DEGRADATION OF MALONIC ACID BY RAT TISSUES*

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In past years, malonate has been extensively used in mammalian systems in vitro as an inhibitor of the citric acid cycle with very little attention given its metabolism per se. Lifson and Stolen (1) demonstrated the conversion of C\textsuperscript{14}-labeled malonate to carbon dioxide in the intact mouse. Later, Lee and Lifson (2) found that the administration to rats of doses of C\textsuperscript{13}-labeled malonate at an inhibiting concentration resulted in the excretion of C\textsuperscript{13}-labeled succinate in the urine. These findings indicate that malonate is an active metabolic substrate in the intact rat. Recently, Wick, Wolfe, and Nakada (3), using eviscerated-nephrectomized rabbits, showed that malonate is a poor inhibitor in vivo of acetate oxidation. Further investigation indicated that an inhibitory intracellular concentration of malonate was not obtained, due to the slow entry of malonate into the cells from the extracellular compartment and the concomitant removal of intracellular malonate by oxidation (3).

Wolfe, Ivler, and Rittenberg (4) and Hayaishi (5) have reported independently on the enzymatic decarboxylation of malonate by purified enzyme systems from Pseudomonas fluorescens. These workers have shown that malonate is activated in the presence of adenosine triphosphate\footnote{The following abbreviations are used in this paper: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CoA, coenzyme A.} and coenzyme A to form malonyl coenzyme A, which is subsequently decarboxylated to acetyl CoA and carbon dioxide.

The present study was undertaken to investigate the metabolism in vitro of malonate by rat tissues and to test for the possible occurrence of the microbial mechanism in the degradation of malonate by mammalian tissues.

Methods

Preparation and Incubation of Tissues—Young male rats (Slonaker strain) were killed by decapitation, and the organs were quickly removed

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and chilled in cold isotonic saline. Slices of liver, kidney, and spleen were prepared, by using the Stadie-Riggs tissue slicer; heart, brain, and lung slices were cut free-hand; testes cells were teased apart; and diaphragms were used intact. Tissue slice experiments were carried out in the conventional manner with the Warburg apparatus, and incubations were generally run at 37° for 2 hours with oxygen in the gas phase. Krebs-Ringer phosphate buffer (pH 7.4) was used for all slice experiments (6). Tissue homogenates were prepared by the Potter-Elvehjem technique in a medium consisting of KCl (70 parts of 1.15 per cent), NaF (10 parts of 1.15 per cent), MgSO₄ (2.5 parts of 3.42 per cent), and phosphate buffer (pH 7.5; 14 parts of 0.1 M). Mitochondria were prepared by the sucrose fractionation procedure of Schneider and Hogeboom (7).

Materials—Methylene C¹⁴-labeled malonate was obtained from the Volk Radiochemical Company; diethyl carboxyl-C¹⁴-labeled malonate was obtained from Tracerlab, Inc., and converted to the disodium salt. Coenzyme A and adenosine triphosphate were purchased from the Pabst Brewing Company.

Hydroxylamine Trapping Experiments—Hydroxylamine trapping experiments were conducted according to the method of Lipmann (8) with the exception that mitochondria were used as the enzyme source. Hydroxamates were determined by the method of Lipmann and Tuttle (9), and paper chromatography of the hydroxamates was carried out by the method of Stadtman and Barker (10).

Acetoacetate Isolation—The acetone moiety of acetoacetate was isolated as the HgSO₄ complex by the method of Van Slyke (11). The isolated acetone complex was decomposed by HCl and the acetone distilled and reprecipitated as the mercury complex.

Results

By using the conversion of malonate-1-C¹⁴ to CO₂ as an index, the distribution of enzymatic activity toward malonate by different organs of the rat was tested. As shown in Table I, slices of kidney, liver, heart, and diaphragm were capable of rapidly converting carboxyl-C¹⁴-labeled malonate to CO₂, whereas spleen, brain, testes, and lung were found to metabolize malonate slowly. A control flask containing substrate and buffer was carried through the experiment to test for the non-enzymatic breakdown of malonate; no such decarboxylation was detectable under these conditions.

Hayaishi (5) reported that, of the various mammalian tissue homogenates tested, only rat kidney homogenate was capable of decarboxylating malonate. In view of our findings it is apparent that the enzyme systems necessary for malonate decarboxylation are not stable to the cell rupture process.
The effect of increasing malonate concentrations on CO$_2$ production from acetate-1-C$^{14}$ and malonate-2-C$^{14}$ by rat kidney slices is given in Fig. 1. Curve 1 confirms the drastic inhibitory effect that malonate has on acetate oxidation in vitro.

### Table I

**Survey of Several Rat Tissues for Ability to Oxidize Malonate**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Respired CO$_2$, per cent added count</th>
<th>Organ</th>
<th>Respired CO$_2$, per cent added count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td>6.8</td>
<td>Testes</td>
<td>0.6</td>
</tr>
<tr>
<td>Liver</td>
<td>18.0</td>
<td>Heart</td>
<td>15.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>27.0</td>
<td>Lung</td>
<td>1.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.7</td>
<td>No tissue</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each flask contained disodium malonate-1-C$^{14}$ (0.005 M), Krebs-Ringer phosphate buffer (pH 7.4) to a total volume of 2 ml., and approximately 300 mg. of tissue slices. (Diaphragms were used intact and testes cells were teased apart.) Incubations were carried out for 1 hour at 37° with air in the gas phase.

Fig. 1. Effect of increasing concentrations of malonate on the oxidation of acetate-1-C$^{14}$, malonate-1-C$^{14}$, and malonate-2-C$^{14}$ by rat kidney slices. Each flask contained approximately 300 mg. of rat kidney slices in a total volume of 2 ml. of Krebs-Ringer phosphate buffer (pH 7.4) plus the substrates. The concentration of acetate-1-C$^{14}$ was 0.005 M. The flasks were incubated for 2 hours at 37° in an atmosphere of oxygen.

The rate of C$^{14}$O$_2$ formation from methylene-labeled malonate (Curve 3) reaches a peak at about 0.003 M malonate concentration and, as the concentration was increased, almost complete self-inhibition was obtained. Such an effect would be expected if methylene-labeled malonate was decarboxylated to methylene-labeled acetate. The methyl-labeled acetate thus formed by the decarboxylation of malonate could then be oxidized via the citric acid cycle. At high concentrations of malonate, methyl-labeled
acetate is being formed but little or no methyl carbon oxidation occurs due to inhibition of the citric acid cycle.

This mechanism is further supported by Curve 2, which indicates how C^{40}O_2 production changes when the concentration of carboxyl-labeled malonate is increased. A peak conversion to C^{40}O_2 occurs around 0.005 M, and at higher concentrations the curve drops, then levels off with only a 50 per cent inhibition of the maximal rate of C^{40}O_2 formation. Thus the optimal concentration could represent a combination of malonate decarboxylation and carboxyl-labeled acetate oxidation. As the concentration of malonate increased, inhibition of the citric cycle occurred, resulting in the lowering of acetate oxidation. The decarboxylation of malonate to the acetate level continued, however, as shown by the leveling off of C^{40}O_2 production from carboxyl-labeled malonate (Curve 2) at a level that was substantially higher than the malonate-inhibited oxidation of carboxyl-labeled acetate (Curve 1).

One of the main uses for malonate in the past has been to block the citric acid cycle in liver preparations, thereby shunting the metabolism of acetate from oxidation into acetoacetate formation. If the postulate that malonate is converted directly to an acetyl derivative is correct, then radioactive malonate, when incubated with rat liver slices, should result in the formation of radioactive acetoacetate. Such an experiment was conducted and the acetone portion of acetoacetate was isolated as the mercuric sulfate acetone complex. The results of this experiment are shown in Table II. The respired CO_2 values were as expected, with about twice as much label from the carboxyl carbons as was found from the methylene carbon. On the other hand, the counts recovered from the acetone showed that twice as much activity was incorporated from the methylene carbon. The acetone portion of acetoacetate was the only part isolated because of the possible non-enzymatic decarboxylation of substrate malonate.

The sequence of reactions leading from malonate to acetoacetate, by use of carboxyl-labeled malonate as an example and by omitting the activating steps for simplicity, can be formulated as follows:

\begin{align*}
(1) \quad & \text{HOOC} - \text{CH}_2 - \text{COOH} \rightarrow \text{CH}_3 - \text{COOH} + ^* \text{CO}_2 \\
(2) \quad & 2\text{CH}_2 - \text{COOH} \rightarrow \text{CH}_3 - \text{CO} - \text{CH}_2 - \text{COOH} \\
(3) \quad & \text{CH}_3 - \text{CO} - \text{CH}_2 - \text{COOH} \rightarrow \text{CH}_3 - \text{CO} - \text{CH}_3 + ^* \text{CO}_2
\end{align*}

Thus carboxyl-labeled malonate would lead to the formation of acetone-labeled malonate only in the carbonyl position. If methylene-labeled malonate were substituted in these equations, the acetone moiety isolated would be labeled in both methyl groups. The ratio of 2:1 for the activities obtained in the acetone from methylene- and carboxyl-labeled malonates,
respectively, is in agreement with the scheme postulated above. In fact, no other theoretical pathway that we could devise, aside from a conversion

<table>
<thead>
<tr>
<th>Table II</th>
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<tbody>
<tr>
<td>Acetoacetate Formation from C\textsuperscript{14}-Malonic Acid by Rat Liver Slices</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>C\textsuperscript{14} per gm. tissue per hr. converted to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Respired CO\textsubscript{2}</td>
</tr>
<tr>
<td></td>
<td>μmoles</td>
</tr>
<tr>
<td>Malonate-1-C\textsuperscript{14}</td>
<td>9.8</td>
</tr>
<tr>
<td>Malonate-2-C\textsuperscript{14}</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
</tr>
</tbody>
</table>

Each flask contained approximately 300 mg. of liver slices suspended in Krebs-Ringer phosphate buffer (pH 7.4) and 0.02 M malonate. Total volume, 3.0 ml.; incubated 2 hours at 37° with O\textsubscript{2} in the gas phase.

<table>
<thead>
<tr>
<th>Table III</th>
</tr>
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<tbody>
<tr>
<td>Complete System Necessary for Maximal Malonate Oxidation by Aged Rat Kidney Mitochondria</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conditions</th>
<th>C\textsuperscript{14} converted to CO\textsubscript{2} per gm. tissue per hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
</tr>
<tr>
<td>Complete system</td>
<td>2.54</td>
</tr>
<tr>
<td>&quot;</td>
<td>minus CoA</td>
</tr>
<tr>
<td>&quot;</td>
<td>ATP</td>
</tr>
<tr>
<td>&quot;</td>
<td>MgCl\textsubscript{2}</td>
</tr>
<tr>
<td>&quot;</td>
<td>glutathione</td>
</tr>
</tbody>
</table>

The complete system contained CoA (50 units), ATP (20 μmoles), malonate-1-C\textsuperscript{14} (20 μmoles), MgCl\textsubscript{2} (40 μmoles), glutathione (20 μmoles), Tris(hydroxymethyl)-aminomethane buffer (pH 7.5, 20 μmoles), mitochondria (1 ml.), and water to make a total volume of 2.0 ml. The mitochondrial suspension was made up so that each ml. contained mitochondria from 0.5 gm. of original tissue (wet weight). The mitochondria were aged at 4° for 5 hours between the second and third centrifugation. Incubations were carried out at 37° for 1 hour.

of malonate to acetate (or acetyl derivative) and CO\textsubscript{2}, would explain the 2:1 ratio obtained.

Preliminary studies with rat kidney homogenates indicated that a loss of malonate decarboxylase activity occurred during the homogenization procedures. This would confirm the low activity found by Hayaishi (5) in this type of preparation. In subsequent studies it was found that the malonate decarboxylase system was associated with the particulate frac-
Rat kidney mitochondria were used as the enzyme source for further investigations.

Initial studies with mitochondria suggested that CoA, ATP, and Mg++ were necessary for maximal conversion of carboxyl-labeled malonate to C¹⁴O₂. However, the differences between the supplemented and unsupplemented controls were not great, and it seemed desirable to deplete the kidney mitochondria of bound CoA and ATP further. This was accomplished by aging the mitochondrial preparation for 5 hours at refrigerator temperatures between the second and third washings. Reexamination clearly indicated that CoA, ATP, and Mg++ were involved in the decarboxylation of malonate by these preparations (Table III).

The involvement of ATP, CoA, and Mg++ in the decarboxylation of malonate suggested that a CoA-activated malonate was found during the course of the reaction. Hydroxylamine was used as a trapping agent to detect the possible formation of such an intermediate. One flask contained all components for the complete system (Table III) plus 0.4 mmole of hydroxylamine. The control flasks contained all the components except CoA. The flasks were incubated at 37° for 30 minutes.

The reaction vessels contained all the components for the complete system (Table III) plus 0.4 mmole of hydroxylamine. The control flasks contained all the components except CoA. The flasks were incubated at 37° for 30 minutes.

The identity of this hydroxamate ester was checked by paper chromatography. An experiment similar to the one given on Table IV was run and, after removal of the mitochondria, the contents of the reaction vessels were evaporated to a small volume, extracted with alcohol, and analyzed by paper chromatography by the method of Stadtman and Barker (10). The $R_f$ values for the known hydroxamates (Table V) were identical with those found by Hayaishi (5). The only hydroxamate spot found from the extract of the incubation mixture had an $R_f$ value that corresponded to the $R_f$ value for malonyl monohydroxamate.

**Table IV**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Preparation</th>
<th>Increase in hydroxamate μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fresh mitochondria</td>
<td>0.30</td>
</tr>
<tr>
<td>2</td>
<td>Aged &quot;</td>
<td>0.85</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The effect of CoA on hydroxamate formation from malonate by rat kidney mitochondria.
The mechanism for malonate degradation appears to be similar to that found in \textit{P. fluorescens} (4, 5), and can be formulated as follows:

\begin{align*}
(1) & \quad \text{Malonate} + \text{CoA} + \text{ATP} \xrightarrow{\text{Mg}^{++}} \text{malonyl-CoA} + \text{ADP} \\
(2) & \quad \text{Malonyl-CoA} \rightarrow \text{acetyl-CoA} + \text{CO}_2 \\
(3) & \quad \text{Acetyl-CoA} \rightarrow \text{acetoacetyl-CoA} \\
(4) & \quad \text{Acetyl-CoA} \xrightarrow{\text{citric acid cycle}} \text{CO}_2
\end{align*}

The requirement for CoA, ATP, and Mg\(^{++}\) for maximal malonate decarboxylase activity and the demonstration of malonyl monohydroxamate formation indicate that step (1) is necessary for malonate metabolism. Whether or not this activation is catalyzed by a specific kinase is not known.

\textbf{Table V}

\begin{center}
\begin{tabular}{|l|c|}
\hline
\textbf{Compound} & \textbf{RF} \\
\hline
Known acetyl hydroxamate & 0.51–0.54 \\
" malonyl dihydroxamate & 0.1–0.15 \\
" \" monohydroxamate & 0.35–0.38 \\
Alcohol extract of hydroxylamine trapping experiment & 0.36 \\
\hline
\end{tabular}
\end{center}

The chromatogram was developed with water-saturated butanol on Whatman No. 1 filter paper. The hydroxylamine trapping experiment was identical with the experiments given in Table IV.

The formation of labeled acetoacetate during the decarboxylation of labeled malonate and the ratio of isotopic carbon recovered in the acetone moieties derived from carboxyl- and methylene-labeled malonates show that acetate or an acetyl derivative is a product of malonyl-CoA degradation. Acetyl-CoA is the most likely acetyl derivative to be formed under these circumstances, and its fate can be accounted for by known routes of degradation.

\textbf{SUMMARY}

1. The extent and pathway for malonate degradation by rat tissues have been studied. Slices of kidney, liver, heart, and diaphragm rapidly metabolized malonate, while spleen, brain, testes, and lung were relatively inactive in this respect.

2. Rat liver slices converted appreciable quantities of C\(^{14}\)-labeled malonate to acetoacetate. The ratio of activities of 2:1 obtained in the acetone moiety from methylene- and carboxyl-labeled malonates, respectively, indicates a direct conversion of malonate to acetate.

3. The requirement for added CoA, glutathione, ATP, and Mg\(^{++}\) for
maximal activity by rat kidney mitochondria indicates that malonyl-CoA formation is necessary for decarboxylation.

4. Increased hydroxamate formation and identification of malonyl-hydroxamate by paper chromatography confirm that malonyl-CoA and subsequent decarboxylation are the degradation pathway for malonate.

**BIBLIOGRAPHY**

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