THE ENZYMATIC SYNTHESIS OF OXALACETATE FROM PHOSPHORYL-ENOLPYRUVATE AND CARBON DIOXIDE*

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The present work is concerned with an enzyme, obtained from an acetone powder of spinach leaves, which catalyzes the carboxylation of phosphoryl-enolpyruvate to yield, under the conditions of isolation here employed, oxalacetate and inorganic phosphate. Pyruvate or pyruvate plus ATP will not substitute for PEP. Reduced triphosphopyridine nucleotide is not added to the reaction mixture and malate does not accumulate. The reaction thus appears to be distinct from the reductive carboxylation of pyruvate to malate by the "malic" enzyme (1). The present reaction may represent a mechanism for the Wood and Werkman reaction in which pyruvate is carboxylated to OA (2). This reaction, as has been shown by Utter and Wood (3), is activated by the addition of ATP. Enzymes catalyzing the Wood and Werkman reaction have now been shown to be present in bacteria (2, 4), pigeon liver (5, 6), and higher plants (7). In two recent communications (8, 9), Utter and Kurahashi describe the reversible formation of OA and ATP from PEP, ADP, and HCO₃⁻ in the presence of an enzyme from pigeon liver. It seems probable that the reaction described by Utter and Kurahashi is the same as that described in the present work, with the addition of a second enzyme catalyzing the transfer of phosphorus from a labile intermediate to ADP. A preliminary report of the present work has appeared (10). Several recent reviews of carboxylation reactions leading to the formation of a dicarboxylic acid are now available (11–13).

Materials

Silver-barium phosphoryl-enolpyruvate was a gift of Dr. Bernard Axelrod and was prepared by the method of Baer and Fischer (14). The K salt was made by decomposition of the silver-barium salt with 1 equiv-

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1 The following abbreviations will be used throughout the present paper: oxalacetate, OA; phosphoryl-enolpyruvate, PEP; adenosinetriphosphate, ATP; adenosinediphosphate, ADP; glutathione, GSH; tris(hydroxymethyl)aminomethane, Tham.
alent each of KCl and K₂SO₄. Glutathione and ADP (chromatographically pure) were obtained from the Schwarz Laboratories and ATP of 95 to 100 per cent purity from the Pabst Laboratories. For use these compounds were adjusted to pH 7.5 with KOH. OA of 85 per cent purity was obtained from the H. M. Chemical Company, Santa Monica, California. A stock solution of NaH¹⁴CO₃ containing 131 μc. per ml. (1.78 × 10⁻⁴ M with respect to HCO₃⁻) was prepared from BaC¹⁴O₃. With the counting technique used (counting at infinite thickness and at a distance of 4.5 cm. from a Geiger-Müller tube, 1.4 mg. per sq. cm.) 0.1 ml. of this solution had an activity of 657,000 c.p.m.

The enzyme used throughout the present investigation was a dialyzed, ammonium sulfate-concentrated extract of spinach leaf acetone powder, purity Stage II, prepared as described by Axelrod et al. (15).

Phosphorus determinations were made by the method of Allen (16). Potassium pyruvate was prepared from freshly distilled pyruvic acid by the method of Robertson (17).

Results

¹⁴CO₂ Incorporation into Oxalacetate—The reaction between PEP and CO₂ was first detected by noting the increased rate of liberation of inorganic phosphate from PEP in the presence of CO₂. Owing, however, to the activity of a phosphatase, an appreciable amount of inorganic phosphate was liberated from PEP in the absence of added CO₂. In order to circumvent the interference caused by this phosphatase the formation of OA was measured directly in subsequent experiments.

By utilizing ¹⁴CO₂ in the reaction mixture, OA synthesis was readily determined. The OA formed was β-decarboxylated with Al⁺⁺⁺ at room temperature and the liberated ¹⁴CO₂ used as a measure of OA. The assay was performed as follows: After completion of the incubation period the reaction was stopped by the addition of 0.25 ml. of 2 N HCl, and unreacted ¹⁴CO₂ was removed by vigorous gassing with 5 per cent CO₂-95 per cent N₂. Aliquots of the acidified reaction mixture were then assayed by the procedure of Krebs and Eggleston (18), which is specific for β-ketonic dicarboxylic acids. A period of time ranging from 4 to 8 hours was allowed for decarboxylation of OA. Liberated ¹⁴CO₂ was collected in KOH solution in the center well of the decarboxylation vessel, and the carbonate together with sufficient carrier carbonate to yield a sample weighing approximately 100 mg. was precipitated as BaCO₃ for counting.

Evidence that the reaction is enzymatically catalyzed is shown in Table I. Heating the enzyme for 2 minutes at 100° resulted in its inactivation. Several replicate experiments are illustrated as an index of reproducibility. The average count in the β-carboxyl of OA for the experiments with un-
treated enzyme is 223,000, which corresponds to 0.60 μM of OA per tube (223,000 counts recovered × 1.78 μM of C¹⁴O₂ per 657,000 counts added). This is a minimal value, as it is assumed that no CO₂ other than that added is present in the incubation mixture. Under conditions in which 0.6 μM of OA was formed, 1.0 μM of inorganic phosphate was liberated from PEP.

**Substrate Specificity**—That the present reaction is highly specific for PEP is shown by the data of Table II. Neither pyruvate alone nor pyruvate plus ATP can replace PEP and both give very low values. Further, the addition of ADP to the PEP-containing treatment did not increase the amount of OA formed. In the absence of data for times shorter than 1000 seconds an effect of ADP upon the reaction rate is not excluded. Some effect of ADP on the rate of OA formation or the amount of OA formed might be expected if PEP were first converted to ATP and pyruvate by means of the ATP-phosphopyruvate transphosphorylase reaction (19).

**Identification of Reaction Product**—Oxalacetate was isolated as the 2,4-dinitrophenylhydrazone from a reaction mixture similar to that described in Table I. Following incubation, acidification with 0.25 ml. of 2 N HCl, and removal of the unreacted C¹⁴O₂, the precipitated protein was removed

### Table I

**Heat Inactivation of Phosphoryl-enolpyruvate Carboxylase Activity**

<table>
<thead>
<tr>
<th>Counts in β-carboxyl of OA per tube.</th>
<th>Experiment A</th>
<th>Experiment B</th>
<th>Experiment C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated enzyme</td>
<td>184,000</td>
<td>229,000</td>
<td>256,000</td>
</tr>
<tr>
<td>Enzyme, boiled 2 min.</td>
<td>4,320</td>
<td>1,520</td>
<td>1,050</td>
</tr>
</tbody>
</table>

Each tube contained 6 μM of PEP, 60 μM of Tham hydrochloride, pH 7.5, 20 μM of MgSO₄, 20 μM of GSH, 1.78 μM of NaHCO₃ (657,000 counts), 0.1 ml. of enzyme. Total volume 1.4 ml. Incubated 1000 seconds at 37°.

### Table II

**Substrate Specificity of Phosphoryl-enolpyruvate Carboxylase Reaction**

<table>
<thead>
<tr>
<th>Counts in β-carboxyl of OA per tube.</th>
<th>Experiment A</th>
<th>Experiment B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate (6 μM)</td>
<td>5,570</td>
<td>2,530</td>
</tr>
<tr>
<td>&quot; (6 μM) + ATP (6 μM)</td>
<td>5,380</td>
<td>4,610</td>
</tr>
<tr>
<td>Phosphoryl-enolpyruvate (6 μM)</td>
<td>202,000</td>
<td>203,000</td>
</tr>
<tr>
<td>&quot; (6 μM) + ADP (6 μM)</td>
<td>206,000</td>
<td>215,000</td>
</tr>
</tbody>
</table>

Reactions conditions as described for Table I except for an additional 0.5 μM of NaHCO₃ per tube. Other additions as indicated. Total volume 1.5 ml.
P402 Incorporation into Malate—Malic acid was isolated from a double portion of reaction mixture, as described for Table I. After acidification and removal of the precipitated protein as previously described, the mixture was lyophilized to dryness. A 200 mg. sample of dl-malic acid was added to the dried residue and the mixture extracted with 2.2 ml. of boiling acetone and filtered. Crystallization was induced by the addition of 7.5 ml. of CHCl₃ (21). The yield was 103 mg. (51 per cent), with a specific activity of 8 c.p.m. One recrystallization yielded 75 mg. of specific activity 1.6 c.p.m., m.p. 127-128°. Thus little or no malate accumulated, as only 320 counts had been fixed in malic acid compared to approximately 200,000 counts found in the oxalacetate when only a single portion of reaction mixture was used.

Location of C¹⁴ in β-Carboxyl of Oxalacetate Hydrazone—The total activity of the oxalacetate hydrazone was determined by wet combustion, collection of the CO₂ in KOH, and precipitation as BaCO₃. The carbonate was counted at infinite thickness in a 1 sq. cm. sample cup at a distance of 1.3 cm. from the Geiger-Müller tube. Activity in the β-carboxyl was determined by collection of the CO₂ liberated when a water solution of the hy-

by centrifugation. 200 mg. of OA and 100 ml. of a saturated solution of 2,4-dinitrophenylhydrazone in 2 N HCl were added. The reaction mixture was left at room temperature for 20 hours, during which time a copious precipitate of golden needles formed.

The hydrazones were collected by filtration and washed with 2 N HCl and a small amount of water. The yield was 231 mg. (49 per cent of theory), with a specific activity of 429 c.p.m. 202 mg. of the crystals were dissolved in approximately 3 ml. of ethyl acetate at room temperature (20), filtered, and recrystallized by the addition of approximately 3 ml. of hexane. The yield was 140 mg. (70 per cent), with a specific activity of 449 c.p.m. A second recrystallization yielded 102 mg. (73 per cent), with a specific activity of 433 c.p.m. A total of 99,000 counts was thus present in the oxalacetate hydrazones or 202,000 after correction for yield. This recovery is in good agreement with the values obtained by β-decarboxylation (Tables I and II).

Elemental analysis of the isolated hydrazones gave C 37.80, H 2.75, N 18.30 per cent; theory, C 38.50, H 2.57, N 17.95 per cent.

A 4.63 mg. sample of the hydrazone was dissolved in 10 ml. of freshly boiled water. Neutralization to pH 8 required 2.07 ml. of 0.0139 N KOH. The theory for oxalacetate 2,4-dinitrophenylhydrazone is 2.14 ml. and for the 2,4-dinitrophenylhydrazone of pyruvic acid, 1.24 ml. A 4.89 mg. sample of the hydrazone was dissolved in 10 ml. of water and heated at 100° for 40 minutes. Neutralization required 1.12 ml. of 0.0139 N KOH. The theory on the assumption that oxalacetate hydrazones had been decarboxylated to pyruvic hydrazones is 1.13 ml.

C¹⁴O₂ Incorporation into Malate—Malic acid was isolated from a double portion of reaction mixture, as described for Table I. After acidification and removal of the precipitated protein as previously described, the mixture was lyophilized to dryness. A 200 mg. sample of dl-malic acid was added to the dried residue and the mixture extracted with 2.2 ml. of boiling acetone and filtered. Crystallization was induced by the addition of 7.5 ml. of CHCl₃ (21). The yield was 103 mg. (51 per cent), with a specific activity of 8 c.p.m. One recrystallization yielded 75 mg. of specific activity 1.6 c.p.m., m.p. 127-128°. Thus little or no malate accumulated, as only 320 counts had been fixed in malic acid compared to approximately 200,000 counts found in the oxalacetate when only a single portion of reaction mixture was used.
drazone (32 mg. in 60 ml.) was refluxed for 40 minutes. All of the radioactivity in the hydrazone was liberated by boiling (Table III). Thus all of the activity fixed was located in the β-carboxyl of the oxalacetate hydrazone.

The aqueous hydrazone solution, after refluxing, was made 2 N with respect to HCl and stored overnight at 2°. Crystals which formed were collected by filtration and washed with a small quantity of 2 N HCl and water. A yield of 20 mg. or 73 per cent of theory for conversion of oxalacetate hydrazone to pyruvic hydrazone was obtained. The melting point was 217–218°, and the mixed melting point with authentic pyruvic hydrazone was 217–218°. As was expected, the product had no detectable radioactivity. The oxalacetate 2,4-dinitrophenylhydrazone thus resembles that of acetoacetate with respect to ease of decarboxylation (20).

### Table III

<table>
<thead>
<tr>
<th>Oxalacetate hydrazone</th>
<th>Treatment</th>
<th>Total counts in BaCO₃</th>
<th>C.p.m. per mg. hydrazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.92</td>
<td>Wet combustion</td>
<td>11,000</td>
<td>2800</td>
</tr>
<tr>
<td>3.85</td>
<td>&quot;</td>
<td>11,700</td>
<td>3040</td>
</tr>
<tr>
<td>32.0</td>
<td>β-Decarboxylation</td>
<td>93,400</td>
<td>2920</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The accompanying reaction mechanism is proposed for the carboxylation of PEP to OA with the liberation of inorganic phosphorus. As here

\[ \text{COOH} \quad \text{COOH} \quad \text{COOH} \quad \text{COOH} \]

\[ \text{C—O—PO₃H₂ + CO₂ } \to \text{C—O—PO₃H₂ } \to \text{C—OH + H₃PO₄ } \Rightarrow \text{C=O} \]

\[ \text{CH₂} \quad \text{CH} \quad \text{CH} \quad \text{CH₂} \]

\[ \text{COOH} \quad \text{COOH} \quad \text{COOH} \quad \text{COOH} \]

postulated, the initial carboxylation product would be phosphoryl-enoloxalacetate, which upon hydrolysis would yield enoloxalacetate. Since there is as yet no direct evidence for the existence of phosphoryl-enoloxalacetate, the above mechanism must be regarded as strictly provisional.

**SUMMARY**

An enzyme has been found in extracts of spinach leaves which catalyzes the carboxylation of phosphoryl-enolpyruvate. Under the assay conditions employed, oxalacetate and inorganic phosphorus are the reaction products. Pyruvate plus adenosinetriphosphate does not replace phosphoryl-
enolpyruvate in this system. Malate does not accumulate in the reaction, which thus appears distinct from the reaction catalyzed by the malic enzyme. A possible relationship to the Wood and Werkman reaction in which pyruvate is carboxylated to oxalacetate is discussed.

BIBLIOGRAPHY

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J. Biol. Chem. 1953, 204:781-786.

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