THE CARBOXYLATION OF PROPIONIC ACID BY LIVER MITOCHONDRIA*

By FELIX FRIEDBERG,† JULIUS ADLER,‡ AND HENRY A. LARDY
(From the Department of Biochemistry and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin)

(Received for publication, July 22, 1955)

The glycogenic effect of propionic acid (1, 2) results from the conversion of propionate carbon into carbohydrate (3, 4) and not from an indirect stimulation of carbohydrate production from other sources. The finding (5, 6) that extracts of mitochondria can convert propionate and bicarbonate to an organic acid identified as succinic acid (6) provided an explanation for this glycogenic effect. The present study with intact mitochondria aims to ascertain the physiological significance of the carboxylation reaction which had been found to occur in soluble extracts.

EXPERIMENTAL

Except as otherwise indicated, the reactions were carried out in Warburg flasks, each containing 0.5 ml. of rat liver mitochondrial suspension (about 2 mg. of N) prepared according to Schneider (7), 3 μmoles of K propionate, 4 μmoles of NaHCO₃ (6.6 X 10⁴ c.p.m., side arm), 30 μmoles of DL-β-hydroxybutyrate, 50 μmoles of phosphate buffer, pH 7.3, 6 μmoles of ATP,¹ 15 μmoles of MgCl₂, and 0.15 M KCl to a volume of 3.0 ml. In some experiments (as indicated), 0.5 ml. of homogenate (10 gm. of rat liver in 10 ml. of 8.5 per cent sucrose) was used in place of mitochondria. Anaerobic conditions, when used, were obtained by flushing the flasks with nitrogen.

The reaction was allowed to proceed at 30° for 20 minutes after tipping the bicarbonate from the side arm, and was then stopped with 2.5 ml. of acid acetone (3 ml. of concentrated HCl per liter of acetone). After centrifugation, a 5.0 μl. aliquot of the protein-free solution was evaporated to

* A more detailed account of this work is presented in the thesis of Julius Adler for the degree of Master of Science, University of Wisconsin, 1954. Supported in part by a grant from the National Institute of Arthritis and Metabolic Diseases (No. A-531), National Institutes of Health, Public Health Service.
† Fellow of the National Foundation for Infantile Paralysis. Present address, Department of Biochemistry, Howard University, Washington, D. C.
‡ National Science Foundation Predoctoral Fellow.
¹ The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CoA, coenzyme A; DNP, dinitrophenol; TPN, triphosphopyridine nucleotide.
CONVERSION OF PROPIONATE TO SUCCINATE

dryness and assayed with a gas flow counter for $^{14}\text{C}_2$ fixed; the remainder was analyzed by paper chromatography (ether-glacial acetic acid-water, 13:3:1) after Denison and Phares (8), and by Dowex 1 chromatography according to Busch, Hurlbert, and Potter (9).

Malic, citric, succinic, fumaric, and aspartic acids were identified by their position on Dowex 1 chromatograms, and, in each experiment, by coincidence on paper chromatograms of authentic unlabeled acids with the radioactive products obtained by column chromatography of the reaction mixture. In addition, the radioactivity in each tube of a malic acid peak was found to coincide with reduced TPN formation catalyzed by a purified malic enzyme (10). The radioactivity of a citric acid peak coincided with citric acid measured according to Perlman, Lardy, and Johnson (11). Aspartic acid was degraded with dimethyl sulfate to fumaric acid (12). Succinic acid has been identified also by chromatography with authentic samples on a silica gel column (6).

The optical antipodes of $\beta$-hydroxybutyric acid were supplied by Dr. Frank Maley, who had resolved them with quinine. Yeast hexokinase was obtained from the Pabst Laboratories. Malic enzyme (10) of high activity was prepared by an unpublished procedure developed by Dr. William Rutter in this laboratory. Other preparations were described in the previous paper (6).

Results

When rat liver mitochondria were incubated with propionate in the reaction mixture described above, only about 1 per cent of the added $^{14}\text{C}$ was fixed (Table I). The additional presence of an oxidizable cosubstrate, however, resulted in the fixation of 10 to 30 per cent of the added 4.0 $\mu$moles of HC$_4$O$_5^-$ in 20 minutes (Tables I and II). A cosubstrate requirement for propionate metabolism has been reported also by other workers (13, 14). The addition of ATP was found necessary for an optimal rate of fixation.

The cosubstrate of choice was $\alpha$-$\beta$-hydroxybutyrate because of its effectiveness in stimulating the carboxylation of propionate, failure by itself to fix appreciable HC$_4$O$_5^-$ (see Table I and (15)), and nearly quantitative conversion to acetoacetate with negligible entry into the citric acid cycle (16). It could, however, be replaced by DL- or L-$\beta$-hydroxybutyrate, succinate, or $\alpha$-ketoglutarate (Table I). Under these conditions $\alpha$-ketoglutarate was itself carboxylated to form $^{14}\text{C}$-citrate (identified chemically as under "Experimental"), a reaction already studied in tissues of the rat and other species (17, 18).

The rôle of the cosubstrate is believed to be the maintenance of a high ATP-ADP ratio by means of oxidative phosphorylation. Evidence in
### Table I

**Carboxylation of Propionate by Rat Liver Mitochondria**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Gas phase</th>
<th>Propionate added</th>
<th>Other additions</th>
<th>Added $\text{HC}^{14}\text{O}_4^{-}$ fixed per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Air</td>
<td>0 µmoles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 µmoles α-ketoglutarate</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 µmoles dl-β-hydroxybutyrate</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0 µmoles</td>
<td>succinate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 µmoles dl-β-hydroxybutyrate</td>
<td></td>
<td>11.0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0 µmoles</td>
<td>dl-β-hydroxybutyrate</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.3 µmoles</td>
<td>+ glucose-hexokinase</td>
<td>21.7†</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>28 µmoles</td>
<td>creatine phosphate + 2 mg. ATP-creatine transphosphorylase</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28 µmoles</td>
<td>+ glucose-hexokinase</td>
<td>11.1</td>
</tr>
<tr>
<td>5</td>
<td>N₂</td>
<td>30 µmoles</td>
<td>+ glucose-hexokinase</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 µmoles dl-β-hydroxybutyrate</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 µmoles dl-β-hydroxybutyrate</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28 µmoles</td>
<td>creatine phosphate + 2 mg. ATP-creatine transphosphorylase</td>
<td>12.7</td>
</tr>
</tbody>
</table>

The reaction mixture is described under "Experimental;" incubation time, 20 minutes.

* The $\text{C}^{14}$ fixed was found predominantly in malic acid.
† The $\text{C}^{14}$ fixed was found approximately equally distributed between the malic and citric acid peaks.

### Table II

**Incorporation of $\text{C}^{14}$ from $\text{HC}^{14}\text{O}_4^{-}$ upon Incubation of Fatty Acids with Mitochondria**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Added counts fixed per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µmoles dl-β-hydroxybutyrate</td>
<td>1.0</td>
</tr>
<tr>
<td>30 &quot; &quot; + 3 µmoles propionate</td>
<td>32.3</td>
</tr>
<tr>
<td>30 &quot; &quot; + 3 &quot; acrylate</td>
<td>1.5</td>
</tr>
<tr>
<td>30 &quot; &quot; + 3 &quot; n-valerate</td>
<td>35.6</td>
</tr>
<tr>
<td>30 &quot; &quot; + 3 &quot; isobutyrate</td>
<td>9.7</td>
</tr>
<tr>
<td>30 &quot; &quot; + 3 &quot; dl-α-methylbutyrate</td>
<td>15.0</td>
</tr>
</tbody>
</table>

The reaction mixture is described under "Experimental;" incubation time, 23 minutes.
favor of this view includes the essentiality of aerobic conditions, and the complete inhibition by $2 \times 10^{-6}$ M dinitrophenol or by a glucose-hexokinase system (Table I). Also, creatine phosphate and ATP-creatine transphosphorylase could replace the cosubstrate, even anaerobically (Table I).

In the absence of any added cosubstrate, mitochondria from liver of guinea pig and beef catalyzed the fixation of CO$_2$ by propionate as rapidly as did rat liver mitochondria fortified with the cosubstrate.

When a whole homogenate of rat liver was used in the system in place of mitochondria, less than 0.5 per cent of the added HC$_3$O$_2^-$ was fixed.

**Distribution**—Under the conditions described, little or no activity was found with mitochondria from rat brain, heart, kidney, lung, skeletal muscle, spleen, and tongue. The system was found present in liver mitochondria from beef, guinea pig, hog, rabbit, rat, and sheep, but not pigeon, and not in fresh or dry bakers' yeast or *Pseudomonas fluorescens* grown on propionate as the sole carbon source. However, extracts of acetone-dried mitochondria from rat kidney or pigeon liver were found to fix HC$_3$O$_2^-$ in the presence of propionate (6).

**Reaction Products**—In aerobic experiments with rat liver mitochondria and β-hydroxybutyrate as cosubstrate, the C$^{14}$ label was found predominantly in malic acid (Fig. 1), even in an experiment of 1 minute duration. Degradation of this C$^{14}$-malic acid to pyruvic acid and CO$_2$ with the malic enzyme released 60 per cent of the radioactivity. The resulting pyruvic acid, trapped as the 2,4-dinitrophenylhydrazone, was also radioactive. Since the label was not exclusively in either carboxyl group, but approximately equally in both, malic acid is probably not the primary compound. The data are consistent with C$^{14}$-malic acid having been derived from C$^{14}$-succinate. Absence of large quantities of C$^{14}$-fumaric or C$^{14}$-succinic acid may be explained by a high rate of their conversion to malate.

When the conversion of succinate to malate was inhibited by anaerobic conditions (Fig. 2) or by addition of malonate (Fig. 3), the radioactivity appeared predominantly in succinic acid. Thus, all data obtained are consistent with the postulate that the primary reaction in mitochondria, as well as in extracts therefrom (6), is propionate + CO$_2$ → succinate.

To gain some information about the reversibility of the carboxylation of propionate, succinate was incubated in the mitochondrial system without propionate. This produced much the same chromatographic pattern as succinate with propionate, which appears in Fig. 4. In both cases the fixed C$^{14}$ was equally distributed between the malic and succinic acid.

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1 In a Dowex 1 chromatogram (9), isosuccinic acid appears in the position of Peak D, Fig. 1, and Peak C, Fig. 4. This fact and other evidence (8) indicate that these and corresponding peaks of Figs. 2 and 3 are partially isosuccinic acid. Succinate is thus the major product under the conditions of our experiments with soluble systems (6) and with mitochondria incubated anaerobically or in the presence of malonate.
fractions; the large succinic acid peak is due to trapping of C\textsuperscript{14}-succinate by the unlabeled pool, but the rapidity of succinate oxidation converts much of the labeled succinate to malate. The total C\textsuperscript{14} fixed by succinate alone is small (Table I). Marshall and Friedberg (19) reported that small amounts of C\textsuperscript{14}O\textsubscript{2} were fixed into succinate by rat liver homogenates to which succinate had been added.

**Fig. 1** Dowex 1 chromatogram of products of propionate carboxylation in the presence of DL-\(\beta\)-hydroxybutyrate. A, aspartic; B, succinic; C, malic; D, citric (in part); E, fumaric acid. Alternate tubes assayed; reaction components as in “Experimental.”

**Fig. 2** Dowex 1 chromatogram of products of propionate carboxylation in the presence of a phosphocreatine system, anaerobic. A, succinic acid; B, malic acid. Alternate tubes assayed; reaction components as under “Experimental” and Table I.

**Fig. 3** Dowex 1 chromatogram of products of propionate carboxylation in the presence of DL-\(\beta\)-hydroxybutyrate and malonate. A, succinic acid; alternate tubes assayed; reaction mixture as under “Experimental;” malonate, 0.01 M.

**Fig. 4** Dowex 1 chromatogram of products of propionate carboxylation in the presence of succinate. A, succinic acid; B, malic acid; C, citric acid (in part); D, fumaric acid. Alternate tubes assayed; reaction mixture as under “Experimental.”

*Precursors of Propionate*—Propionate is thought to be formed in animals by the oxidation of odd-numbered fatty acids (20) and by the metabolism of certain amino acids (20-23). To determine whether these pathways occur in mitochondria, \(n\)-valerate, valine, and isoleucine were incubated in the system without propionate. As can be seen from Table II, \(n\)-valerate caused incorporation of C\textsuperscript{14} as readily as did propionate itself.
Little or no fixation was obtained with valine and isoleucine. However, when isobutyric and $\alpha$-methylbutyric acids, which result from the oxidation of these amino acids, were tested, incorporation of $^{14}C$ occurred (Table II). The initial steps of valine and isoleucine metabolism thus seem to occur slowly or not at all in mitochondria under these conditions, but the branched chain fatty acids, once obtained, appear to give rise to propionate readily, and thence further to dicarboxylic acids. In the case of each fatty acid mentioned in Table II, the distribution of radioactivity in a Dowex 1 chromatogram was entirely similar to that obtained with propionate. The identity of the malic acid peak in an $n$-valerate experiment was established by reaction with triphosphopyridine nucleotide in the presence of malic enzyme, as described in "Experimental." The small amount of aspartate formed was identified by the correspondence of position of radioactivity with added aspartic acid in three paper chromatographic systems. The acid produced by treating the original aspartate fraction with dimethyl sulfate (12) was identified as fumaric acid by chromatography (8).

Slight fixation was obtained with acrylate (Table II). Only very slight or questionable fixation could be obtained with $\alpha$-ketobutyrate or $\beta$-alanine.

**DISCUSSION**

The possibility that propionate is carboxylated by way of pyruvate in the system under study appears unlikely. Wood (24) has pointed out that accumulation of $^{14}C$-succinate in the presence of malonate cannot occur by the carboxylation of pyruvate. In addition, pyruvate showed little activity in our system, and inosine triphosphate did not replace ATP. The carboxylation of propionate to succinate thus represents a new CO$_2$-fixing reaction in animals.

The inertness of a methyl group $\beta$ to a carbonyl function makes the direct carboxylation of the $\beta$ position of propionyl CoA appear unlikely. Consideration of this led to the postulation of an acrylic intermediate (5). Although acrylate reacts extremely slowly in our system, it produces much the same chromatographic pattern as propionate itself. Possibly acrylate is only slowly activated in rat liver, and acrylyl CoA, once formed, would be effective either directly or after reduction to propionyl CoA. It is of interest that Green et al. (25) have demonstrated the reduction of butyryl CoA dehydrogenase by propionyl CoA, indicating the probable formation of acrylyl CoA.

Isosuccinate (26, 27) may be an intermediate in the carboxylation of propionate (27); the mechanism of isomerization of isosuccinate to succinate remains to be elucidated. The propionate carboxylation system
resembles in many respects the decarboxylation of malonate by extracts of *Pseudomonas fluorescens* (28, 29).

The wide-spread occurrence of the enzyme system which catalyzes the carboxylation of propionic acid is indicated (a) by its presence in *Propionibacterium pentosaceum* (30) and the photosynthetic *Chlorobium thiosulfatophilum* (31), where it was first independently reported (30, 31), and in *Veillonella gazogenes* (32) and *Micrococcus lactilyticus* (33); (b) by our distribution data, and (c) by the recent report of Pennington (34) that utilization of propionate by epithelium of sheep rumen is stimulated by CO₂, and that succinate accumulates when propionate is metabolized in the presence of malonate.

Under the conditions of these experiments, the mitochondria from 1 gm. of liver (8.75 mg. of N (7)) fixed 5.25 μmoles of HC¹⁴O₃⁻ in 20 minutes. Assuming that the reaction is twice as fast at 38° as at 30° and that the liver comprises 5 per cent of the body weight, the liver mitochondria of a 150 gm. rat could carboxylate 235 μmoles of propionate in an hour. Although slightly higher rates of propionate utilization have been reported for the intact rat (3, 4), it should be recalled that, in the experiments *in vitro*, the measured carboxylation of propionate may be lower than the actual rate. The HC¹⁴O₃⁻ added to the reaction mixture would have been diluted by carbon dioxide produced from the oxidation of succinate via the Krebs cycle, and some of the C¹⁴ fixed would have returned to the HC¹⁴O₃⁻ pool by this same pathway.

**SUMMARY**

1. Rat liver mitochondria, incubated aerobically with 4 μmoles of HC¹⁴O₃⁻, propionate, 2-oxo-β-hydroxybutyrate, ATP, Mg⁺⁺, and phosphate buffer, fixed 20 to 30 per cent of the added label in 20 minutes. When propionate was omitted, less than 1.5 per cent was fixed. 2-oxo-β-Hydroxybutyrate could be replaced by α- or β-hydroxybutyrate, succinate, α-ketoglutarate, or by ATP-creatine transphosphorylase, even anaerobically. Glucose-hexokinase and 2 X 10⁻⁴ M dinitrophenol inhibited the uptake of HC¹⁴O₃⁻ completely.

In the absence of any added oxidizable cosubstrate, mitochondria from liver of guinea pig and beef catalyzed the fixation of CO₂ by propionate as rapidly as did rat liver mitochondria fortified with a cosubstrate.

2. Chromatography of the deproteinized reaction mixture showed that C¹⁴ had been incorporated predominantly into malate when the 2-oxo-β-hydroxybutyrate system was employed aerobically. In the presence of malonate or anaerobically, the label appeared predominantly in succinate.

3. Incubation of n-valerate, isobutyrate, and DL-α-methylbutyrate with
mitochondria resulted in fixation of C\textsuperscript{14} from HC\textsubscript{14}O\textsubscript{3}, and the chromatographic distribution was similar to that obtained with propionate.

**BIBLIOGRAPHY**

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