats were used. Rats 81, 87, and 92 were from the Memphis colony (of Wistar origin). All the other rats were obtained from the Holtzman Company. Three animals (Rats 81, 87, and 92) were given a diet containing 1 to 1.5 g of cyclohexyl-β-alanine over a period of 4 days, during which time urine was collected. On the second day each rat received, by intraperitoneal injection, 5.9 μc (18 μmole) of sodium glutarate-3-C¹⁴. The 4-day urine was made alkaline, extracted with ether, acidified, and continuously extracted with ether for 24 hours. Ether was removed from the acid extract and the colored residue was subjected to chromatography on Celite (8) with ether as the mobile phase. The
Data obtained from rats given glutaric acid-3-C\textsuperscript{14} and large dose of acetonedicarboxylic, \(\beta\)-hydroxyglutaric, or acetoacetic acid

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Rat's weight</th>
<th>Catabolite acid administered</th>
<th>Amount administered as mmoles</th>
<th>Glutamic acid-3-C\textsuperscript{14} given</th>
<th>Injec- (%)</th>
<th>Injection time</th>
<th>Duration of experiment</th>
<th>Total radioactivity</th>
<th>Total catabolite isolated</th>
<th>(\beta)-Hydroxyglutaric peak</th>
<th>Acetone from catabolite</th>
<th>Acetate from catabolite</th>
<th>Carboxyl of acetate from acetone</th>
<th>Iodoform from acetone</th>
<th>Carboxyl from acetone</th>
<th>Carboxyl from acetoacetate</th>
<th>Urine excretion data</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>250</td>
<td>Acetonedicarboxylic</td>
<td>2.5</td>
<td>23.0</td>
<td>7.9</td>
<td>45</td>
<td>4.4</td>
<td>0.5</td>
<td>0</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>370</td>
<td>Acetonedicarboxylic</td>
<td>2.6\textsuperscript{a}</td>
<td>11\textsuperscript{b}</td>
<td>36</td>
<td>9</td>
<td>2.2</td>
<td>0.9</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>153</td>
<td>320</td>
<td>(\beta)-Hydroxyglutaric</td>
<td>2.5\textsuperscript{a}</td>
<td>12.8\textsuperscript{c}</td>
<td>56</td>
<td>55</td>
<td>2.8</td>
<td>1.2</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>253</td>
<td>(\beta)-Hydroxyglutaric</td>
<td>2\textsuperscript{d}</td>
<td>11.8\textsuperscript{d}</td>
<td>47</td>
<td>25</td>
<td>3.8</td>
<td>1.2</td>
<td>24.6</td>
<td>0.3\textsuperscript{e}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>139</td>
<td>170</td>
<td>Acetate</td>
<td>2.5</td>
<td>4.7</td>
<td>58</td>
<td>13</td>
<td>0.9</td>
<td>0.3</td>
<td>0.5</td>
<td>1710</td>
<td>483</td>
<td>0.5</td>
<td>135</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>123</td>
<td>Acetate</td>
<td>2.5</td>
<td>12.8</td>
<td>42</td>
<td>19</td>
<td>2.3</td>
<td>0.4</td>
<td>0.05(0.05)\textsuperscript{f}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\textsuperscript{a}\) Too low for accurate measurement. These samples may have had no radioactivity.

\(\textsuperscript{b}\) The glutaric acid-3-C\textsuperscript{14} was given 15 minutes before the acetonedicarboxylic acid.

\(\textsuperscript{c}\) This animal died of suffocation. The exact time of death is not known.

\(\textsuperscript{d}\) Given in 2 doses, 3.5 hours apart.

\(\textsuperscript{e}\) Figures are based on CO\textsubscript{2} release; those in parentheses are based on recovery of the mercury-acetone complex.

The results are presented in Tables I, II, and III. All the data have been corrected for any dilutions which were made during isolation or degradation.

**Formation of Urinary Acetyl Groups**—The data presented in Table III clearly substantiate our earlier conclusion (1) that carbon 3 of glutarate is catabolized via the carboxyl position of acetate.
Oxidation of glutaric acid-3-C\(^{14}\) by rat liver mitochondria

Incubations were conducted at 30° at pH 7.3 for 3 hours in standard Warburg flasks. Each mixture had a final volume of 3 ml, and contained, (exceptions are described in footnotes) in pmoles, the following: phosphate, 30; MgCl\(_2\), 12; ATP, 12; cytochrome c, 0.03; sucrose, 250 to 500; Tris buffer, 2. Each mixture also contained the mitochondria from 0.5 g of rat liver and 36 pmoles of glutaric acid-3-C\(^{14}\) (2.36 mc).

<table>
<thead>
<tr>
<th>Ex. RUN</th>
<th>Trancene added</th>
<th>C(^{14})O(_2) formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>pmoles</td>
<td>% C(^{14}) added to incubation mixture</td>
</tr>
<tr>
<td>1a</td>
<td>None</td>
<td>1.8</td>
</tr>
<tr>
<td>1b(^a)</td>
<td>None</td>
<td>8.2</td>
</tr>
<tr>
<td>2a(^a)</td>
<td>None</td>
<td>5.4</td>
</tr>
<tr>
<td>3a(^a)</td>
<td>None</td>
<td>1.4 0.00(^a) 0.74(^a)</td>
</tr>
<tr>
<td>4a(^a)</td>
<td>None</td>
<td>3.5</td>
</tr>
<tr>
<td>2b(^a)</td>
<td>Acetoneicarboxylic</td>
<td>60 0.0 0.4 0.46</td>
</tr>
<tr>
<td>4b(^a)</td>
<td>Acetoneicarboxylic</td>
<td>60 1.0 0 0.15</td>
</tr>
<tr>
<td>4c(^a)</td>
<td>Acetoneicarboxylic</td>
<td>10 2.5 0.04 0.42</td>
</tr>
<tr>
<td>4d(^a)</td>
<td>Acetoneicarboxylic</td>
<td>10 0.6 0.05 0.46</td>
</tr>
<tr>
<td>5a(^a)</td>
<td>Acetoneicarboxylic</td>
<td>10 0.4 0.4 0.4</td>
</tr>
<tr>
<td>5b(^a)</td>
<td>Acetoneicarboxylic</td>
<td>10 0.4 0.4 0.4</td>
</tr>
<tr>
<td>6b(^a)</td>
<td>(\beta)-Hydroxyglutaric</td>
<td>60 2.8 2.8 0.8</td>
</tr>
<tr>
<td>6a(^a)</td>
<td>(\beta)-Hydroxyglutaric</td>
<td>60 1.0 0.03 0.0</td>
</tr>
<tr>
<td>6b(^a)</td>
<td>(\beta)-Hydroxyglutaric</td>
<td>60 0.9 0.04 0.0</td>
</tr>
<tr>
<td>7a(^a)</td>
<td>Acetoctic</td>
<td>35 0.3 0.13 0.41 0.35 0</td>
</tr>
<tr>
<td>7b(^a)</td>
<td>Acetoctic</td>
<td>35 0.3 0.12 0.40 0.34 0</td>
</tr>
<tr>
<td>7c(^a)</td>
<td>Acetoctic</td>
<td>17 1.3 0.07 0.42 0</td>
</tr>
<tr>
<td>8a(^a)</td>
<td>Acetoctic</td>
<td>34 0.7 0.10 0.57 0</td>
</tr>
<tr>
<td>8b(^a)</td>
<td>Acetoctic</td>
<td>51 0.5 0.09 0.51 0</td>
</tr>
<tr>
<td>8a(^a)</td>
<td>Acetoctic</td>
<td>17 1.9 0.08 0.63 0</td>
</tr>
<tr>
<td>8b(^a)</td>
<td>Acetoctic</td>
<td>17 0.6 0.14 0.71 0</td>
</tr>
</tbody>
</table>

\(^{a}\) Fumarate, 1.5 pmoles per flask was added.

\(^{b}\) Instead of 36 pmoles, 6 pmoles of glutaric acid were added.

\(^{c}\) From acetoneicarboxylic acid added as carrier.

\(^{d}\) Acetone was prepared from \(\beta\)-hydroxyglutaric acid obtained from the reduction of acetoneicarboxylic acid (See "Experimental Procedure").

\(^{e}\) Instead of 36 pmoles, 42 pmoles of glutaric acid were added.

Radioactivity in urinary acetylcylohexyl-L-alanine after administration of glutaric acid-3-C\(^{14}\)

<table>
<thead>
<tr>
<th>Rat 81</th>
<th>Rat 87</th>
<th>Rat 92</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mcuc/mole</td>
<td>mcuc/mole</td>
</tr>
<tr>
<td>Acetylcyclohexyl-L-alanine</td>
<td>41.5</td>
<td>21.0</td>
</tr>
<tr>
<td>Carbon 1 of acetyl</td>
<td>30.3</td>
<td>17.7</td>
</tr>
<tr>
<td>Carbon 2 of acetyl</td>
<td>0.4</td>
<td>lost</td>
</tr>
</tbody>
</table>
the carboxyl carbon of acetoacetate and the acetoacetyl moiety of acetoacetate. Current concepts concerning the metabolism of malonyl-CoA indicate that a malonyl derivative is formed in considerable amount if malonyl-CoA is formed from glutarate, it would have ever, the data presented here appear to rule out the possibility and resynthesis of acetoacetyl-CoA does not completely ran-
tween endogenous and carrier acid may not have occurred (20-
butyrate samples contained significant radioactivity. Of inter-
incubated with rat liver mitochondria, ethylmalonate and
3-Cl4 and either acetoacetic, acetonedicarboxylic, or P-hydroxy-
fi-hydroxyglutaric acids contained no detectable Ci4.

These results indicate that glutarate is converted to acetate
1. Acetoacetic acid was labeled in the carbonyl and carboxyl
carbonyl and carboxyl ratio being at least 3:1.
3. The three middle carbons of acetonedicarboxylic and

Addendum—Recently, we have administered to rats, and
incubated with rat liver mitochondria, ethylmalonate and glutarate-3-C14. Ethylmalonic acid was isolated from urine or
the mitochondrial incubation mixtures, decarboxylated to bu-
tyric acid, and the latter assayed for radioactivity. None of the
butyrate samples contained significant radioactivity. Of inter-
est is the recent demonstration that extracts of Pseudomonas
fluorescens convert glutaryl-CoA to CO2 and two moles of acetyl-
CoA (34).

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