SYNTHESIS OF SUCCINATE FROM PROPIONATE AND BICARBONATE BY SOLUBLE ENZYMES FROM LIVER MITOCHONDRIA*

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Propionic acid has long been known (1, 2) to be a glycogenic substance in animals, but the pathway by which it is converted to intermediates of carbohydrate metabolism has remained obscure. Lorber et al. (3) demonstrated that the α- and β-carbon atoms of propionate are randomized completely before incorporation into liver glycogen, while those of lactate are not (4). In liver slices, complete randomization of the α- and β-carbon atoms of propionate occurs during the formation of lactate (5) and acetate (6). Therefore the postulated direct oxidation by animal tissue of propionate, through acrylate and lactate, to pyruvate (7, 8) does not appear to occur to any appreciable extent in the intact animal or in surviving liver. It has been postulated (3, 5, 6) either that propionate is converted to a symmetrical 4-carbon acid by CO₂ fixation or that the pyruvate, which might be produced by direct oxidation, is rapidly equilibrated with an unknown symmetrical intermediate.

Succinate is decarboxylated to propionate and CO₂ by Propionibacteria and related bacteria (9-11), and the reverse reaction may account for the incorporation of C¹⁴O₂ into succinate by these organisms (12) and for the photosynthetic production of succinate from propionate and CO₂ by Chlorobium thiosulphatophilum (13). In the present study, which was undertaken to test what at that time was a logical but hypothetical pathway for propionate metabolism (14), the direct conversion of propionate to succinate by CO₂ fixation has been demonstrated with soluble enzymes from mammalian liver. Certain aspects of the work have been reported elsewhere (14-16), while the following paper (17) describes the occurrence of this reaction in intact mitochondria.

EXPERIMENTAL

Acetone-desiccated rat or beef liver mitochondria, and aqueous extracts thereof, were prepared as described previously (18). The extracts, usually

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prepared with 0.05 M Tris buffer, pH 7.4, were centrifuged at 18,000 × g for 30 minutes, and the clear supernatant fluid was retained. In preliminary experiments, 0.2 to 0.4 ml. of the enzyme solution was incubated, usually for 20 minutes, at 20° under N₂ with 20 μmoles of K propionate, 1.2 μmoles of ATP, 1.5 μmoles of MgSO₄ or MnSO₄, 1.5 μmoles of HC₁₄Ο₃⁻, and 30 μmoles of Tris buffer, pH 7.4, in a total volume of 1 ml. The reaction was stopped by adding 0.2 ml. of 9 N H₂SO₄. After heating in a boiling water bath for 5 minutes to facilitate removal of denatured protein, any remaining C¹⁴O₂ was swept out by addition of particles of solid CO₂. The protein was removed by centrifuging and washed once. The combined supernatant liquid and washings were continually extracted with ether for 18 hours. The solvent was evaporated, and the invisible residue was taken up in alcohol for plating in preparation for radioactivity measurements. For some of the studies conducted after it had been established that virtually all of the radioactivity fixed was in succinic acid, counts were made on aliquots of the C¹⁴O₂-free reaction mixture.

Propionyl CoA was prepared by the method of Simon and Shemin (19).

**Results**

Aqueous extracts of acetone-dried liver mitochondria of the rat, ox, pigeon, and guinea pig were found to effect a rapid incorporation of C¹⁴O₂ into the non-volatile acid fraction when ATP, propionate, and a divalent cation were present (Table I, System A).

The reaction rate was enhanced by additions of coenzyme A, and a nearly absolute requirement for this coenzyme could be demonstrated by aging (20) the preparation at 24° (Fig. 1) or by treating the fresh extract with Dowex 1 (21). With fresh preparations of enzyme, propionyl CoA was 2 to 4 times more effective than propionate; however, it was only slightly more effective than propionate plus CoA. With propionyl CoA as the substrate (System B, Table I), the same reaction components were required. It is noteworthy that ATP was required when propionyl CoA was used, indicating an energy requirement for the CO₂-fixing process in addition to that for propionate activation. A slight but reproducible fixation of radioactivity occurred when the enzyme was incubated at pH 7.4 with HC¹⁴Ο₃⁻, ATP, and CoA. The propionate carboxylation proceeded equally well aerobically or anaerobically.

The reaction could not be demonstrated in whole mitochondria (17) from rat kidney or pigeon liver, but extracts of acetone powders of each of these types of mitochondria were approximately as active as extracts of rat liver mitochondria. As shown in Table II, the necessary combina-

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1 The following abbreviations are used: Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; CoA, coenzyme A.
tion of enzymes appears to be confined to the mitochondrial fraction of a sucrose homogenate of rat liver. The small amount of C\textsuperscript{14} fixed by the nuclear fraction may be the result of the presence of some mitochondria, since the nuclear fraction was washed only twice.

### Table I

**Fixation of HC\textsuperscript{14}O\textsubscript{3} in Presence of Propionate**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>System A</th>
<th>System B</th>
<th>Added C\textsuperscript{14} fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m.</td>
<td>c.p.m. × 10\textsuperscript{-3}</td>
<td>per cent</td>
</tr>
<tr>
<td>Complete system</td>
<td>3600</td>
<td>3160</td>
<td>25</td>
</tr>
<tr>
<td>No enzyme</td>
<td>0</td>
<td>8</td>
<td>0.2</td>
</tr>
<tr>
<td>&quot; Mg\textsuperscript{++}&quot;</td>
<td>200</td>
<td>12</td>
<td>0.2</td>
</tr>
<tr>
<td>&quot; ATP&quot;</td>
<td>36</td>
<td>24</td>
<td>0.2</td>
</tr>
<tr>
<td>&quot; propionate&quot;</td>
<td>48</td>
<td>50</td>
<td>0.4</td>
</tr>
<tr>
<td>&quot; propionyl CoA&quot;</td>
<td></td>
<td>1100</td>
<td>9</td>
</tr>
<tr>
<td>Propionate instead of propionyl CoA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

System A, as described under "Experimental." The complete system fixed approximately 10 per cent of the counts added; incubated 30 minutes at 20°. System B contained, per ml., 2 μmoles of bicarbonate with a higher C\textsuperscript{14} concentration, 7 μmoles of MgCl\textsubscript{2}, 6 μmoles of ATP, 25 μmoles of Tris buffer, pH 8.5, propionyl CoA, and enzyme; incubation 20 minutes at 37°. A different enzyme preparation was used in each of the two experiments.

**Fig. 1.** Dependency of propionate carboxylation on CoA in aged preparation; extract aged 3.5 hours at 24°, then 3.5 hours at 1°; components as in System B, Table I.

The reaction rate was proportional to the amount of enzyme added up to 0.4 ml. (incubation time, 10 minutes at 37°); it decreased slightly with 0.7 ml. With propionate as substrate, the pH optimum for HC\textsuperscript{14}O\textsubscript{3} fixation by beef and rat mitochondrial extracts was between 6.5 and 7.5. With propionyl CoA as substrate, the optimum was shifted to pH 7.25 to 8.75; the rate of the reaction dropped sharply above and below this range. The data of Table III demonstrate the high degree of specificity for pro-
pionate. The minute amounts of C\textsuperscript{14} fixed in the presence of butyrate, \(\alpha\)-chloropropionate, and \(\alpha\)-methylbutyrate might have resulted from traces of propionate in these samples. Methylbutyrate may have given rise to a small amount of propionate by oxidation (17). The apparent \(K_m\) for propionate was found to be about 0.003 M.

Table II

<table>
<thead>
<tr>
<th>Liver fraction*</th>
<th>(\text{C}^{14}\text{O}_2) fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>170</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>743</td>
</tr>
<tr>
<td>Microsomes</td>
<td>9</td>
</tr>
<tr>
<td>Soluble fraction†</td>
<td>13</td>
</tr>
</tbody>
</table>

Reaction mixture as described under "Experimental." 1.5 mmoles of Mn\textsuperscript{++}; incubated 15 minutes at 20°.

* An aqueous extract of 10 mg. of acetone powder was used in each case.
† Since sucrose would have interfered with the acetone precipitation of the supernatant fraction, the latter was prepared from a homogenate prepared in 0.15 M KCl.

Table III

<table>
<thead>
<tr>
<th>Acid added</th>
<th>(\text{C}^{14}\text{O}_2) fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{c.p.m.})</td>
</tr>
<tr>
<td></td>
<td>(\text{c.p.m.})</td>
</tr>
<tr>
<td>None</td>
<td>35</td>
</tr>
<tr>
<td>Acetate</td>
<td>16</td>
</tr>
<tr>
<td>Propionate</td>
<td>2100*</td>
</tr>
<tr>
<td>Butyrate</td>
<td>65</td>
</tr>
<tr>
<td>Succinate</td>
<td>20</td>
</tr>
</tbody>
</table>

Conditions as described in the text.

* 6 per cent of the HC\textsuperscript{14}O\textsuperscript{-} added.

The reaction indicates an absolute requirement for ATP (Table I; Fig. 2) with an optimum of approximately 1 \(\mu\)mole per ml. when either Mn\textsuperscript{++} or Mg\textsuperscript{++} is present at 0.001 M. When the concentration of ATP was increased above that of the divalent cation, the reaction was progressively inhibited. A 3-fold higher concentration of ATP inhibited the fixation by more than 50 per cent. Increasing the metal ion concentration in this region (Fig. 2) reversed the inhibition, indicating that the ratio of metal to ATP governs the activation and inhibition of the enzyme. These observations led to a more extensive study of this phenomenon with ATP-creatine transphosphorylase (22).
The requirement for a divalent cation depends on the thoroughness with which the enzyme is dialyzed. Magnesium and manganese salts were approximately equally effective in supporting the reaction, and both were optimally effective at concentrations approximately equal to that of ATP (Fig. 3).

Identification of Product—At least 90 per cent of the C\textsuperscript{14} fixed by the complete system was recovered in the non-volatile, ether-extractable acid fraction. This fraction was mixed with a pool of fumaric, succinic, and malic acids and resolved on a silica gel chromatograph according to Marvel and Rands (23). The results are presented in the upper part of Fig. 4. The recovery of the carrier acids was virtually quantitative. No detectable amounts of C\textsuperscript{14} were found in the fumaric or malic acid fractions, while in the succinic fraction the isotope concentration closely paralleled the amount of acid present. At the time these experiments were performed (1952), the Marvel-Rands system was the best available for chromatography of dicarboxylic acids. In their procedure, abrupt changes were made in the composition of the eluting solvent, which accounts for the lack of symmetry in the elution pattern.

The oxidation-reduction balance of the propionate carboxylation reaction under anaerobic conditions might lead to succinate production even...
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if some other acid were the primary product formed. To examine this possibility the experiment was repeated with the inclusion of 0.01 M malonate to prevent any fumarate being reduced to succinate. The results are shown on the bottom half of Fig. 4. The added malonic acid was recovered quantitatively but, again, all of the isotope was found in the succinic acid fraction.

More recently, the reaction mixture has been deproteinized, freed of C\textsuperscript{14}O\textsubscript{2}, and the acids separated by chromatography on Dowex 1 according to Busch, Hurlbert, and Potter (24), and by paper chromatography after Denison and Phares (25). Both of these procedures indicate that succinate is the major product, but give evidence for the occurrence of minor components containing radioactivity. In agreement with Flavin (26), we have obtained no evidence for the formation of isosuccinic acid by soluble extracts from rat liver. Isosuccinic acid has been found to be produced from propionate in rat liver slices (27) and in heart extracts (26).

Reversibility of Propionate Carboxylation in Extracts of Mitochondria—The fact that succinate does not stimulate HCO\textsuperscript{14}O\textsubscript{3}\textsuperscript{-} uptake in this system (Table III) indicates that the over-all reaction is not readily reversible. One reason for this might be the absence of significant amounts of the succinate-activating enzyme. Separation and analysis of the propionate...
remained in the reaction mixture after incubation indicated that it con-
tained less than 1 per cent as much C\textsuperscript{14} as had been fixed into succinate.

Synthetic succinyl CoA was slowly decarboxylated by this system. When 3.5 \(\mu\)moles of carboxyl-C\textsuperscript{14}-succinyl CoA were incubated for 40 minutes in the regular system, 1.2 per cent of the radioactivity was re-
covered as CO\textsubscript{2} in a KOH trap.\textsuperscript{2} A deacylated (20 hours; 30\textdegree) sample yielded only a third as much C\textsuperscript{14}O\textsubscript{2}.

**Influence of Biotin Deficiency on Carboxylation of Propionate**—A variety of CO\textsubscript{2} fixing reactions in animal tissue and in microorganisms is dependent

<table>
<thead>
<tr>
<th>Table IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Influence of Biotin Deficiency on Carboxylation of Propionate</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>State of biotin nutriture</th>
<th>Protein used</th>
<th>C\textsuperscript{14}O\textsubscript{2} fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3</td>
<td>1108</td>
</tr>
<tr>
<td></td>
<td>Biotin-deficient</td>
<td>3</td>
<td>229</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>3</td>
<td>1295</td>
</tr>
<tr>
<td></td>
<td>Biotin-deficient</td>
<td>3</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>Deficient, injected with 100 (\gamma) biotin daily for 3 days</td>
<td>3</td>
<td>2117</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>3</td>
<td>1158</td>
</tr>
<tr>
<td></td>
<td>Biotin-deficient</td>
<td>6</td>
<td>2260</td>
</tr>
<tr>
<td></td>
<td>Deficient + 100 (\gamma) biotin daily for 3 days</td>
<td>3</td>
<td>1475</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3771</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Biotin-deficient</td>
<td>3</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.3</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Biotin-deficient + control</td>
<td>3 + 0.3</td>
<td>643</td>
</tr>
</tbody>
</table>

on an adequate supply of biotin (see (16)). As reported briefly before (16), extracts of mitochondria from biotin-deficient rats carboxylate propionate at a greatly reduced rate as compared with preparations from normal rats (Table IV). The defective carboxylation in the biotin-deficient rats can be completely corrected by administration of biotin for a period of 3 days. In fact, such replete animals yielded preparations which were more active than those from control animals (Experiments 2 and 3, Table IV). Rapid restoration of CO\textsubscript{2}-fixing ability by administration of biotin to deficient rats has also been observed for the citrulline-synthesizing system (16, 28). Although propionyl CoA has not been tested with the biotin-deficient

\textsuperscript{2} This experiment was conducted by Dr. Felix Friedberg. The succinyl CoA was kindly contributed by Dr. Rao Sanadi.
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preparation, it is unlikely that the defect is in the propionate-activating reaction, since the metabolism of straight, short chain fatty acids proceeds normally in mitochondria from biotin-deficient rats (29). The activity of a biotin-deficient preparation could be greatly enhanced by the addition of a small quantity of a control preparation which alone fixed only a negligible amount of C\textsuperscript{14} (Experiment 4, Table IV). Additions of biotin or biocytin to the deficient preparation were without effect. Carbamyl glutamate, which enhances citrulline synthesis by biotin-deficient preparations (30), did not restore propionate carboxylation by the deficient extracts.

Observation on Miscellaneous Inhibitors and Activators—The carboxylation of propionate was strongly inhibited by octanoate, probably because the latter is converted to octanoyl CoA, leaving none of the coenzyme for propionate activation. At 0.003 M, the inhibition amounted to 42 per cent. Malonate, at 0.01 M, inhibited 50 to 60 per cent. Inhibition by succinate was variable; in some experiments 0.01 M succinate inhibited 50 per cent.

The rate of propionate carboxylation by dialyzed preparations was not enhanced by diphosphopyridine nucleotide, triphosphopyridine nucleotide, or glutathione. Supplementing the regular reaction mixture with 130 units of a highly purified preparation of "malic enzyme" (31) did not affect the C\textsuperscript{14} fixation with either normal or biotin-deficient extracts.

DISCUSSION

The direct carboxylation of propionate to succinate, followed by the oxidation of the latter through fumarate, malate, and oxalacetate to pyruvate, provides a pathway for the conversion of propionate to carbohydrate. Such a pathway would result in the observed (3, 5, 6) distribution of propionate carbon atoms. It will be demonstrated in the following paper that liver mitochondria are capable of carboxylating relatively large amounts of propionate.

The requirements for the carboxylation of propionate by mitochondrial extracts resemble those for the decarboxylation of succinate by extracts of Micrococcus lactilyticus (10), Propionibacterium pentosaceum, and Veillonella gazogenes (11). Whiteley (10) and Delwiche, Phares, and Carson (11) have found evidence for the involvement of propionyl CoA and succinyl CoA, and have demonstrated a requirement for ATP (10, 11), CoA (10, 11), and possibly cocarboxylase (10).

SUMMARY

Extracts of acetone-dried rat, ox, or guinea pig liver mitochondria catalyzed the carboxylation of propionate to succinate. The reaction required
ATP and Mg$^{2+}$ or Mn$^{2+}$. Optimal activity was obtained when the ratio
adenosine triphosphate-metal ion was approximately 1:1. A requirement
for coenzyme A could be demonstrated with aged preparations or by treating
fresh preparations with Dowex 1. Propionyl CoA was more effective than
propionate but the system still required ATP with this substrate. Acids
closely related to propionic did not support the fixation of HC$_4$O$_4^-$. The
product was identified as succinate by three chromatographic pro-
cedures.

Preparations from biotin-deficient rats were much less effective than
those from normal rats in catalyzing the carboxylation of propionate. This
metabolic defect was repaired by injected biotin.

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