BIOSYNTHESIS OF DICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION

II. FURTHER STUDY OF THE PROPERTIES OF THE "MALIC" ENZYME OF PIGEON LIVER*

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The isolation, partial purification, and some of the properties of an enzyme from pigeon liver which catalyzes Reactions 1 and 2 have already been described (1).

\[
\begin{align*}
(1) & \quad l\text{-Malate} + \text{TPN}_{\text{ox}} \xrightleftharpoons{\text{Mn}^{++}} \text{pyruvate} + \text{CO}_2 + \text{TPN}_{\text{red}}. \\
(2) & \quad \text{Oxalacetate} \xrightleftharpoons{\text{Mn}^{++}} \text{pyruvate} + \text{CO}_2
\end{align*}
\]

This enzyme is provisionally referred to as "malic" enzyme. The present paper deals with further observations on the kinetics and other properties of the purified enzyme.¹

The enzyme is totally inactive in the absence of Mn++. Mg++ can replace Mn++ but is less effective. Further evidence has been obtained indicating that free oxalacetate is not an intermediate in Reaction 1 and that both Reactions 1 and 2 are catalyzed by one and the same enzyme. Additional evidence has also been obtained showing that the dismutation between malate and pyruvate (2, 3), represented by Reaction 3, is catalyzed

\[
\begin{align*}
(3) & \quad l\text{-Malate} + \text{pyruvate} \xrightleftharpoons{\text{Mn}^{++}, \text{TPN}} \text{pyruvate} + \text{CO}_2 + \text{lactate}
\end{align*}
\]

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† On leave of absence from the Department of Biochemistry, College of Medicine, University of Sao Paulo, Brazil.

¹ Solid line arrows (→) will be used to indicate ready reversibility of a reaction; one solid and one dash arrow (--->) to indicate a reversible reaction which occurs predominantly in the direction of the solid arrow; and one solid and one dotted arrow (---) to indicate that, while the reaction is probably reversible, progress in the direction of the dotted arrow has not been demonstrated. TPN = triphosphopyridine nucleotide; DPN = diphosphopyridine nucleotide.
by a combination of "malic" enzyme and lactic dehydrogenase. As would be expected from the dependence of Reaction 1 on TPN, cytochrome c is rapidly reduced by l-malate in a system containing pigeon liver "malic" enzyme, cytochrome reductase (4), Mn++, and TPN.

Further work on the biosynthesis of malate from pyruvate and CO₂, with purified pigeon liver "malic" enzyme, is presented in Paper III (5).

Results

Preparation and Purity of Enzyme—The enzyme was prepared as previously described (1). The combined extracts of 600 gm. of acetone powder contained 980,000 units of specific activity about 5. The final preparation contained 49,600 units of specific activity 500. The yield was thus 5 per cent. It has since been found that, by carrying out the entire procedure on smaller batches (100 gm.) of acetone powder, a similar degree of purification is reached with fewer steps and much higher yields. Preparations of specific activity 600 to 700 with 25 to 30 per cent yield have been obtained in this way.² The fraction of specific activity 500 was dialyzed at 0° against 0.01 M phosphate buffer, pH 7.4, and lyophilized. This resulted in a decrease of the specific activity of the enzyme to 350; the activity of the dry powder, which was stored at 0° in a vacuum over calcium chloride, dropped gradually to about 100 over a period of several months. Solutions of this powder were used for the experiments reported in this paper.

Data illustrating the more or less complete removal of several enzymes originally present in the acetone powder extract are shown in Table I. Similar data have already been presented (1), but those of Table I are more complete; they include glutamic dehydrogenase activity, which had not been previously determined, and activity tests of the various contaminating enzymes at each major step in the purification of the "malic" enzyme. The test for "malic" enzyme (Reaction 1) and oxalacetic carboxylase (Reaction 2) activity were carried out as described above Tables II and III respectively. The optical tests for the other enzymes were as previously described (3). As can be seen from the ratio of other enzymes to "malic" enzyme shown in Table I, both glutamic and isocitric dehydrogenases were completely removed and malic dehydrogenase to a considerable extent. However, the removal of lactic dehydrogenase was still incomplete. We should like to emphasize again the remarkable constancy of the ratio of oxalacetic carboxylase to "malic" enzyme activity (O/E ratio, Table I) throughout purification. This ratio is higher than that previously reported (1) because at pH 4.5, at which the oxalacetic carboxylase tests are now being carried out, the activity of the enzyme is about 20 per cent higher

than at pH 5.0. In the more recent preparations referred to above,\textsuperscript{2} of specific activity 700, the O/E ratio was also 1.2; i.e., it remained constant over a 140-fold purification. These preparations also showed a more complete removal of malic and lactic dehydrogenase, the ratio of malic dehydrogenase to “malic” enzyme averaging 0.15 and that of the lactic dehydrogenase to “malic” enzyme averaging 0.6.

The parallel loss of “malic” enzyme and oxalacetic carboxylase activity

**Table I**

*Activity of Various Enzymes at Different Purification Stages of Pigeon Liver “Malic” Enzyme*

The figures in parentheses represent the number of tests averaged.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>“Malic” enzyme</td>
<td>Oxal-</td>
</tr>
<tr>
<td></td>
<td>(E)</td>
<td>acetic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carboxy-</td>
</tr>
<tr>
<td>Aqueous extract\textsuperscript{†}</td>
<td>5</td>
<td>6.6</td>
</tr>
<tr>
<td>1st ethanol fractionation</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Refractionated with ethanol\textsuperscript{‡}</td>
<td>48</td>
<td>66</td>
</tr>
<tr>
<td>Eluted from alumina gel\textsuperscript{§}</td>
<td>190 (5)</td>
<td>236 (2)</td>
</tr>
<tr>
<td>Refractionated with ethanol and alumina gel\textsuperscript{§}</td>
<td>350 (5)</td>
<td>435</td>
</tr>
</tbody>
</table>

* Tested in the presence of added TPN.
† No glucose dehydrogenase found at levels tested.
‡ Solution of lyophilized enzyme.

when the purified enzyme is heated reinforces the evidence that both activities reside in the same protein. Thus, a dilute solution of enzyme in 0.004 M phosphate buffer, pH 7.4, lost half of its “malic” and half of its oxalacetic carboxylase activity when heated for 5 minutes at 50°; both activities vanished when heated for the same time at 60°. In both cases the lactic dehydrogenase activity of the preparation remained unchanged.

When tested at their respective pH optima and at approximately the same temperature in the presence of optimum substrate concentrations, the rates of the forward Reactions 1 and 2 (per mg. of enzyme protein) are nearly identical. If the extinction coefficient of TPN\textsubscript{red.} (6) is taken as ε
6.22 \times 10^6 \text{ (sq. cm. \times mole}^{-1} \text{), 1 enzyme unit, as previously defined (1), corresponds to an early rate of reduction of TPN of 0.0048 \times 10^{-6} \text{ mole per minute at 25° under the conditions of our standard test. A "malic" enzyme specific activity of 700 corresponds then to a rate of reduction of TPN, or a rate of evolution of CO}_2, of 700 \times 0.0048 \times 10^{-6} = 3.36 \times 10^{-6} \text{ mole per minute per mg. of protein. The same preparation has an oxalacetic carboxylase specific activity of 840; i.e., it catalyzes the evolution of 84 c.mm. (or 3.75 \times 10^{-6} \text{ mole}) of CO}_2 from oxalacetate per minute per mg. of protein at 25°. The above values correspond to a rate of conversion of about 350 moles of malate or oxalacetate per 100,000 gm. of protein per minute. This relatively low degree of activity may indicate that our preparations of pigeon liver "malic" enzyme are still rather impure.}

**Effect of pH**—The pH-activity curves of Reactions 1 and 2 are shown in Fig. 1. In determining the activity of oxalacetic carboxylase at pH values above 5.1, complete evolution of the CO}_2 produced by decarboxylation was insured by tipping acid from the side bulb of the Warburg vessels in the usual manner. Both reactions have sharp pH optima. The optimum for Reaction 1 is at pH 7.5, while that for Reaction 2 is at pH 4.5. It is clear from the curves that at pH 7.5 the enzyme catalyzes Reaction 1 exclusively, whereas at pH 4.5 only Reaction 2 is catalyzed. This shows that free oxalacetate cannot be an intermediate in Reaction 1. This conclusion is further supported by the fact that, whereas malonate strongly inhibits decarboxylation of oxalacetate by the pigeon liver enzyme, it does not significantly inhibit Reaction 1 at similar concentrations (see also Korkes et al. (7)). The final proof that free oxalacetate is not an intermediate in Reaction 1 was provided by experiments with C^{14}O}_2 (8). Through Reaction 1, C^{14}O}_2 is readily incorporated in the carboxyl of malate $\beta$ to the alcoholic group, but little or no isotope is incorporated into oxalacetate if this compound is present along with the components of the reaction.2

**Kinetics**—Although Reaction 1 is readily reversible (1), its equilibrium position is so far to the right that, under the conditions of the standard optical test, it proceeds until practically all of the TPN is reduced. It has not yet been possible to determine the equilibrium constant because of the slight contamination of the enzyme with lactic dehydrogenase (1). The forward reaction follows roughly first order kinetics with respect to TPN until about 70 to 80 per cent of the nucleotide is reduced; at this point it becomes slower than first order. As is shown in Table II, the first order velocity constant, up to 80 per cent TPN reduction, is proportional to the enzyme concentration over a 5-fold range of enzyme dilutions.

While the spontaneous decarboxylation of oxalacetate is kinetically of first order (9, 10), the enzymatic decarboxylation follows zero order kinetics
as long as the enzyme is saturated with substrate (Table III). This is understandable if, in accordance with the Michaelis-Menten theory, the velocity of enzymatic decarboxylation of oxalacetate is proportional to the concentration of an enzyme-Mn-oxalacetate complex.

Within the range of enzyme concentrations illustrated in Table III the

![Fig. 1. pH-activity curves of pigeon liver enzyme. Standard tests for “malic” enzyme (Curve 1) and oxalacetic carboxylase (Curve 2) activity except that, in the latter case, 2.5 μM of MnCl₂ and 76 μM of oxalacetate were present. Final volume, 2.0 cc.; temperature, 23° and 25° respectively. The manometric determination of oxalacetic carboxylase activity at pH values above 5.1 was carried out as described in the text. The buffers used were as follows: pH 3.8 to 5.1, acetate; pH 5.5 to 6.7, citrate; pH 7.0 to 8.5, veronal. At pH 7.5 veronal and glycylglycine buffers gave identical results for “malic” enzyme activity. Final concentration of buffer, 0.025 M in the “malic” enzyme tests and either 0.1 M acetate or citrate or 0.05 M veronal in the oxalacetic carboxylase tests. Freshly prepared solutions of oxalacetic acid were brought to the desired pH with dilute, CO₂-free sodium hydroxide. The pH values plotted are initial ones. Except in the case of the oxalacetic carboxylase tests, in which the final pH values were about 0.2 unit higher, the actual pH values of the reaction mixtures (as determined at the end with the glass electrode) agreed well with the calculated ones. Lyophilized enzyme; specific activity (“malic” enzyme) at time of use, 325.

concentration of oxalacetate needed to saturate the enzyme has been found to be about $1.4 \times 10^{-2}$ M. The drop in the rate of CO₂ evolution after 20 and 15 minutes in the samples with 0.081 and 0.121 mg. of enzyme protein, respectively, is due mainly to the oxalacetate concentration falling below the saturation level. Since oxalacetate also undergoes spontaneous decarboxylation, the concentration of the β-keto acid in the experiments of Table III, at the time when the rate of CO₂ evolution dropped, was prob-
ably below 1.3 \times 10^{-2} \text{ m}. Table III shows that, under the conditions chosen, the rate of CO_2 evolution during the first 15 minutes is proportional to the enzyme concentration over a 7-fold range of enzyme dilutions.

In previous experiments (1) the effect of metallic ions on Reactions 1 and 2 had been studied with relatively crude enzyme fractions of specific activity 100 to 150. Although markedly activated by addition of metal, these preparations were not inactive in its absence. With purer enzyme preparations, addition of Mn^{++} or Mg^{++} is indispensable for activity of both Reaction 1 and 2. Fig. 2 illustrates the dependence of enzyme activity on the concentration of either Mn^{++} or Mg^{++}. Half maximum rates of Reaction 1 and 2 are reached in each case when the concentration of Mn^{++} is about 5 \times 10^{-6} \text{ m}. In the case of Mg^{++} the half saturation concentration for Reaction 1 is about 5 \times 10^{-4} \text{ m}. The curve expressing the dependence of Reaction 2 on the concentration of Mg^{++} is anomalous, owing perhaps to excessive complex formation between Mg^{++} and oxalacetate at the higher concentration of cation required in this case.

The affinity of the pigeon liver "malic" enzyme for l-malic acid is very high. Fig. 3 illustrates the dependence of the rate of Reaction 1 in the forward direction on the concentration of l-malic acid. Half maximum rate is reached when the malate concentration is 5 \times 10^{-5} \text{ m}. Thus, the

### Table II

**Rate of Reduction of TPN by l Malic Acid As Function of "Malic" Enzyme Concentration**

Standard optical test, 0.025 M glycylglycine buffer, pH 7.4, 3 \mu M of MnCl_2, 0.135 \mu M of TPN_{ox}, 1.5 \mu M of l-malate, enzyme, and water. Final volume, 3.0 cc. Corex or silica cells, 1.0 cm. diameter. No TPN in control cell. Reaction started by addition of enzyme. Temperature, 23\degree. Lyophilized enzyme; specific activity at time of use, 240. Protein values corrected to correspond to initial specific activity of fraction (500) before lyophilization. Initial TPN_{ox} concentration, c_0 = 0.038 \times 10^{-4} \text{ mole per cc.}

<table>
<thead>
<tr>
<th>Enzyme concentration</th>
<th>k^*</th>
<th>(k) Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg. protein per cc.</td>
<td>min.^{-1}</td>
<td></td>
</tr>
<tr>
<td>0.0024</td>
<td>0.18</td>
<td>75.0</td>
</tr>
<tr>
<td>0.0034</td>
<td>0.22</td>
<td>64.8</td>
</tr>
<tr>
<td>0.0050</td>
<td>0.36</td>
<td>72.0</td>
</tr>
<tr>
<td>0.0068</td>
<td>0.49</td>
<td>72.0</td>
</tr>
<tr>
<td>0.0102</td>
<td>0.68</td>
<td>66.5</td>
</tr>
</tbody>
</table>

* First order velocity constant, \(k = \frac{1}{t} \ln \left(\frac{c_0}{c_t}\right)\), where \(t\) = time in minutes, \(c_0 = \text{initial TPN}_{ox}\) concentration, \(c_t = \text{TPN}_{ox}\) concentration at time \(t\). Extinction coefficient of TPN_{red.} at 340 \mu taken as \(e = 6.22 \times 10^6\) (sq. cm. \times mole^{-1}) (6).
dissociation constant of the enzyme-l-malic acid and the enzyme-Mn complex is the same. The spontaneous decarboxylation of oxalacetic acid makes it difficult to obtain an accurate estimate of the half saturation and saturation values of the protein-oxalacetic acid complex in Reaction 2. As stated above, the latter value was found to be in the neighborhood of $1.4 \times 10^{-3}$ mole of oxalacetate per liter; the half saturation value is about $10^{-3}$ mole per liter.

### Table III

**Rate of Decarboxylation of Oxalacetic Acid by Pigeon Liver Enzyme As Function of Enzyme Concentration**

Standard manometric test, 0.1 M acetate buffer, pH 4.5, 1.0 $\mu$M of MnCl$_2$, 0.083 $\mu$M of TPN, and 19 $\mu$L (425 c.mm.) of oxalacetate ($1.9 \times 10^{-2}$ M). Final volume, 1.0 cc. Gas, air; temperature, 25°. Same enzyme as in Table II. Protein values corrected in the same way. Reaction started by tipping in enzyme from side bulb of small Warburg vessels (capacity, about 6 cc.) after temperature equilibration. Measurement of CO$_2$ evolution started at 5 minutes following addition of enzyme to reaction mixture. CO$_2$ evolution due to enzyme obtained by subtracting that due to spontaneous decarboxylation of oxalacetate which was measured in parallel runs with neither enzyme nor TPN.

<table>
<thead>
<tr>
<th>Enzyme concentration, mg. protein per cc.</th>
<th>0.0162</th>
<th>0.0405</th>
<th>0.061</th>
<th>0.121</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min.)</td>
<td>CO$_2$ evolution</td>
<td>CO$_2$</td>
<td>CO$_2$ evolution</td>
<td>CO$_2$</td>
</tr>
<tr>
<td></td>
<td>c.mm.</td>
<td>c.mm.</td>
<td>c.mm.</td>
<td>c.mm.</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>1.0</td>
<td>10.0</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>10.0</td>
<td>1.0</td>
<td>22.0</td>
<td>2.2</td>
</tr>
<tr>
<td>15</td>
<td>15.0</td>
<td>1.0</td>
<td>32.0</td>
<td>2.13</td>
</tr>
<tr>
<td>20</td>
<td>21.0</td>
<td>1.05</td>
<td>42.0</td>
<td>2.1</td>
</tr>
<tr>
<td>25</td>
<td>26.0</td>
<td>1.04</td>
<td>51.0</td>
<td>2.04</td>
</tr>
</tbody>
</table>

| CO$_2$ / $t \times$ mg. protein           | 61.5    | 52.0   | 52.5*    | 50.0†  |

* Calculated for 20 minutes.
† Calculated for 15 minutes.

**Inhibitors of Oxalacetate Decarboxylation**—It was previously reported (11) that malate strongly inhibits the enzymatic decarboxylation of oxalacetate by crude pigeon liver preparations. This inhibition has now been studied with the purified enzyme; typical results are illustrated in Table IV. It is apparent that $d$-malate is as inhibitory as $l$-malate, whereas, as previously noted, fumarate is practically non-inhibitory. Malonate is a stronger inhibitor than is malate. Evans et al. (12) first noticed mala-
nate inhibition of the decarboxylation of oxalacetate by pigeon liver extracts. Later Lwoff et al. (13) observed with intact cells of a mutant strain of Moraxella lwofii, that malonate (as well as malate) inhibited the decarboxylation of oxalacetate but not the oxidative decarboxylation of malate, and interpreted this observation to mean that oxalacetate was not an intermediate in the latter reaction. Although malate, malonate, and succinate may be regarded as structural inhibitors of the enzymatic

![Figure 2](http://www.jbc.org/)

**Fig. 2.** "Malic" and oxalacetic carboxylase activities of pigeon liver enzyme as a function of the concentration of Mn++. Standard tests for "malic" enzyme and oxalacetic carboxylase activity. Curve 1, (○) "malic" activity with Mn++, (●) "malic" activity with Mg++, (▲) oxalacetic carboxylase activity with Mn++; Curve 2, (△) oxalacetic carboxylase activity with Mg++. Same enzyme as in Fig. 1.

**Fig. 3.** "Malic" enzyme activity as a function of the concentration of L-malic acid. Standard tests (each with 8 enzyme units). Specific activity of enzyme at time of use, 260.

decarboxylation of oxalacetate, the inhibition seems to be non-competitive, since it is not lessened by increasing the concentration of substrate.

**Malate-Pyruvate Dismutation**—Evidence has already been presented (3) that the malate-pyruvate dismutation (Reaction 3) is catalyzed by "malic" enzyme together with lactic dehydrogenase, and it was pointed out that this TPN-linked reaction is possible because of the fact that lactic dehydrogenase can react with TPN, although at a much lower rate than with DPN. The earlier experiments with purified pigeon liver "malic" enzyme, carried out at pH 5.2, definitely showed that the amount of lactic dehydrogenase contaminating the preparation was not sufficient for maxi-
Table IV

Inhibitors of Decarboxylation of Oxalacetic Acid by Pigeon Liver Enzyme

Conditions as in Table III, except that some experiments were carried out at pH 5.2. Final concentration of oxalacetate, 0.019 M. Enzyme activity (corrected for spontaneous decarboxylation of oxalacetate) calculated for the period between 5 and 10 minutes after tipping in the enzyme. Activity in the absence of inhibitor taken as 100.

<table>
<thead>
<tr>
<th>Initial pH</th>
<th>Inhibitor</th>
<th>Relative activity</th>
<th>Inhibition per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>0.0025 M l-malate</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>4.5</td>
<td>0.005 &quot; &quot;</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>4.5</td>
<td>0.005 &quot; dl-malate&quot;</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>5.2</td>
<td>0.001 &quot; l-malate&quot;</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>5.2</td>
<td>0.005 &quot; &quot;</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>5.2</td>
<td>0.01 &quot; &quot;</td>
<td>37</td>
<td>63</td>
</tr>
<tr>
<td>5.2</td>
<td>0.01 &quot; dl-malate&quot;</td>
<td>37</td>
<td>63</td>
</tr>
<tr>
<td>5.2</td>
<td>0.02 &quot; d-malate&quot;</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>5.2</td>
<td>0.01 &quot; malonate&quot;</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>5.2</td>
<td>0.01 &quot; succinate&quot;</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>5.2</td>
<td>0.01 &quot; fumarate&quot;</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>5.2</td>
<td>0.01 &quot; acetoacetate&quot;</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>5.2</td>
<td>0.01 &quot; pyruvate&quot;</td>
<td>92</td>
<td>8</td>
</tr>
</tbody>
</table>

Table V

Dismutation between L-Malate and Pyruvate with "Malic" Enzyme and Lactic Dehydrogenase at pH 7.4

The complete samples contained 0.05 M glycylglycine buffer, pH 7.4, 0.135 μM of TPN, 2.5 μM of MnCl₂, 30 μM (672 c.mm.) of L-malate, 60 μM of pyruvate, lactic dehydrogenase (6000 units), and "malic" enzyme of specific activity 325 at time of use (245 units in Experiment 1; 225 units in Experiment 2). Final volume, 2.0 cc.; gas, air; temperature, 25°. Reaction started by tipping in "malic" enzyme from the side bulb of Warburg vessels after temperature equilibration. CO₂ evolution determined by tipping in sulfuric acid at the end of the experiment under correction for the CO₂ present initially in control samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CO₂ evolution in 30 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Complete</td>
<td>c.mm.</td>
</tr>
<tr>
<td>No lactic dehydrogenase</td>
<td>322</td>
</tr>
<tr>
<td>Complete</td>
<td></td>
</tr>
<tr>
<td>No &quot;malic&quot; enzyme</td>
<td></td>
</tr>
<tr>
<td>&quot; malate</td>
<td></td>
</tr>
<tr>
<td>&quot; pyruvate</td>
<td></td>
</tr>
<tr>
<td>&quot; TPN, 0.15 μM DPN</td>
<td></td>
</tr>
</tbody>
</table>
mum rates of dismutation, since increased rates were obtained on addition of lactic dehydrogenase. However, when it was found that at pH 5.2 the rate of Reaction 1 is but a small fraction of that at pH 7.4, a repetition of the dismutation experiments at the latter pH was desirable. The results, illustrated in Table V, clearly show that both “malic” enzyme and lactic dehydrogenase are required.

Reaction with Cytochrome c—The reduction of ferricytochrome c by the “malic” enzyme system in the presence of cytochrome reductase (4) is illustrated in Figs. 4 and 5. Fig. 4 shows the effect of the concentration

Fig. 4. Reduction of cytochrome c by l-malic acid in the presence of “malic” enzyme, TPN, and cytochrome reductase. 0.025 M glycyl-glycine buffer, pH 7.4, 1.5 μM of MnCl₂, 0.0067 μM of TPN, 1.5 μM of l-malate, 0.07 μM of cytochrome c, and varying amounts of cytochrome reductase and pigeon liver enzyme (specific activity at time of use, 100). Final volume, 1.5 cc.; gas, air; temperature, 22°. Corex cells, 0.5 cm. diameter. Wave-length, 550 mμ. Curves 1 to 4, reaction started by addition of 3.75 units of “malic” enzyme; Curve 5, reaction started by addition of 0.75 unit of “malic” enzyme. Cytochrome reductase; Curve 1, 10 μ; Curve 2, 25 μ; Curve 3, 50 μ; Curves 4 and 5, 75 μ. Curve 6, all components but one present at zero time; at 2 minutes, system completed by addition of either 3.75 units of “malic” enzyme (▲), 75 μ of cytochrome reductase (O), l-malate (Δ), or TPN (□).

Fig. 5. Rate of reduction of cytochrome c by l-malic acid as a function of the TPN concentration. Samples with 3.75 units of “malic” enzyme, 75 μ of cytochrome reductase, and varying amounts of TPN; otherwise as in Fig. 4. Reaction started by addition of “malic” enzyme.
of reductase and "malic" enzyme on the rate of the reaction (Curves 1 to 5), as well as the indispensability of each of the components of the system (Curve 6). Fig. 5 illustrates the dependence of the reaction rate on the concentration of TPN. The latter data indicate the suitability and high sensitivity of this system for the quantitative assay of TPN, since amounts of TPN as low as 0.02 μ can be accurately and specifically determined. A method of TPN assay by use of the glucose-6-phosphate dehydrogenase-cytochrome reductase-cytochrome c system has been described by Haas et al. (14). Cytochrome c was prepared by the method of Keilin and Hartree (15). The sample of cytochrome reductase was one previously used (16), kindly supplied by Dr. Kurt I. Altman.

**DISCUSSION**

The results presented in this paper support the view that Reaction 1 is catalyzed either by a single enzyme or by enzymes which are so closely associated as to form a single functional unit. The term "malic" enzyme is used to avoid confusion of this type of enzyme, which catalyzes a reversible oxidative decarboxylation of L-malic acid by pyridine nucleotide, with malic dehydrogenase, which catalyzes a reversible oxidation of L-malic acid by pyridine nucleotide (Reaction 4). The TPN-specific "malic" enzyme of pigeon liver does not catalyze the reduction of oxalacetate by

\[
\text{TPN}_{\text{red.}} + \text{DPN}_{\text{red.}} \rightarrow \text{L-malate} + \text{DPN}_{\text{ox.}} \quad \text{or} \quad \text{TPN}_{\text{ox.}}
\]

within the range pH 4.5 to 7.5 and, therefore, is not a TPN-malic dehydrogenase. In fact, at the optimum pH for catalysis of Reaction 1 (pH 7.5) the "malic" enzyme is neither a malic dehydrogenase nor an oxalacetic carboxylase, since at this pH it also fails to catalyze the decarboxylation of oxalacetate. It may be again emphasized that a mixture of Mn++, DPN, or TPN, purified malic dehydrogenase of pig heart, and purified oxalacetic carboxylase of *Micrococcus lysodeikticus*, each enzyme being devoid of "malic" enzyme activity, fails to catalyze Reaction 1 in either direction (1, 3). Little interaction between these two enzymes systems would be expected if the intermediate substrate

\[\text{Note added at proof correction.} \quad \text{Herbert (20) has recently succeeded in purifying the } M. \text{ lysodeikticus oxalacetic carboxylase (which catalyzes Reaction 2 from left to right) to a very high degree. With a combination of this highly active enzyme, malic dehydrogenase, and DPN he was able to obtain oxidative decarboxylation of malate. This positive result was undoubtedly made possible by the use of large amounts of carboxylase. Nevertheless, under similar conditions he failed to obtain the reverse reaction, i.e. reductive carboxylation of pyruvate, as measured by oxidation of DPN}_{\text{red.}} \text{ in the presence of high concentrations of pyruvate and CO}_2 \text{ (Society for Experimental Biology, Symposium on carbon dioxide fixation, Sheffield, July 1-5, 1950).}\]
(oxalacetate) were present at any time at concentrations much lower than is required to half saturate each enzyme. That such may be the case for the particular enzyme combination under discussion is not surprising, because, as is well known, the equilibrium position of the malic dehydrogenase system is far in the direction of reduction of oxalacetate to malate, while that of the oxalacetic carboxylase system is so far in the direction of decarboxylation of oxalacetate that there is at present no direct proof of the reversibility of this reaction (8).

As an alternative to the earlier hypothesis that enzyme-bound oxalacetate would be an intermediate in Reaction 1 (1), the "malic" enzyme might be assumed to catalyze the reaction as indicated in Reaction 5. A somewhat similar explanation was proposed by Weil-Malherbe (17) for the oxidative decarboxylation of α-ketoglutaric acid.

\[
\begin{align*}
\text{C-CH}_2\text{-C-COOH} + \text{TPN}^+ & \rightleftharpoons \text{C-CH}_2\text{-C-COOH} + \text{TPNH} + \text{H}^+ \\
\text{l-Malic acid} & \rightleftharpoons \text{Oxalacetic hydrate lactone} \\
\text{CO}_2 + \text{CH}_2\text{C(OH)-COOH} & \longrightarrow \text{Enol-pyruvic acid}
\end{align*}
\]

The reaction catalyzed by the "malic" enzyme is analogous in many respects to the reversible oxidative decarboxylation of d-isocitric acid to α-ketoglutaric acid and CO₂. Although the latter reaction was provisionally assumed to be catalyzed by the interaction of two enzymes, isocitric dehydrogenase and oxalosuccinic carboxylase (18), there are indications that only one enzyme, similar in nature to the "malic" enzyme, may be involved (19).

**SUMMARY**

1. Further evidence is presented indicating that one single enzyme or functional enzyme unit is involved in the catalysis of the reversible oxidative decarboxylation of l-malic acid and the decarboxylation of oxalacetic acid by highly purified preparations of the "malic" enzyme of pigeon liver. The former reaction has a sharp optimum at pH 7.5, but falls to zero at pH 4.5. The latter reaction has a sharp optimum at pH 4.5, but falls to zero at pH 7.5. At pH 7.5 the enzyme catalyzes neither the decarboxylation of oxalacetate nor its reduction by TPN. Thus at neutral pH the enzyme is neither an oxalacetic carboxylase nor a malic dehydrogenase, and free oxalacetate cannot be an intermediate in the reversible oxidative decarboxylation of malate.
2. The presence of Mn++ is indispensable for activity in each of the reactions catalyzed by the "malic" enzyme; Mg++ is much less effective than Mn++. The saturation and half saturation values of the protein-Mn++, protein-malate, and protein-oxalacetate complexes have been determined. The affinity of the enzyme for D-malic acid is very high.

3. The enzymatic decarboxylation of oxalacetic acid is strongly inhibited by both D- and L-malate and by malonate.

4. Further evidence is presented in support of the previous finding that the TPN-linked dismutation between L-malate and pyruvate, to yield pyruvate, CO₂, and lactate, is catalyzed by the pigeon liver "malic" enzyme plus lactic dehydrogenase.

5. The reduction of cytochrome c by L-malic acid, in the presence of "malic" enzyme, Mn++, TPN, and cytochrome reductase, is described.

6. The nature of the "malic" enzyme is discussed in the light of the above and previous findings.

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The various preparations used were obtained as described in previous papers (1, 3).

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BIOSYNTHESIS OF DICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION: II. FURTHER STUDY OF THE PROPERTIES OF THE "MALIC" ENZYME OF PIGEON LIVER
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