ENZYMATIC DECARBOXYLATION OF OXALIC ACID*

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Although it has been recognized that oxalic acid is decomposed by a variety of microorganisms (2–6), relatively little is known of the details of oxalic acid metabolism. The present study represents an attempt to establish the mechanism of oxalate degradation at the enzyme level by the use of a bacterium isolated from soil.

Partially purified enzyme preparations obtained from the organism catalyzed the decarboxylation of oxalic acid with the formation of formic acid and carbon dioxide. The system requires substrate quantities of ATP¹ and catalytic amounts of acetate, CoA, ThPP, and magnesium ions. Acetate, CoA, ATP, and magnesium ions are required for the formation of acetyl CoA (Equation 1) which is in turn utilized according to the over-all reaction described by Equation 2.

\[
\begin{align*}
(1) \quad & \text{ATP} + \text{CoA} + \text{acetate} \xrightarrow{\text{Mg}} \text{acetyl CoA} + \text{ADP} \text{or AMP} + \text{Pi} \text{or PP} \\
(2) \quad & \text{Acetyl CoA} + \text{oxalate} \xrightarrow{\text{ThPP}} \text{acetate} + \text{formate} + \text{CO}_2 + \text{CoA}
\end{align*}
\]

Evidence will be presented which suggests that Equation 2 is the sum of Equations 3, 4, and 5.

\[
\begin{align*}
(3) \quad & \text{Acetyl CoA} + \text{oxalate} \rightleftharpoons \text{oxalyl CoA} + \text{acetate} \\
(4) \quad & \text{Oxalyl CoA} \xrightarrow{\text{ThPP}} \text{[formyl CoA]} + \text{CO}_2 \\
(5) \quad & \text{[Formyl CoA]} + \text{H}_2\text{O} \rightarrow \text{formate} + \text{CoA}
\end{align*}
\]

Materials and Methods

Oxalic acid-C¹⁴, formic acid-C¹⁴, acetic acid-1-C¹⁴, and diethyl malonate-1-C¹⁴ were obtained from the Isotopes Specialties Company, Inc., Burbank,

* A preliminary account of this work was presented before the American Chemical Society (1).

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¹ The following abbreviations are used: ATP, ADP, and AMP, adenosine tri-, di-, and monophosphates, respectively; CoA, coenzyme A; GSH, reduced glutathione; ThPP, thiamine pyrophosphate; Pi, inorganic phosphate; PP, inorganic pyrophosphate; GTP, UTP, CTP, and ITP, the triphosphates of guanosine, uridine, cytidine, and inosine, respectively; Tris, tris(hydroxymethyl)aminomethane.
California. Malonic acid was prepared by the saponification of diethyl malonate. Monoethyl oxalate was prepared by saponification of diethyl oxalate by the method of Freund (7). Acetyl phosphate was synthesized by a modification of the method of Avison (8). The method of Simon and Shemin (9) as detailed by Kaufman et al. (10) was used for the synthesis of acetyl CoA. All other compounds used here are commercially available.

Protein was determined by the method of Lowry et al. (11). The formation of acethydroxamic acid was followed as described by Lipmann and Tuttle (12).

Radioactivity was measured with a windowless gas flow counter. For the estimation of radioactivity on paper chromatograms a low background Robinson gas flow counter (13) was used.

**Growth Conditions**—Enrichment cultures were prepared with a medium similar to that of Bhat and Barker (5). The organism which was isolated from a single colony after several transfers was found to be a small, motile, strictly aerobic, gram-negative rod which has tentatively been classified as a member of the genus, *Bacterium*.

The organism was grown in 6 liter Erlenmeyer flasks containing 1.5 liters of medium which were aerated on a reciprocal shaker. The medium included the following constituents in gm. per liter: K$_2$HPO$_4$, 1.5; NaH$_2$PO$_4$, 0.5; MgSO$_4$·7H$_2$O, 0.2; NH$_4$NO$_3$, 1; NaCl, 0.1; yeast extract, 0.6; ammonium oxalate, 2. Although the organism can grow without the addition of yeast extract, the yield of cells in its absence is very low. The bacterium may be grown on yeast extract alone or on acetate. Under these conditions, however, the cell-free extract does not decarboxylate oxalic acid.

Growth was allowed to take place at 25°C for 24 hours, and the cells were collected by centrifugation, washed twice with 0.9 per cent saline, and frozen. The yield of cells was approximately 0.4 gm. (wet weight) per liter of oxalate-yeast extract medium.

**Enzyme Preparation**—Cell-free extracts could be obtained either by grinding the frozen cells with alumina, utilizing the procedure of McIlwain (14), or by sonic disintegration of a suspension of the cells in phosphate (0.1 M)-glutathione (0.005 M) buffer at pH 6.9 in a 10 kc Raytheon sonic oscillator. The latter method was preferred for large batches of material. Generally, 15 gm. of cells in 100 ml. of buffer were subjected to sonic oscillation for 10 minutes, and the suspension was centrifuged at 2°C for 20 minutes at 15,000 X g. All fractionation procedures were conducted at 0-2°C.

The supernatant fluid, containing approximately 12 to 15 mg. of protein per ml., was treated with 15 ml. of a 1 per cent solution of protamine sul-

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*Personal communication from Dr. A. Kornberg.*
fate per 100 ml. of crude extract. After removal of the precipitate, the solution was treated with 19.4 gm. of ammonium sulfate per 100 ml. of supernatant fluid, and the precipitate was discarded. The precipitate formed upon the further addition of 15 gm. of ammonium sulfate was dissolved in one-fourth of the original volume of buffer containing phosphate (0.05 M) and glutathione (0.005 M) at pH 6.9. Unless otherwise specified such ammonium sulfate fractions were used throughout this study.

In some cases the ammonium sulfate fraction was dialyzed for a total of 4 hours against two changes of a phosphate (0.05 M)-glutathione (0.005 M) solution at pH 6.9. 10 ml. of enzyme were dialyzed against 250 ml. of buffer.

Standard Assay System—Oxalic acid-C¹⁴ was used as substrate, and enzyme activity was followed by assay of C¹⁴-carbon dioxide formed. Carbon dioxide produced by the reaction was trapped in alkali by a modification of a method originally described by Kornberg et al. (15).

The components of the incubation mixture were placed in the main chamber of a Thunberg tube and included the following (expressed in micromoles) in a total volume of 1 ml.: potassium phosphate at pH 6.9, 50; potassium fluoride, 50; magnesium chloride, 5; glutathione, 5; acetate, 10; ATP, 5; CoA, 0.1; ThPP, 0.05; oxalate-C¹⁴, 1. Potassium fluoride served to inhibit phosphatases which were present. The side arm of the Thunberg tube contained 0.5 ml. of 0.3 N KOH.

The reaction was started by the addition of the enzyme preparation after which the tube was closed and incubated at 35° for 40 minutes. The reaction was stopped by the addition of 0.3 ml. of 0.5 N KOH to the main chamber, and the Thunberg tube was again sealed and evacuated with a water aspirator.

Approximately 0.3 ml. of 18 N H₂SO₄ was placed in the evacuation outlet. Because of the partial vacuum in the Thunberg tube, the acid will flow into the main chamber when the side arm opening coincides with that of the evacuation outlet. Before all the acid had entered, the tube was closed and incubated at 60° for 30 minutes. After cooling to room temperature a 0.03 ml. aliquot of the alkali from the side arm was plated on a planchet, dried, and counted.

Results

When dialyzed ammonium sulfate fractions were incubated with oxalic acid, only minimal carbon dioxide formation could be detected. Supplementation of such incubation mixtures with ATP, CoA, ThPP, and MgCl₂ and with acetate allowed the formation of carbon dioxide from oxalate. Under standard assay conditions the concentrations of the required components were varied independently of each other with the results illustrated
in Figs. 1 and 2. Of interest is the stimulation by potassium fluoride (Fig. 1, A) which is known to inhibit phosphatases (17).

ATP is replaced by ITP but not by equimolar quantities of GTP, CTP, UTP, or AMP. ADP is only 5 per cent as active as ATP, and it is presumed that this activity is due to myokinase which is present in the enzyme preparation. At high concentrations CoA proved inhibitory (Fig. 2, A). It should be noted that the standard assay system contains CoA at a concentration (5 × 10⁻⁵ M) below the optimal level. The enzyme system is saturated at an oxalate concentration below 5 × 10⁻⁴ M. Such a concentration-activity curve is not reproduced for the magnesium ion requirement as the enzyme preparation contained sufficient active material to allow 40 per cent of the maximal activity in the absence of exogenous magnesium. The system was saturated at a magnesium chloride concentration of 1 × 10⁻³ M.

The activity of the oxalate-decarboxylating system at various pH values is shown in Fig. 3. The optimal pH is at 6.9. Enzyme activity under anaerobic conditions, i.e. in a helium atmosphere, is equal to that obtained in air.

As illustrated in Fig. 4, carbon dioxide formation is a linear function of time.
W. B. Jakoby, E. Ohmura, and O. Hayaishi

Fig. 2. Decarboxylation activity as a function of (A) CoA concentration and (B) acetate concentration. Conditions as in Fig. 1.

Fig. 3. The effect of pH on the system decarboxylating oxalic acid; 0.1 M buffer.

Specificity and Reversibility—Under the experimental conditions employed C\textsuperscript{14}-formate and acetate-1-C\textsuperscript{14} did not give rise to radioactive carbon dioxide. Malonate-1-C\textsuperscript{14} was decarboxylated at approximately 25 per cent of the rate of oxalate decarboxylation. However, it remains to be estab-
lished whether a single enzyme system is responsible for the activity on both dicarboxylic acids.\textsuperscript{3}

That the system is not readily reversible was demonstrated by an experiment in which the usual assay components were incubated with non-radioactive oxalate in the presence of approximately 2 $\mu$moles of C\textsuperscript{14}-carbon dioxide ($1 \times 10^6$ c.p.m.). When approximately 0.5 $\mu$ mole of oxalate had been decarboxylated, the reaction mixture was made 0.1 N with respect to hydrochloric acid, and aliquots were plated directly. No incorporation of C\textsuperscript{14} was observed either in this experiment or in one in which a further supplement of formate was included. In the latter case the non-radioac-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Time-course of decarboxylation, 0.2 $\mu$ mole of oxalate was added at zero time to each of two series of tubes. At Arrow 1 a further addition of 0.2 $\mu$ mole of oxalate was made to one series.}
\end{figure}

tive oxalate system was supplemented with 2 $\mu$ moles of C\textsuperscript{14}-formate ($1.2 \times 10^{-5}$ c.p.m.).

Products of Reaction and Stoichiometry—Exhaustive incubation of oxalate with the complete system led to the formation of 1 mole of carbon dioxide per mole of oxalate added. The other product of the reaction could be separated by ion exchange chromatography on Dowex 1-Cl. 2 $\mu$ moles of oxalic acid (476,000 c.p.m.) were incubated at 32$^\circ$ under standard assay conditions in each of two Thunberg tubes. After 2 hours the reaction in one tube was terminated by the addition of alkali and was assayed for carbon dioxide in the usual manner. 0.83 $\mu$ mole (98,000 c.p.m.) of carbon dioxide was found to have been formed. The contents of the second tube

\textsuperscript{3}More recently fractions of the oxalate system have been obtained which show negligible activity toward malonate.
were applied directly to a column of Dowex 1-Cl. After washing the column with 30 ml. of water, it was eluted with a 0.02 M sodium chloride-0.015 N hydrochloric acid solution. The elution pattern as followed by the appearance of radioactivity is shown in Fig. 5. 1.16 μmoles of oxalate (276,000 c.p.m.) were recovered as well as 0.82 μmole (97,000 c.p.m.) of a more readily eluted compound. The latter was found to be a volatile acid and was identified as formic acid by oxidation of an aliquot of the eluate with mercuric acetate (18). Of the 16,650 c.p.m. subjected to oxidation 16,200 c.p.m. were recovered as carbon dioxide. Authentic formic acid yielded an identical elution pattern.

![Elution pattern of products of oxalate decarboxylation.](image)

**Fig. 5.** Elution pattern of products of oxalate decarboxylation. Oxalic acid (476,000 c.p.m.) was incubated under standard assay conditions for 2 hours and transferred to a 1 cm. × 5 cm. Dowex 1-chloride column. The material was eluted with a NaCl (0.02 M)-HCl (0.015 N) solution, the eluate brought to pH 10, and aliquots assayed for radioactivity. Carbon dioxide which was determined separately accounted for 98,000 c.p.m.

When 0.5 μmole of oxalate was subjected to enzyme action under similar incubation conditions, the reaction went to completion. Equimolar quantities of formate and carbon dioxide were recovered; oxalate was not found.

*Rôle of Acetate*—Incubation of the complete system in the presence of hydroxylamine (200 μmoles) and acetate (10 μmoles) led to the formation of a hydroxamic acid which was identified by chromatography (19) as acethydroxamic acid. In the absence of acetate and in the presence of oxalate, hydroxamic acid-reacting material was not formed.

If the rôle of CoA, ATP, and magnesium ions is solely to produce acetyl CoA, then oxalate decarboxylation should be dependent only on ThPP
ENZYMATIC OXALATE DECARBOXYLATION

and acetyl CoA. That this is, in fact, the case is shown by the data of Table I. The further addition of CoA proves inhibitory. The requirement for ThPP and acetyl CoA is again demonstrated (Table II) when acetyl phosphate and CoA replace acetyl CoA. From the data in Fig. 6 it is evident that stoichiometric amounts of acetyl phosphate are required for oxalate decarboxylation.

**Table I**

*Replacement of Acetate by Acetyl CoA*

The incubation mixture in a volume of 1 ml. (in micromoles) consisted of potassium phosphate at pH 6.9, 100; KF, 50; GSH, 5; ThPP, 0.05; oxalate, 2.

<table>
<thead>
<tr>
<th>Additions</th>
<th>CO₂ (µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl CoA, 0.4 µmole</td>
<td>0.16</td>
</tr>
<tr>
<td>1.0 µmole</td>
<td>0.28</td>
</tr>
<tr>
<td>- ThPP, 0.05 µmole</td>
<td>0.07</td>
</tr>
<tr>
<td>+ Mg, 5 µmoles</td>
<td>0.28</td>
</tr>
<tr>
<td>+ ATP, 5 µmoles</td>
<td>0.25</td>
</tr>
<tr>
<td>+ CoA, 0.2 µmole</td>
<td>0.14</td>
</tr>
</tbody>
</table>

**Table II**

*Replacement of Acetate by Acetyl Phosphate*

The incubation mixture in a volume of 1 ml. (in micromoles) consisted of potassium phosphate at pH 6.9, 100; KF, 50; GSH, 5; ThPP, 0.05; CoA, 0.2; acetyl phosphate, 5; oxalate, 2.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.23</td>
</tr>
<tr>
<td>- Acetyl phosphate</td>
<td>0.02</td>
</tr>
<tr>
<td>- CoA</td>
<td>0.07</td>
</tr>
<tr>
<td>- ThPP</td>
<td>0.13</td>
</tr>
<tr>
<td>- Mg</td>
<td>1.21</td>
</tr>
<tr>
<td>+ Arsenate (20 µmoles)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Although the presence of oxalate had no effect on the total quantity of hydroxamate formed when acetate was incubated with ATP, CoA, and magnesium ions, evidence of oxalhydroxamic acid formation has been obtained. In one experiment 5 µmoles of CoA were used to supplement the standard incubation mixture. After incubation for 1 hour hydroxylamine was added, and the hydroxamic acids were extracted with ethanol by the
method of Stadtman and Barker (19). Aliquots of the final extract were chromatographed with phenol-water (1:1 volt per volt) (20) as the developing solvent. In this system oxalmonohydroxamic acid prepared chemically from monoethyloxalate (21) and enzymatically as described above had an identical $R_F$ of 0.08. Acethydroxamate in this solvent system was localized at an $R_F$ of 0.65. Formyl hydroxamate ($R_F = 0.50$) was not found.

![Fig. 6](image1)

**Fig. 6.** Stoichiometry of acetyl phosphate requirement. In addition to 0.7 μmole or 1.4 μmoles of acetyl phosphate the incubation mixtures contained 4 mg. of enzyme and the following (in micromoles) in a total volume of 1 ml.: potassium phosphate at pH 6.9, 100; KF, 50; GSH, 5; ThPP, 0.15; CoA, 0.1; oxalate, 2. Theoretical carbon dioxide formation is indicated by dashed lines.

**Fig. 7.** Paper chromatogram of radioactive oxalhydroxamic acid. The details are discussed in the text. The incubation was conducted with (○) and without (●) exogenous ThPP.

**Role of ThPP**—In a similar experiment, 5 μmoles of CoA were added to each of two tubes containing the usual incubation mixture, except that exogenous ThPP was absent from one tube. Both mixtures were incubated for 1 hour, treated with hydroxylamine, and extracted as described above. As $^{34}$-oxalic acid was used, the presence of oxalhydroxamate was detected by measuring radioactivity on the paper chromatogram. The results are detailed in Fig. 7. Approximately twice as much oxalhydroxamate was present in the experiment in which exogenous ThPP was not added as in the presence of excess ThPP. It appears, therefore, that ThPP is a limiting factor in the further metabolism of the “activated” oxalate.
DISCUSSION

The work of several laboratories has disclosed an oxidative mechanism for carbon dioxide formation from oxalate (Equation 6) which enjoys ubiquitous distribution among mosses (22-25) and certain plants (22, 26).

\[ \text{Oxalate} + \text{O}_2 \rightarrow 2\text{CO}_2 + \text{H}_2\text{O} \]

The data presented here reveal a novel reaction responsible for the decarboxylation of oxalic acid. In contrast to the oxidative reactions found in plants the decarboxylation reaction catalyzed by the bacterial enzymes proceeds anaerobically and requires acetate, ATP, CoA, magnesium ions, and ThPP.

It has been demonstrated that acetyl CoA is an obligatory requirement for oxalate decarboxylation, and it appears likely that oxalyl CoA is formed as the product of a CoA transfer reaction between acetyl CoA and oxalate (Equation 3). Analogous reactions have been described for CoA transfer to fatty acids (27) and dicarboxylic acids (28-31). The demonstration of oxalhydroxamic acid in the present system is taken as evidence for the formation of oxalyl CoA.

That ThPP is an obligatory requirement of the oxalate decarboxylation system has been shown, and it is proposed that ThPP is directly involved in the decarboxylation of monooxalyl CoA (Equation 4). Supporting this suggestion is the finding that in the absence of exogenous ThPP, oxalhydroxamate accumulates in quantities greater than in the presence of ThPP. Previous work by Whiteley (32) on the decarboxylation of succinyl CoA to propionyl CoA suggested a ThPP requirement for that system.

Whether or not formyl CoA is a primary product of the decarboxylation reaction has not been established. It is to be noted, however, that evidence does exist for the participation of formyl CoA in other enzymatic reactions (27, 33) and that formyl CoA appears to be an extremely unstable compound. Indeed, the synthesis of both formyl CoA and oxalyl CoA has been attempted with negative results. Although a mixed anhydride of formic acid (34) could be formed, the addition of water to such a system resulted in the immediate formation of the free acids. Stoichiometric quantities of acetyl phosphate were consumed for oxalate decarboxylation whereas catalytic amounts would be expected to be required were a cyclic mechanism operative. Such cyclic mechanisms have been demonstrated for the decarboxylation of succinate (30) and malonate (31) in which cases the products of the reactions, i.e. propionyl CoA and acetyl CoA, respectively, are utilized as CoA donors for the formation of succinyl CoA and malonyl CoA, respectively. It must, therefore, be concluded that either formyl CoA does not serve as a donor for the transferase reaction (Equa-
tion 3) or that its extreme lability leads to the hydrolysis of formyl CoA if it is formed.

The data which have been presented and discussed above are consistent with Equations 3, 4, and 5 for the decarboxylation of oxalic acid in the presence of acetate, CoA, ATP, magnesium ions, and ThPP.

SUMMARY

A soluble enzyme system has been obtained from a bacterium isolated from soil which catalyzes the anaerobic decarboxylation of oxalic acid. Adenosine triphosphate, coenzyme A, acetate, magnesium ions, and thiamine pyrophosphate are required for decarboxylation. When acetyl CoA is substituted, only ThPP is required. The data are in accord with the following mechanism: (1) transfer of CoA from acetyl CoA to oxalate to form oxalyl CoA and (2) decarboxylation of oxalyl CoA in the presence of ThPP. The decarboxylation of oxalic acid in this system results in the formation of equimolar quantities of carbon dioxide and formate.

BIBLIOGRAPHY

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