METABOLISM OF PROPIONIC ACID IN ANIMAL TISSUES

I. ENZYMATIC CONVERSION OF PROPIONATE TO SUCCINATE*

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The fate of propionic acid in the animal body is of interest since this compound appears to be a product of some metabolic reactions. In ruminants propionic acid is produced through bacterial fermentations in the rumen (1) and can be incorporated into higher odd-numbered fatty acids which have been found to occur in natural fats (2). Propionic acid may normally also arise not only from the β oxidation of odd-numbered fatty acids but from the degradation of branched aliphatic amino acids (3-5).

Although propionic acid had long been known to undergo oxidation in the animal body, the nature of the intermediary reactions was obscure. Some investigators considered that the primary attack was by α oxidation, via the sequence propionate → acrylate → lactate → pyruvate (6-8). However, experiments on the distribution of C14 from labeled propionate in tissue glycogen (9) and acetyl groups (10), or in lactate (11), showed a complete randomization of the carbon atoms originating from the α or β position of propionate. When propionate was reisolated, no redistribution of C14 was found to have occurred between its α- and β-carbons (10). These results suggested that propionate was irreversibly converted to pyruvate by way of a symmetrical intermediate such as would be formed if propionate or acrylate were first carboxylated to succinate or fumarate, followed by oxidation to oxalacetate and decarboxylation of the latter. This view finally received direct support through the finding of Lardy and Peanasky (12) that extracts of acetone-dried rat liver mitochondria catalyzed an

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ATP-dependent\(^1\) fixation of \(\text{CO}_2\) by propionate to form succinate. A marked dependence on \(\text{CO}_2\) of propionate oxidation by sheep rumen epithelium, as well as a production of succinate in the presence of malonate, was observed by Pennington (13).

The above observations largely mirrored studies of the mechanism of conversion of pyruvate to propionate by propionic acid bacteria. Thus, the assumption that propionate might arise through the sequence pyruvate \(\rightarrow\) lactate \(\rightarrow\) acrylate \(\rightarrow\) propionate was disproved by Barker and Lipmann (14), who found that lactate was not an intermediate, and previous work of Wood et al. (15) indicated that propionate might arise via fixation of \(\text{CO}_2\) by pyruvate, followed by decarboxylation of a symmetrical dicarboxylic acid intermediate. Sheep rumen organisms (16) and propionic acid bacteria (17) were later found to bring about a quantitative anaerobic conversion of succinate to propionate and \(\text{CO}_2\). With cell-free extracts of *Micrococcus lactilyticus*, Whitley (18) obtained evidence that decarboxylation of succinate to propionate proceeds via succinyl CoA, as well as some indications that the extracts can convert propionate to succinate by \(\text{CO}_2\) fixation. Similar observations, with extracts of propionic acid bacteria, were reported by Delwiche et al. (19). The demonstration by Larsen (20) of a photochemical formation of \(^{14}\text{C}\text{succinate}\) from \(^{14}\text{O}_2\) and propionate by green photosynthesizing bacteria is also of interest in this connection.

In preliminary reports from this laboratory (21, 22) evidence has been presented that the major pathway of propionate oxidation in animal tissues involves the sequence propionate \(\rightarrow\) ATP + CoA \(\rightarrow\) propionyl CoA \(\rightarrow\) ATP + CO\(_2\) \(\rightarrow\) methylmalonyl (isosuccinyl) CoA \(\rightarrow\) succinate, followed by oxidation of succinate via the citric acid cycle. The conversion of propionate to methyl malonate has been independently observed by Katz and Chaikoff (23) in rat liver slices. This paper presents supplementary evidence for the above sequence and describes previously unpublished experimental details, while Papers II and III (24, 25) are concerned with further studies of the enzymatic steps leading from propionyl CoA to succinate.

*Formation of Methyl Malonate*

Dialyzed pig heart extracts fix \(^{14}\text{O}_2\) when incubated with propionate, provided that ATP, CoA, and Mg\(^{++}\) are added (Table I, Experiment 1). The requirement of ATP and Mg\(^{++}\) had been observed by Lardy and Peanasky (12) for the formation of \(^{14}\text{C}\text{succinate}\) from propionate and \(^{14}\text{O}_2\) by rat liver mitochondrial extracts.

\(^1\) The following abbreviations are used: adenosine 5'-mono-, di-, and triphosphate, AMP, ADP, and ATP; pyrophosphate, PP; coenzyme A, CoA; reduced glutathione, GSH; tris(hydroxymethyl)aminomethane, Tris; ethylenediaminetetraacetic acid, EDTA.
The requirement of CoA for the carboxylation of propionate suggested that propionyl CoA was a reactant. Synthetic propionyl CoA proved to be much more effective than propionate + CoA even with crude heart extracts (Experiment 2, Table I). This suggests that propionate becomes effective as a CO₂ acceptor only after conversion to propionyl CoA. Such conversion undoubtedly occurs via Reaction 1, catalyzed by acetic thio-

\[
\text{Propionate + CoA + ATP} \rightleftharpoons \text{propionyl CoA + AMP + PP}
\]

kinase (26, 27) which is also active with propionate. Of particular interest was the fact that, when propionyl CoA was used in place of propionate + CoA, ATP was still required (Experiment 2, Table I) for methyl malonate synthesis (22), indicating that it played some additional role in the carboxylation reaction beyond its function in Reaction 1. As reported in a preliminary note from this laboratory (28) and discussed in more detail in Paper II (24), ATP appears to be involved in the activation of CO₂ for carboxylation. The substrate role of propionyl CoA and the additional requirement for ATP have been confirmed by Lardy and Adler (29).

**Identification of Methyl Malonate**—In the experiments of Table I, fixation of C^{14}O₂ was determined on aliquots of the reaction mixture after depro-
teinization with sulfuric or perchloric acid. The samples were placed on stainless steel planchets, and their radioactivity was determined with a windowless flow counter after drying over a steam bath; blanks due to residual $\mathrm{C}^{14}O_2$ were very low. For identification of the radioactive products, aliquots of the deproteinized reaction mixtures were submitted to continuous ether extraction overnight. The ether was dried and evaporated; the residue was taken up in a small volume of ethyl acetate and applied, along with aqueous solutions of known dicarboxylic acids as markers, on Whatman No. 1 filter paper. Ascending chromatography was

TABLE II

Identification of $\mathrm{C}^{14}$-Labeled Dicarboxylic Acids by Recrystallization with Carrier Succinic or Methylmalonic Acid

In Experiments 1 and 2, the labeled dicarboxylic acid was derived from $\mathrm{C}^{14}O_2 +$ propionate on incubation with pig heart extract (conditions similar to those of Experiment 1, Table I, complete sample). In Experiment 3, the labeled dicarboxylic acid was derived from the action of rat liver acetone powder extract on biosynthetic $\mathrm{C}^{14}$-labeled methyl malonate (conditions similar to those of Experiment 1, Table IV, second sample). The labeled acids were isolated by ether extraction, paper chromatography, and elution as described in the text. Crystallizations with carrier succinic acid were from acetone-petroleum ether, with carrier methylmalonic acid from ethyl acetate-benzene. Counting at infinite thickness.

<table>
<thead>
<tr>
<th>No. of recrystallizations</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Product recrystallized with succinic acid</td>
<td>Product recrystallized with methylmalonic acid</td>
<td>Product recrystallized with succinic acid</td>
</tr>
<tr>
<td>Before recrystallization</td>
<td>80</td>
<td>112</td>
<td>960</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>114</td>
<td>960</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>108</td>
<td>980</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>112</td>
<td></td>
</tr>
</tbody>
</table>

carried out at first in a solvent system of $n$-butanol saturated with 3.0 M formic acid. Spots were located by spraying with 0.04 per cent brom phenol blue, adjusted to pH 6.7, in 95 per cent ethanol, and by autoradiography. With this solvent system essentially all of the $\mathrm{C}^{14}$ fixed in a non-volatile form migrated to the position of succinate. However, the eluted product did not yield any labeled fumarate or malate on incubation with succinoxidase, and, upon the first recrystallization with carrier succinic acid, its specific radioactivity dropped to one-tenth of the original value (Experiment 1, Table II).

Since methylmalonic acid appeared to be the most likely alternative, a

2 The acids were eluted from the paper with 50 per cent aqueous ethanol.
solvent system was used which, contrary to the one described above, separated methylmalonic acid from succinic acid. This system, consisting of isoamyl alcohol saturated with 4.0 M formic acid, was found to achieve complete resolution of a mixture of malic, malonic, succinic, methylmalonic, and fumaric acids (Strip 1, Fig. 2). With this solvent the enzymatically formed radioactive compound appeared in the position of methylmalonic acid.

Further identification of the radioactive reaction product as methylmalonic acid was obtained by recrystallization to constant specific radioactivity with carrier methylmalonic acid (Experiment 2, Table II). A

### Table III

**Recovery of C\textsuperscript{14} in Products of Pyrolysis of Biosynthetic Carboxyl-Labeled Methylmalonic Acid**

Carboxyl-labeled C\textsuperscript{14}-methylmalonic acid, prepared under the conditions of Experiment 1 in Table I (complete sample), was isolated as described in the text and recrystallized five times with carrier methylmalonic acid from ethyl acetate-benzene. The specific radioactivity of the product was 112 c.p.m. per mmole. 1.0 mmole was heated to 160\(^\circ\)C in a stream of nitrogen, and the products were collected and isolated as described in the text. Counting at infinite thickness.

<table>
<thead>
<tr>
<th>Pyrolysis products</th>
<th>Specific radioactivity (c.p.m. per mmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount</td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Barium carbonate</td>
<td>0.92</td>
</tr>
<tr>
<td>Silver propionate</td>
<td>0.54(\dagger)</td>
</tr>
</tbody>
</table>

* Assuming equal self-absorption for methylmalonic acid, barium carbonate, and silver propionate and equal distribution of C\textsuperscript{14} between the two products.

\(\dagger\) Accidental loss.

sample thus recrystallized was subjected to pyrolysis in a stream of nitrogen (30); the evolved propionic acid and CO\textsubscript{2} were collected in dry ice\textsuperscript{3} and barium hydroxide traps and isolated as silver propionate and barium carbonate, respectively. The radioactivity was equally distributed between the propionate and the CO\textsubscript{2} (Table III). Succinic acid is stable under these conditions (30). Another sample was converted to 5-methylbarbituric acid by heating with urea in a mixture of glacial acetic acid and acetic anhydride (31). The derivative retained the radioactivity although it could not be recrystallized to constant specific radioactivity. This may have been due to oxidation by air (32), as it was observed that the crystalline form changed on recrystallization.

The demonstration that carboxylation of propionate yields methyl

\textsuperscript{3} The propionic acid was collected at the bottom of a U-shaped Pyrex tube immersed in a dry ice-ethanol mixture.
Fig. 1. Activated methyl malonate as the carboxylation product of propionyl CoA. All the reaction mixtures contained (in micromoles) Tris buffer, pH 7.0, 100 MgCl₂, 10; GSH, 5; ATP, 8; and pig heart extract with 15 mg. of protein. In addition, the sample for Strip 1 contained (in micromoles) propionyl CoA, 2.5; KH₂¹⁴O (9 × 10⁶ c.p.m.), 10; and hydroxylamine, 100; the sample for Strip 2, propionate, 5; CoA, 1.5; KH₂¹⁴O₃, 10; and hydroxylamine, 100; the sample for Strip 3, same as that for Strip 2 but no hydroxylamine; the samples for Strips 4 and 5, CoA, 1.5; and methyl malonate-¹⁴C, either a trace amount (Strip 4) or 0.5 (Strip 5). Final volume, 1.5 ml. After incubation for 1 hour at 30°, each sample received 1.0 mmole of hydroxylamine, and the incubation was continued for a further 10 minutes. The samples were prepared for chromatography essentially by the method of Stadtman and Barker (34). The reaction was terminated by addition of 35 ml. of ethanol, and the precipitate was removed by centrifugation. After evaporating the supernatant fluid on the steam bath, and then in a vacuum to dryness, the residue was extracted with 2 ml. of ethanol, the supernatant fluid was evaporated to dryness, and the residue was extracted with 0.2 ml. of ethanol. 0.1 ml. aliquots of the supernatant solution were used for chromatography in the isoamyl alcohol-formic acid system. The figure is a photograph of autoradiograms obtained from the paper chromatograms. The origin is at the bottom, and the position of the authentic compounds is shown on the strip at the left. The spot near the origin on this strip is the most prominent spot given by methylmalondihydroxamic acid (see under "Preparations"). The strips were cut horizontally because the autoradiograms of methylmalonic and methylmalonhydroxamic acids were obtained on different sheets of x-ray film.
malonate, obtained independently by Katz and Chaikoff (23), provided the first clue to the biological significance of this compound, discovered in normal rat urine 21 years ago (33).

**Immediate Product of Propionyl CoA Carboxylation**—As previously reported (22), the immediate product of carboxylation of propionyl CoA appears to be an activated form of methyl malonate. Fig. 1 (Strips 1 to 3) shows that, on addition of hydroxylamine, much of the C\(^{14}\) fixed after incubating heart extracts with C\(^{14}\)O\(_2\), ATP, and propionate + CoA or propionyl CoA is present as methylmalonmonohydroxamic acid. In all the experiments of Fig. 1 each of the samples (final volume 1.5 ml.) received 1.0 mmole of hydroxylamine after incubation for 1 hour at 30°, and incubation was continued for a further 10 minutes before preparation for chromatography. In addition, the samples corresponding to Strips 1 and 2 each contained 100 \(\mu\)moles of hydroxylamine throughout the incubation. In these samples there was some accumulation of unlabeled propionhydroxamic acid along with labeled methylmalonhydroxamic acid. The experiments corresponding to Strips 4 and 5 of Fig. 1 are controls in which an amount of C\(^{14}\)-labeled methyl malonate of the same order of magnitude as is formed from propionate and CO\(_2\) replaced these compounds. In these experiments there was no detectable formation of labeled methylmalonhydroxamic acid. This eliminates the possibility that methyl malonate is formed first and activated subsequently. The results of further work (25) point to methylmalonylmono CoA as the intermediate.

**Formation of Succinate**

Under optimal conditions for the synthesis of methyl malonate, no succinate was formed by the pig heart preparations. Since the carboxylation of propionate to methyl malonate did not, at first sight, appear to be helpful in explaining the randomization of the \(\alpha\) and \(\beta\)-carbons of propionate mentioned in the introduction, and in view of the fact that succinate had been found to be produced by the carboxylation of propionate in liver extracts (12), the C\(^{14}\)O\(_2\) fixation experiments were repeated with extracts of liver and other animal tissues. These experiments showed (22) that both C\(^{14}\)-methyl malonate and C\(^{14}\)-succinate are produced on incubation of propionyl CoA and C\(^{14}\)O\(_2\) with dialyzed extracts of rat heart, liver, or kidney, or sheep kidney, in the presence of ATP and Mg\(^{++}\). Methylmalonic and succinic acids were identified by paper chromatography (and autoradiography) in the isoamyl alcohol-formic acid solvent system, and succinic acid was further identified by recrystallization with carrier to constant specific radioactivity. Strip 2 of Fig. 2, from an experiment with an ace-

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4 In confirmation of Boyland and Levi (33) we have found that methyl malonate is less than one-tenth as effective as malonate as inhibitor of succinoxidase.
tone powder extract of rat liver, shows the formation of a relatively large amount of succinate and a smaller amount of methyl malonate from $\text{C}^{14}\text{O}_2$ and propionyl CoA, along with an as yet unidentified component of $R_F$ slightly greater than that of fumarate. Strip 1, from a parallel experiment, shows that malonate causes a relatively greater accumulation of methyl malonate at the expense of succinate. On the other hand, in analogy with pig heart extracts, the supernatant fluid obtained from rat liver homogenates by the method of Schneider and Hogeboom (35) formed only $\text{C}^{14}$-methyl malonate from propionyl CoA and $\text{C}^{14}\text{O}_2$ (Strip 3, Fig. 2).

**Isomerization of Methyl Malonate to Succinate**—The above results suggested that methyl malonate (or methylmalonyl CoA) might be formed

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**Fig. 2.** Formation of $\text{C}^{14}$-labeled methyl malonate and succinate in rat liver extracts. Drawings of paper chromatograms, in the isoamyl alcohol-formic acid solvent system, paired with photographs of their autoradiograms. Except for the enzyme, the composition of the reaction mixtures and other conditions for Strips 1 to 3 were the same as those in Experiment 2 (complete sample, Table I). The incubation corresponding to Strip 1 was carried out in the presence of 0.01 M malonate. The rat liver enzymes were as follows: Strips 1 and 2, acetone powder extract with 10 mg. of protein; Strip 3, supernatant fluid of homogenate, prepared after Schneider and Hogeboom (35), with 6 mg. of protein. For Strip 4, the conditions were the same as those in Experiment 1 (second sample, Table IV). After incubation, the organic acids were extracted with ether from acid-deproteinized samples and spotted on the paper together with known dicarboxylic acids as carriers.
first and subsequently isomerized to succinate. This hypothesis received support from the finding (22) that, in the presence of ATP, CoA, and Mg++, C^{14}-labeled methyl malonate was converted to C^{14}-succinate (see also Table IV) by all tissue preparations capable of forming both methyl malonate and succinate from propionyl CoA, but not by pig heart extracts (which form only methyl malonate), and by the fact that this conversion was inhibited by malonate. As shown in Table II (Experiment 3), the product obtained from incubation of carboxyl-labeled methyl malonate with rat liver extract was identified as succinate by recrystallization with carrier succinic acid. As illustrated in Fig. 2 (Strip 4), this isomerization frequently proceeded to completion and was essentially the only reaction which methyl malonate could be shown to undergo, even in crude extracts. The need for ATP and CoA suggested activation of methyl malonate to methylmalonyl CoA as a preliminary step in its conversion to succinate.

The above results and those reported previously (22), in which the time-course of formation of both methyl malonate and succinate from propionyl CoA was followed in the absence and presence of malonate, lend support to the view that propionyl CoA is carboxylated to methylmalonyl CoA and
TABLE IV

Conversion of Variously Labeled Preparations of C\textsuperscript{14}-Methyl Malonate to Succinate

The reaction mixture contained (in micromoles) glycine buffer, pH 9.0, 100; MgCl\textsubscript{2}, 10; GSH, 8; ATP, 8; CoA, 1; extract of fresh or acetone-dried rat liver with 15 mg. of protein; and C\textsuperscript{14}-methyl malonate and other additions as indicated. Final volume, 1.5 ml. Incubation at 25°. Direct counting of acid-deproteinized aliquots, as described in the text, gave the total radioactivity present. Counting after heating an aliquot for 10 minutes at 100° with a few drops of 3 per cent KMnO\textsubscript{4}, and removing any excess permanganate by reduction with a few drops of methanol, gave the radioactivity due to succinate, which is resistant to the permanganate treatment, while methyl malonate (as well as malate and fumarate) is destroyed by oxidation to volatile products. The precipitated MnO\textsubscript{2} was removed by centrifugation before counting. The results are expressed in counts per minute per 1.5 ml. of reaction mixture.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>C\textsuperscript{14}-Methyl malonate</th>
<th>Other additions</th>
<th>Incubation time</th>
<th>Radioactivity</th>
<th>Methyl malonate converted to succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Source</td>
<td>Amount</td>
<td>μmol</td>
<td>μmol</td>
<td>min.</td>
</tr>
<tr>
<td>1</td>
<td>C\textsuperscript{14}O\textsubscript{2} + propionate</td>
<td>Trace</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&quot; + &quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>&quot; + &quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>CO\textsubscript{2} + propionate 1 C\textsuperscript{14}</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>&quot; + &quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>&quot; + &quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>Synthetic (CH\textsubscript{3}-labeled)</td>
<td>0.2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>0.2</td>
<td>&quot;</td>
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<td></td>
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<td>0.2</td>
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<td>60</td>
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<td></td>
<td>&quot;</td>
<td>0.2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>60</td>
</tr>
</tbody>
</table>

Methyl malonate was converted to succinate by 53% to 100%.
the latter is then converted to succinate (see also Beck et al. (25)). Further support for this view is provided by the effect of hydroxylamine on the formation of labeled methyl malonate and succinate from C\textsuperscript{14}O\textsubscript{2} and propionyl CoA in rat liver extracts. As shown in Fig. 3, in the presence of small, increasing concentrations of hydroxylamine, there is a proportionally greater formation of methyl malonate at the expense of succinate. This effect, which resembles that of malonate, suggests that hydroxylamine traps the intermediary methylmalonyl CoA and prevents its further conversion to succinate.

Preliminary Experiments on Mechanism of Isomerization Reaction—It has been reported previously (22) that the conversion of methyl malonate (or methylmalonyl CoA) to succinate does not seem to involve decarboxylation to propionate (or propionyl CoA) followed by recarboxylation to form succinate, since there was little reduction of the radioactivity of the C\textsuperscript{14}-succinate produced from trace amounts of C\textsuperscript{14}-methyl malonate by rat liver extracts in the presence of pools of bicarbonate, propionate, or propionyl CoA. Table IV shows the results of experiments with three kinds of labeled methyl malonate, (1) carboxyl-labeled prepared enzymatically from C\textsuperscript{14}O\textsubscript{2} and propionate, (2) carboxyl-labeled prepared enzymatically from CO\textsubscript{2} and propionate-1-C\textsuperscript{14}, and (3) methyl-labeled prepared synthetically. The addition of non-labeled compounds was essentially without effect in Experiment 1. In Experiments 2 and 3 there was some depression of the formation of C\textsuperscript{14}-succinate, particularly in the presence of propionate or propionyl CoA. It is clear, however, that for a decarboxylation-recarboxylation mechanism there should have been considerably more depression than has been observed. On the other hand, the results do not rule out a transcarboxylation mechanism of the kind indicated by Reaction 2, in which carboxyl migration would occur on the enzyme and propionyl CoA

(2) Methylmalonyl CoA + propionyl CoA \rightleftharpoons propionyl CoA + succinyl CoA

would act catalytically as a relatively undissociable, enzyme-bound reactant. All that can be said at present is that methyl malonate is converted to succinate in a more or less direct fashion.

DISCUSSION

The oxidation of propionate via succinate appears to be a major pathway of propionate metabolism in animal tissues; this is now supported by considerable experimental evidence. In addition to the direct evidence that propionate is converted to dicarboxylic acids (12, 21–23, 29), the oxidation of propionate by liver homogenates (22) and rumen epithelium (13) has been shown to require CO\textsubscript{2}. Moreover, the work mentioned in the intro-
duction (9-11) and the more recent results of Wolfe (36) with rabbit liver homogenates appear to rule out a major role for an \( \alpha \) oxidation pathway via acrylate and lactate.\footnote{It is of interest that Stadtman (37) has found that \( \alpha \) oxidation of propionyl CoA to acrylyl CoA occurs in cell-free extracts of \textit{Clostridium propionicum}.} It may also be mentioned that according to Friedberg \textit{et al.} (38) the rate of carboxylation of propionate by liver mitochondria can account for the rate of propionate utilization observed in the intact animal.

The novel features in the dicarboxylic acid pathway disclosed by previous work (22) and that reported in this and Papers II and III (24, 25) are (a) the formation of an activated form of methyl malonate (isosuccinate) rather than succinate as the immediate product of carboxylation of activated propionate and (b) the conversion of activated methyl malonate to succinate. The carboxylation of the methylene group of activated propionate, rather than of the less reactive methyl group, might have been predicted on chemical grounds. However, the isomerization of methyl malonate to succinate is a reaction as yet without precedent in chemistry or biochemistry.

\textit{Preparations}

\textit{Pig Heart Extracts}—Pig hearts, obtained immediately after death, were packed in ice, trimmed of fat, blood clots, and connective tissue, and passed twice through an electric mincer. All operations were performed at about 0\(^\circ\). 2 volumes of 0.02 \( \text{M} \) potassium phosphate buffer, pH 7.4, containing 0.001 \( \text{M} \) cysteine, were added to 1 volume of mince, and the suspension was stirred mechanically for 40 minutes. The extract was squeezed by hand through eight thicknesses of cheesecloth, and the filtrate was dialyzed overnight against 50 or more volumes of 0.02 \( \text{M} \) Tris buffer, pH 7.4, containing 0.001 \( \text{M} \) cysteine. A precipitate which formed was discarded after centrifugation, and the supernatant fluid was stored at \(-8^\circ\). Protein was determined spectrophotometrically with correction for the nucleic acid content (39).

\textit{Rat Liver Acetone Powder Extract}—Acetone powders of freshly removed rat livers were prepared as described previously for pigeon liver (40). The powder was extracted by grinding in a mortar for 15 minutes with the gradual addition of 6 volumes of 0.02 \( \text{M} \) potassium phosphate buffer, pH 7.0, containing 0.2 \( \text{M} \) KCl. The supernatant fluid, after centrifugation, was dialyzed according to the procedure used for the heart extracts and stored at \(-18^\circ\). The liver extracts used in some experiments were prepared by homogenizing freshly removed rat livers in a Waring blendor with 5 volumes of 0.04 \( \text{M} \) Tris buffer, pH 7.4, containing 0.001 \( \text{M} \) cysteine, and removing the insoluble residue by centrifugation at 30,000 \( \times \) g in a Servall angle centrifuge at 4\(^\circ\).
CoA Thio Esters—Propionyl CoA was prepared by the procedure described by Simon and Shemin (41) for the preparation of succinyl CoA. Sufficient propionic anhydride was added to a fresh solution of CoA at pH 7.5 to cause complete disappearance of the free sulfhydryl group (42). The free propionic acid formed was removed by three extractions, each with 5 volumes of ether, at 0° and pH 3.0, and the amount of thio ester present was determined as hydroxamic acid (43), after removing the ether in a vacuum. Stored at pH 5 to 6 at −18°, the material appeared stable for months.

Synthesis of Labeled Compounds—Methyl-labeled methyl malonate was prepared from C14-methyl iodide and sodium diethyl malonate (44). After saponification, extraction with ether, and recrystallization from acetone, the free acid was radiographically pure. We are indebted to Dr. H. Castro-Mendoza for this preparation. Methyl malonate-1-C14 was prepared enzymatically with pig heart extract in two ways, either from KHC14O3 and propionate or from propionate-1-C14 and KHCO3, and was purified by chromatography as described in the experimental section.

Other Preparations—Methylmalonic (and propylmalonic) acids were prepared from the diethyl ester (Sapon organic chemicals) by saponification, ether extraction, and repeated crystallization from acetone or acetone-petroleum ether. Methylmalonmonohydroxamic acid was prepared by the alkaline hydroxylamine method (45, 46) from crystalline potassium monoethylmethyl malonate which, in turn, was prepared from the diethyl ester (47). Preparation of methylmalondihydroxamic acid from methylmalonic diethyl ester was attempted by the above method and also by adding diethyl ester to anhydrous hydroxylamine in ethanol in the presence of sodium ethoxide. By these methods products were obtained which gave multiple spots on paper chromatography. The most prominent of these spots, which appeared only several days after spraying with ferric chloride, is indicated in Fig. 1. Its identity has not been ascertained. The “methylmalondihydroxamic acid” preparations decomposed to yield products migrating to the position of methylmalonmonohydroxamic acid on chromatography in the isoamyl alcohol-formic acid solvent system. Sigma crystalline ATP and Pabst CoA were used in most experiments. Propionate-1-C14 and other materials were obtained commercially.

SUMMARY

Propionate is oxidized in animal tissues by a path involving (a) conversion to propionyl coenzyme A, (b) adenosine triphosphate-dependent carboxylation to methylmalonyl (isosuccinyl) coenzyme A, and (c) quantitative conversion of the carbon skeleton of methyl malonate to succinate which is then oxidized via the citric acid cycle. The conversion of methyl malonate to succinate does not take place by decarboxylation of the α-car-
bon followed by recarboxylation on the β-carbon of propionate, but by some relatively direct route. The oxidation of propionate via dicarboxylic acids seems to be a major pathway of propionate metabolism in animal tissues.

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