INTERRELATIONSHIPS OF OXALACETIC AND L-MALIC ACIDS IN CARBON DIOXIDE FIXATION*

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Studies of the incorporation of CO₂ into the dicarboxylic acids have centered on two reactions: (a) the reversible oxidative decarboxylation of l-malic acid (Reaction 1) as demonstrated in extracts of pigeon liver (1) and plants (2), and (b) the incorporation of isotopic CO₂ into oxalacetate during its decarboxylation (Reaction 2) by bacteria (3) or extracts of pigeon liver (4).

\[
\text{"malic enzyme"} \\
\text{l-Malate + TPN}_{\text{ox}} \xrightarrow{\text{enzyme}} \text{CO}_2 + \text{pyruvate + TPN}_{\text{red}}. \quad (1)
\]

\[
\text{"oxalacetate carboxylase"} \\
\text{Oxalacetate} \xrightarrow{\text{enzyme}} \text{CO}_2 + \text{pyruvate} \quad (2)
\]

Reaction 1 requires TPN, as shown, while the incorporation of CO₂ into oxalacetate during Reaction 2 is catalyzed by ATP in pigeon liver extracts (4, 5), although not in bacterial preparations (6).

The relationship between the two reactions has been obscure, although some information is available. It has been reported that ATP has no effect on Reaction 1 (1) and that TPN cannot replace ATP in Reaction 2 (5). Ochoa et al. (1) believe that it is unlikely that Reaction 1 is a summation of Reaction 2 and an oxidation-reduction reaction similar to that catalyzed by malic dehydrogenase, since oxalacetate cannot be substituted for pyruvate and CO₂ in reversing Reaction 1.

Recently Veiga Salles et al. (7) suggested that Reaction 1 may be the primary reaction for CO₂ fixation in dicarboxylic acids and that the reversal

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1 The following abbreviations have been used throughout: TPN = triphosphopyridine nucleotide, DPN = diphosphopyridine nucleotide, ATP = adenosinetriphosphate, DPNase = diphosphopyridine nucleosidase.

2 Utter, M. F., unpublished results.
of Reaction 2 may be a summation of Reaction 1 and the action of malic dehydrogenase action as shown below:\(^3\)

\[
\begin{align*}
C^*O_2 + \text{pyruvate} + \text{TPN}_{\text{red.}} & \rightleftharpoons \text{malate}^* + \text{TPN}_{\text{ox.}} \quad (1, a) \\
\text{Malate}^* + \text{TPN}_{\text{ox.}} & \rightarrow \text{malic dehydrogenase} \\
& \rightarrow \text{oxalacetate}^* + \text{TPN}_{\text{red.}} \quad (3)
\end{align*}
\]

\[
C^*O_2 + \text{pyruvate} \rightleftharpoons \text{oxalacetate}^* \quad (1, a + 3)
\]

By this proposed mechanism CO\(_2\) would enter oxalacetate by way of malate, although the malate need be present in catalytic amounts only.

The foregoing suggestion had the following experimental bases: (a) purified "malic enzyme" fixed CO\(_2\) in oxalacetate only when malic dehydrogenase was added, (b) catalytic amounts of malate replaced ATP in the fixation CO\(_2\) in oxalacetate by unpurified enzyme preparations under certain conditions, in accord with the idea that the function of ATP was linked to the formation of malate from endogenous materials, and (c) a non-correlation between the activity of oxalacetate carboxylases as measured by decarboxylation and by ability to fix CO\(_2\) in oxalacetate had been reported (8, 9). None of the above points constitutes direct evidence for the hypothesis that Reaction 1 is the primary fixation reaction in the incorporation of labeled CO\(_2\) in oxalacetate in the oxalacetate carboxylase reaction, although (a) demonstrates that oxalacetate may be formed via malate.

In the present investigation the relationship between oxalacetate and l-malate in CO\(_2\) fixation has been studied, with particular emphasis on the possible rôle of malate as a precursor of oxalacetate in the oxalacetate carboxylase reaction. The relative specific activities of the two acids have been determined under experimental conditions favoring Reaction 1 (i.e., the presence of TPN) and Reaction 2 (i.e., the presence of ATP), respectively.

The results do not support the thesis that malate is a precursor of oxalacetate in Reaction 2 or that oxalacetate is a precursor of malate in the reaction catalyzed by "malic enzyme."

Materials

Pigeon Liver Preparations—Acetone powders were prepared from pigeon liver as described previously (4). The powders deteriorate slowly even when kept cold and dry in so far as the reactions under study are concerned. For use the powder was mixed thoroughly with 8 parts of water or 0.02 M potassium phosphate buffer (pH 7.8) and extracted for 15 minutes at 38°C. After centrifugation, the supernatant was dialyzed for 5 to 6 hours against

\(^3\) The incorporation of a labeled C atom in the molecule is indicated without regard to the position by an asterisk in the above equations.
0.9 per cent potassium chloride at 5-7°. The dialyzed extract was stored overnight in the frozen state before use.

**Brain DPNase**—In some experiments, the extract was treated prior to dialysis with a preparation of mouse brain capable of destroying DPN and other substances. Brain DPNase was prepared by homogenizing one or more freshly excised brains in 10 volumes of 0.004 M phosphate buffer (pH 7.5), centrifuging for 15 minutes at 3000 r.p.m. in a Servall angle centrifuge, and washing the precipitate twice with 0.02 M phosphate buffer (pH 7.8). After the final centrifugation, the residue was mixed with the same amount of pigeon extract, incubated for 30 minutes at 38°, and the brain particles removed by centrifugation. The pigeon liver extract was then dialyzed as described.

**Reaction Components**—Oxalacetic acid was prepared from the commercial ethyl ester by hydrolysis (10) and recrystallized twice from acetone and benzene.

The l-malic acid used was obtained by two recrystallizations of the commercial acid from acetone and benzene.

The sodium salt of ATP was prepared from ATP isolated from rabbit muscle as the barium salt (11). In some experiments, a commercial sodium salt was used without demonstrable alteration of results.

TPN was isolated from pork liver by the chromatographic procedure described by LePage and Mueller (12). Spectrophotometric assay with Zwischenferment (13) indicated a purity of about 40 per cent.

**Methods**

**Procedure for Fixation Experiments**—Unless otherwise noted, the experiments were run in Warburg vessels with enzyme preparation in one side arm and NaHCO₃ in the other. The main chamber contained the other components, including oxalacetate neutralized to pH 6.2 to 6.4 with Na₂PO₄ just before use. After an N₂ atmosphere had been established in the vessel and temperature equilibrium attained, the contents of the side arm were tipped in, yielding a well buffered solution with a final pH of 7.2 to 7.3. At the end of the incubation period, the reaction was stopped by tipping in H₂SO₄ sufficient to give a concentration of about 0.1 N. After centrifugation the reaction mixture was analyzed as described in the next subsections.

**Determination and Degradation of Oxalacetate**—The decarboxylation of oxalacetate to CO₂ and pyruvate by Al⁺⁺⁺, as described by Krebs and Eggleston (14), was used for the determination of oxalacetate and also as a degradation procedure to obtain the carboxyl group β to the carbonyl group. In the latter process, the residual C³⁴O₂ was removed by a rapid passage of tank CO₂ through the mixture, followed by CO₂-free air. The
solution was then placed in vessels containing Al+++ and potassium acid phthalate in the side arms and a 2.5 N solution of carbonate-free NaOH in the center well, and the decarboxylation reaction carried out. The collected CO₂ was washed from the center well with dilute NaOH and plated directly on paper disks by a method similar to that described by Henriques et al. (15). Frequent control runs have shown that no appreciable C¹⁴O₂ remains after the rinsing procedure and also that the manometric and plating procedures give zero values under the conditions described when oxalacetate is omitted. In a few experiments in which it was desirable to assess the C¹⁴ content of the α-carboxyl of oxalacetate, the pyruvate obtained from the Al+++ decarboxylation was oxidized to CO₂ and acetic acid by ceric sulfate (16) in Warburg flasks and the CO₂ collected and plated as described above. This method is not specific when run on a reaction mixture following decarboxylation by Al+++ and yields CO₂ not only from pyruvate formed in the Al+++ reaction but also from the pyruvate formed previously, as well as from malate and lactate.

Degradation of l-Malate—In most of the experiments, the C¹⁴ content of the carboxyl group of malate β to the carbinol group was obtained by the use of the malate-adapted strain of Lactobacillus arabinosus described by Korkes and Ochoa (17). This process was preceded by a partial purification of malate by chromatographic means to remove other substances attacked by the cells (e.g. fumarate and pyruvate). An aliquot of the acidified and centrifuged reaction mixture was made 3 N with respect to H₂SO₄, heated in a boiling water bath for 25 minutes to destroy oxalacetate, and the solution incorporated into Celite (Johns-Manville, No. 535, 2 gm. per ml. of solution) which had previously been washed thoroughly with ether. The Celite mixture was packed into a small glass column with an inside diameter of 1.2 cm. above a base of 1 gm. of Celite made up with 0.5 ml. of 3 N H₂SO₄. Fumarate and pyruvate were eluted from the column with 5 per cent butanol in chloroform. With a 5 gm. column, 50 ml. of the eluant are sufficient to remove 100 μM each of fumarate and pyruvate. The malate was then eluted with ether (about 200 ml. per 100 μM). After the addition of a small amount of water, the ether was removed at room temperature with a jet of dry air and the water layer adjusted carefully to pH 4.5 with NaOH. The solution was then placed in a Warburg vessel containing 0.1 × 10⁻⁴ M Mn++, 1 ml. of the Lactobacillus suspended in 0.5 M phosphate buffer (pH 4.5), and 2.5 N NaOH in the center well. After replacement of the air with N₂, the malate was fermented to CO₂ and lactate and the CO₂ plated as BaCO₃.

In some cases, the α-carboxyl of the malate was obtained by an oxidation by Ce⁴⁺⁺⁺⁺ of the lactate resulting from the malate fermentation after acidification, boiling, and removal of the cells by centrifugation. Controls
without malate were always included in these degradations, but no significant amount of \( \text{BaCO}_3 \) results from endogenous sources with well washed cells. However, the \( \text{Ce}^{++} \) oxidation has a small blank value (0.5 to 1.5 mg. of \( \text{BaCO}_3 \)) due to oxidizable substances extracted from the cells. The blank values have been subtracted in all cases in which \( \text{Ce}^{++} \) oxidation of lactate has been performed.

In some experiments, the \( \alpha\text{-COOH} \) of malate was obtained directly as CO by treatment with concentrated H\(_2\)SO\(_4\) at 50° for 2 hours, followed by a gradual warming to 80°. The CO was converted to CO\(_2\) (18) and plated as BaCO\(_3\). The method is similar to that used by Weinhouse et al. (19) for obtaining the tertiary carboxyl of citrate. The malate degradation yields theoretical amounts of CO (measured as CO\(_2\)) from known samples of malate. A very small amount of CO\(_2\) is also obtained, presumably from the \( \beta\text{-COOH} \), but the CO\(_2\) can be removed prior to the oxidation of the CO.

The determination of malate by the \textit{Lactobacillus} fermentation method involves a possible loss in radioactivity from the \( \beta\)-carboxyl and a concomitant gain in the \( \alpha\)-carboxyl through randomization by equilibration with fumarate by the pathway shown below.

\[
\text{POOH-CHz.CHOH.COOH} \rightarrow \text{C\text{\textsuperscript*}OOH-CH:CH.C\text{\textsuperscript*}OOH} \rightarrow \text{C\text{\textsuperscript*}O}_2 + \text{CHz.CHOH.C\text{\textsuperscript*}OOH}
\]

Theoretically, the values determined for the \( \beta\)-carboxyl might be as low as one-half the actual values if randomization were complete. An experiment was carried out to learn the extent of the error introduced by the degradative procedures. C\(^{14}\)Malate was prepared biologically by incubating the pigeon liver enzyme with NaHC\(_{14}\)O\(_3\), TPN, and malate. The malate was recovered, purified, and degraded chemically to determine the C\(^{14}\) content of the two carboxyl groups by use of the concentrated H\(_2\)SO\(_4\) method, described previously, to determine the \( \alpha\)-carboxyl group and by calculation of the maximum C\(^{14}\) content of the \( \beta\)-carboxyl groups as the difference between the total counts in the malate molecule, as determined by persulfate oxidation (20), and the \( \alpha\)-carboxyl groups. A second aliquot of the malate was then degraded with \textit{Lactobacillus}. The loss of activity from the \( \beta\)-carboxyl by randomization during the bacterial degradation was found to be between 9 and 13 per cent. An error of this magnitude will not alter conclusions based on large differences in specific activity.

\textit{Chromatography}—In a few cases larger amounts of malate or oxalacetate have been purified by chromatographic procedures. The substances were extracted from the deproteinized and acidified reaction mixtures after
incorporation in Celite. After evaporation of the ether, the small aqueous residues were lyophilized, taken up in butanol-chloroform, and chromatographed on silica gel columns (21). The methods are modifications of the technique described by Isherwood (22). Certain relevant details are included in the description of the individual experiments under "Results and discussion."

Counting—The BaCO₃ was counted in finite thickness with an end window Geiger-Müller tube with an efficiency of about 10 per cent under the geometry obtaining and corrected for self-absorption. The samples were counted sufficiently long to insure a counting error of ±3 per cent. The results are reported either in terms of the actual corrected counts observed or as specific activity (counts per minute per mg. of carbon).

### Table I

Replacement of ATP by Malate and TPN in Oxalacetate Carboxylase Reaction

<table>
<thead>
<tr>
<th>Additions</th>
<th>BaCO₃ (mg)</th>
<th>Total counts</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10.8</td>
<td>26</td>
<td>41</td>
</tr>
<tr>
<td>2.5 μM ATP</td>
<td>11.3</td>
<td>382</td>
<td>556</td>
</tr>
<tr>
<td>3 μM l-malate</td>
<td>10.2</td>
<td>21</td>
<td>34</td>
</tr>
<tr>
<td>1.5 μM TPN</td>
<td>10.7</td>
<td>37</td>
<td>57</td>
</tr>
<tr>
<td>3 μM l-malate + 1.5 μM TPN</td>
<td>10.6</td>
<td>54</td>
<td>83</td>
</tr>
</tbody>
</table>

Each experiment contained the following in a total volume of 2.0 ml., in addition to the substances noted in the table: 0.4 ml. of enzyme, 120 μM of oxalacetate (neutralized to pH 6.4 with Na₂PO₄), 4 μM of MnCl₂, and 100 μM of NaHC⁴O₃ containing 35,500 counts. Incubated for 4 minutes at 38°.

* Counts per minute per mg. of C.

**RESULTS AND DISCUSSION**

Substitution of l-Malate for ATP—If the hypothesis that oxalacetate is formed via malate is correct, catalytic amounts of this substance should be able to replace ATP. The results of such a study are presented in Table I. The experimental time was short in order to permit better differentiation between reactions with different rates. It is clear that under these conditions malate exerts no stimulatory effect, TPN alone a slight effect, and the two combined a relatively small effect when compared with ATP.

In experiments not shown here, it has been found that the discrepancy between the ATP and TPN malate-catalyzed fixations lessens with longer incubation periods. On the other hand, even in longer experiments, the addition of catalytic amounts of malate without TPN caused only a slight
stimulation of CO₂ fixation over that in the control to which no additions were made.

CO₂ Fixation into Oxalacetate and Malate with ATP—Although the results of Table I indicate that catalytic amounts of malate will not replace ATP even in the presence of TPN, for a more critical examination of the role of malate in the oxalacetate carboxylase reaction it was necessary to study the relative specific activities of the two acids during different stages of CO₂ fixation.

Table II gives the results of such an experiment. Approximately equal amounts of oxalacetate and malate were incubated with NaH¹⁴CO₃, ATP, and a dialyzed enzyme preparation which contained oxalacetate carboxylase, malic dehydrogenase, “malic enzyme,” and other enzymes. If malate is a precursor of oxalacetate, the specific activity of the former substance must be at least as high as that of the latter at all times. The experiment is complicated somewhat by the action of malic dehydrogenase,

Table II

<table>
<thead>
<tr>
<th>Treatment of enzyme</th>
<th>Time of incubation</th>
<th>Oxalacetate</th>
<th>Malate</th>
<th>Ratio, oxalacetate to malate specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis</td>
<td>min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>356</td>
<td>649</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>365</td>
<td>810</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>347</td>
<td>947</td>
<td>376</td>
</tr>
<tr>
<td>Pretreatment with</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>brain DPNase fol-</td>
<td>4</td>
<td>323</td>
<td>623</td>
<td>32</td>
</tr>
<tr>
<td>lowered by dialysis</td>
<td>8</td>
<td>421</td>
<td>908</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>411</td>
<td>1088</td>
<td>97</td>
</tr>
</tbody>
</table>

Each vessel contained the following in a total volume of 4.0 ml.: 0.8 ml. of enzyme, 160 µM of oxalacetate (neutralized to pH 6.3 with Na₂HPO₄), 150 µM of l-malate, 4 µM of Na ATP, 8 µM of MnCl₂, and 200 µM of NaH¹⁴CO₃ containing approximately 48,000 counts. One-half of the contents of each vessel was used for oxalacetate degradation and one-half for degradation of l-malate. The total counts are calculated on the basis of 2 ml. of reaction mixture.

* The values in parentheses represent the total counts and specific activity of the α-COOH of malate as determined by Ce⁴⁺⁺⁺ oxidation.
which tends to bring the two acids into equilibrium and thus obscure any differences. As shown by the results, however, after a relatively short incubation period, the specific activity of the oxalacetate is more than 3 times as high as that of the malate, making it unlikely that the latter is a precursor in the reaction. It is to be noted that the ratio of specific activities of the two acids approaches 1 after longer incubation periods. Presumably, this effect is due in the main to the action of malic dehydrogenase, although part of the CO₂ may enter malate directly by way of the "malic enzyme."

Although the enzyme preparations had been dialyzed, it is known that it is difficult to remove all traces of DPN by dialysis, probably because of the close attachment of the DPN to its apoenzymes. In brain homogenates it can be shown that even the DPN attached to its apoenzymes, such as glyceraldehyde phosphate dehydrogenase, is destroyed in a very short time after disruption of cellular structure (23). The DPN is inactivated by splitting off nicotinamide (24). Accordingly, an attempt was made to remove the DPN remaining in the pigeon liver extract by use of brain DPNase, thus slowing or preventing equilibration between oxalacetate and malate. In the experiments shown in the lower section of Table II, the enzyme was pretreated, but all other conditions were identical with the experiment with extract which had undergone dialysis. Since the DPNase is present in well washed particles of the brain homogenate, it is removed by centrifugation following the treatment. The treatment with the brain preparation had little effect on the C₁⁴ content of the oxalacetate, as is shown by comparison of the values in the two sections of Table II, but the incorporation into malate was much slower. Consequently, the resulting ratios of activities of oxalacetate to malate were increased by a factor of 3 or more. In similar experiments, the ratio of oxalacetate to malate has always been 9 or greater after a short incubation time. These results with the DPNase-treated enzyme leave no doubt that malate as such is not a precursor of oxalacetate in the oxalacetate carboxylase reaction catalyzed by ATP.

CO₂ Fixation in Oxalacetate and L-Malate with TPN—The demonstration that the addition of ATP to pigeon liver extracts directed the fixation of C₁⁴O₂ into oxalacetate raised the question of the effect of TPN under similar conditions. The results reported in Table III are from experiments in which the ATP was replaced with TPN. All other experimental details were unchanged from those of Table II. It will be noted that, in the experiments with the dialyzed preparations, the results are the converse of those with ATP. After a short incubation, the specific activity of the oxalacetate is less than a third that of the malate. With longer incubation periods, the ratio again approaches 1, presumably because of the action of
malic dehydrogenase. Treatment of brain DPNase did not alter the ratios, as was the case in Table II.

The results of this experiment and similar ones not shown here make it highly improbable that oxalacetate as such is a precursor of malate in the CO₂ fixation reaction catalyzed by TPN. These experiments with unpurified enzyme preparations capable of fixing CO₂ in both malate and oxalacetate are in confirmation of the results of Veiga Salles et al. (7) with purified "malic enzyme."

**Fixation of CO₂ with Simultaneous Addition of TPN and ATP**—Since the foregoing experiments indicate that the addition of ATP favors the incorporation of C¹⁴O₂ in oxalacetate and the addition of TPN favors the incorporation in malate, it was of interest to determine the effect of the

<table>
<thead>
<tr>
<th>Treatment of enzyme</th>
<th>Time of incubation (min.)</th>
<th>p-COOH of residual acids</th>
<th>Ratio, oxalacetate/malate Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oxalacetate</td>
<td>Malate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total counts</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Dialysis</td>
<td>4</td>
<td>62</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>122</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>218</td>
<td>597</td>
</tr>
<tr>
<td>Pretreatment with</td>
<td>4</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td>brain DPNase fol-</td>
<td>8</td>
<td>83</td>
<td>173</td>
</tr>
<tr>
<td>lowed by dialysis</td>
<td>16</td>
<td>136</td>
<td>323</td>
</tr>
</tbody>
</table>

The reaction components were the same as in Table II, except that ATP was omitted and 1 μM of TPN was added.

The simultaneous addition of both substances. The results of such an experiment are shown in Table IV. Various concentrations of ATP and TPN were added singly and in combination to a dialyzed preparation which had not been pretreated with DPNase. The incubation time was short in order to minimize equilibration through malic dehydrogenase. As in Table II, the addition of ATP caused a preponderant fixation in oxalacetate. The increase in specific activity in the oxalacetate over the control was about 11-fold compared with a 2-fold increase in the specific activity of the malate. Replacement of ATP by TPN caused a greater increase in the malate, as expected. In the presence of the same amount of both substances, the specific activities of both acids were increased, with a ratio of about 1.3 in favor of oxalacetate. When the ratio of ATP to TPN was increased 4-fold (Experiment 5) over that of Experiment 4, a
slight increase in the ratio occurred, but when the ATP:TPN ratio was lowered to one-quarter that of Experiment 4 (Experiment 6), the ratio was changed markedly in favor of the malate.

The above results show that the fate of the fixed C\textsuperscript{14}O\textsubscript{2} depends not only on the nature of the cofactor added but also upon the relative concentrations of the two substances when both are present.

An additional interesting fact should be mentioned. In the experiments of Table IV in which ATP was present (2, 4-6) the distribution of the counts between oxalacetate and malate was dependent upon the amount of TPN present. However, the sum of the counts present in the two acids is seen to be constant and independent of the distribution.

**TABLE IV**

Effect of Simultaneous Addition of ATP and TPN on Fixation of CO\textsubscript{2} in Oxalacetate and Malate

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>β-COOH of residual acids</th>
<th>Ratio, oxalacetate malate Specific activity</th>
<th>Sum of counts in oxalacetate and malate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>TPN</td>
<td>Total counts</td>
<td>Specific activity</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>0.25</td>
<td>25</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>0.13</td>
<td>306</td>
<td>576</td>
</tr>
<tr>
<td>3</td>
<td>0.6</td>
<td>0.25</td>
<td>58</td>
<td>121</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>0.25</td>
<td>230</td>
<td>456</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>0.5</td>
<td>176</td>
<td>328</td>
</tr>
</tbody>
</table>

The experimental conditions were those described in the legend to Table III, except that ATP and TPN were added, yielding the concentration indicated above. The enzyme preparation was treated only by dialysis and the incubation time was 4 minutes in every case.

An attempt has been made to learn whether CO\textsubscript{2} is fixed in substances other than malate and oxalacetate. In a short time fixation experiment in which oxalacetate, malate, and ATP were present, the total counts fixed were found to be 496 as determined by persulfate oxidation of the deproteinated reaction mixture. In a similar aliquot the β-carboxyls of oxalacetate and l-malate were found to contain 461 and 45 counts respectively. The results make it unlikely that any C\textsuperscript{14}-containing substances other than oxalacetate and malate accumulate during short term experiments.

Identification of Oxalacetate and Malate—In the previous experiments determinations of the specific activity of malate and oxalacetate were
carried out on aliquots of the reaction mixture. Although known interfering substances were not present in either case, some uncertainty regarding the source of the radioactivity remained, since the substances had not been rigidly purified.

In view of these considerations, a large scale experiment was carried out in which the oxalacetate and malate were purified and identified. The conditions were such that the bulk of the activity was directed toward oxalacetate by use of the following experimental conditions: addition of

**Table V**

Purification of Oxalacetate and Malate Obtained by $^{14}$O$_2$ Fixation in Presence of ATP

<table>
<thead>
<tr>
<th>Experiment No. and description of fraction analyzed</th>
<th>Portion of molecule</th>
<th>Oxalacetate Specific activity</th>
<th>Malate Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Aliquots from reaction mixture</td>
<td></td>
<td>369,396</td>
<td>30.9, 30.3</td>
</tr>
<tr>
<td>Decarboxylation by Al+++ &quot; &quot; Lactobacillus</td>
<td>$\beta$-COOH</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Analysis of cuts from oxalacetate chromatograph</td>
<td></td>
<td>416,432</td>
<td>58.3, 58.8</td>
</tr>
<tr>
<td>Fraction A</td>
<td>&quot;</td>
<td>429,444</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; B</td>
<td>&quot;</td>
<td>440,450</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; C</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>2. Aliquots from reaction mixture</td>
<td></td>
<td>601,592</td>
<td>60.2, 57.6</td>
</tr>
<tr>
<td>Decarboxylation by Al+++ &quot; &quot; Lactobacillus</td>
<td>&quot;</td>
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</tr>
<tr>
<td>Analysis of malate after purification, as diphenacyl ester</td>
<td>601,592</td>
<td>60.2, 57.6</td>
<td></td>
</tr>
<tr>
<td>Decarboxylation by Lactobacillus</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>CO from H$_2$SO$_4$ degradation</td>
<td>$\alpha$-COOH</td>
<td>2.4</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

In Experiments 1 and 2 the following components were present in two 150 ml. Warburg vessels, with a combined volume of 48 ml.: 9.6 ml. of enzyme (pretreated with brain DPNase and then dialyzed), 2.80 mM of oxalacetate, 2.7 mM of l-malate, 72 $\mu$M of Na$_4$ ATP, 48 $\mu$M of MnCl$_2$, 2.4 mM of NaHCO$_3$ containing 860,000 counts. Incubated for 4 minutes at 38°.

ATP, omission of TPN, pretreatment of the enzyme with brain DPNase and a short incubation period.

After deproteinization, aliquots were taken for analysis of the oxalacetate and malate in the usual way. The results, reported in terms of specific activity, are presented in Experiment 1 of Table V. The ratio of specific activities of the oxalacetate to malate was more than 12 in accordance with expectations.

The remainder of the deproteinized solution was extracted with ether after mixing with Celite and chromatographed on a silica gel column. To
obtain oxalacetate it is necessary to increase the $\text{H}_2\text{SO}_4$ concentration used in preparation of the column and eluants from 0.5 to 3 N, since the oxalacetic acid is far more stable in the stronger acid.

The column was first treated with 10 per cent butanol in chloroform to remove pyruvic acid. Oxalacetate was then eluted with 15 per cent butanol in chloroform. The over-all yield from the extraction and chromatographic procedures was about 50 per cent. The eluant containing the oxalacetate was combined into three approximately equal fractions representing the first, middle, and final portions of the band. After extraction from the eluant with dilute alkali, duplicate aliquots were degraded by the Al$^{++}$ method. Analysis of the CO$_2$ thus obtained demonstrated that the specific activity of the oxalacetate in the various portions of the oxalacetate band was relatively constant. The constancy is a good indication that the substance eluted is not contaminated by substances capable of decarboxylation which possess specific activities higher or lower than that of oxalacetate itself. The specific activity of the chromatographed oxalacetate was slightly higher than that found on the original unfractionated reaction mixture.

From the chromatographic experiments it seems certain that C$^{14}$O$_2$ is fixed in oxalacetate or a compound which is converted to oxalacetate during the handling procedures. The latter possibility will be considered more fully later.

In a comparable experiment (No. 2, Table V) after analysis of aliquots of the original reaction mixture, the malate from the remainder of the solution was purified by chromatography on a silica gel column and the diphenacyl ester prepared. After three recrystallizations the ester had a melting point of 109° (uncorrected), the same as that of a sample prepared from authentic $l$-malate, and the mixed melting point showed no depression. The ester was saponified, the alcohol removed, and the malate degraded in the usual manner with Lactobacillus. The specific activity of the $\beta$-carboxyl of the purified malate was the same as that obtained from an aliquot of the original reaction mixture. This experiment effectively identifies malate and also establishes the validity of the shorter method of malate purification.

The C$^{14}$ content of the $\alpha$-carboxyl of the purified malate was obtained by degradation of another aliquot by the $\text{H}_2\text{SO}_4$ method described previously. The C$^{14}$ content of the $\alpha$-carboxyl is negligible under these particular experimental conditions, as shown in the last line of Table V.

Possible Mechanisms of CO$_2$ Fixation in Oxalacetate and Malate

The foregoing experiments present evidence that malate is not a precursor of oxalacetate in CO$_2$ fixation catalyzed by ATP and likewise that
oxalacetate is not a precursor of malate in the TPN-catalyzed fixation. These conclusions, based upon comparison of specific activities, involve the assumption that an intermediate formed metabolically will mix with the carrier pool of that substance. This assumption seems plausible in experiments in which no cellular structural elements are involved, but the possibility remains that an intermediate is formed which fails to dissociate from the enzymatic surface.

On the assumption just discussed, the results may be explained by either of the two following hypotheses: (a) CO$_2$ is fixed in oxalacetate and malate by two separate pathways requiring ATP and TPN, respectively, with the two products interconvertible by malic dehydrogenase, or (b) CO$_2$ is fixed in a precursor common to both acids, which is converted principally to oxalacetate in the presence of ATP or to malate in the presence of TPN (see Scheme 1). Although the experiments presented do not permit a

![Scheme 1](http://www.jbc.org/)

definite conclusion concerning these alternatives, one observation that may have an important bearing may be mentioned. In Table IV, ATP was present in Experiments 2, 4, 5, and 6 together with TPN in amounts ranging from 0 to 0.5 $\mu$M per ml. Although the distribution of counts between oxalacetate and malate was dependent upon the TPN concentration, the sum of the counts fixed in the two acids was remarkably constant. The constancy of the total fixed radioactivity is in accord with a mechanism embodying a common precursor rather than with a system employing two separate fixation reactions.

Although the experiments of Table V indicate that the major product of CO$_2$ fixation in the presence of ATP is oxalacetate or a substance easily converted to oxalacetate, the possibility that the oxalacetate might be inhomogenous has been examined by milder and slower decarboxylative procedures.
In Table VI, C\textsuperscript{14}-oxalacetate was prepared by incubating the pigeon liver enzyme with NaH\textsubscript{14}CO\textsubscript{3}, Mn\textsuperscript{++}, ATP, and oxalacetate. At the end of a short incubation period, the fixation of C\textsuperscript{14}O\textsubscript{2} was stopped by adjustment of the pH to 5.4 and a sweeping out of the C\textsuperscript{14}O\textsubscript{2}. After an aliquot of the oxalacetate had been removed for analysis, the remaining material was allowed to decarboxylate enzymatically, since the liver enzymes are active at this pH. At intervals, aliquots were removed and degraded by Al\textsuperscript{+++}. If the oxalacetate is a homogenous substance, the specific activities of the various samples should be identical. The results show, however, that the specific activity increases as the decarboxylation proceeds. This

\begin{table}[h]
\centering
\caption{Inhomogeneity of Oxalacetate As Shown by Specific Activity of Residual Acid after Enzymatic Decarboxylation}
\begin{tabular}{|c|c|c|}
\hline
Time of enzymatic decarboxylation & Oxalacetate decarboxylated & \textit{β}-COOH, residual oxalacetate \\
\hline
min. & per cent & c.p.m. per mg. C \\
\hline
0 & & 1288 \\
15 & 31.4 & 1543 \\
30 & 46.3 & 1653 \\
45 & 61.6 & 1720 \\
\hline
\end{tabular}
\end{table}

C\textsuperscript{14}-Oxalacetate was prepared with a pigeon liver extract which had been frozen 12 hours on dry ice, incubated at 25° for 4 hours, and then dialyzed for 5 hours at 7°. The following constituents were present in a total volume of 24.0 ml.: 3.0 ml. of extract, 1.40 mM of oxalacetate (neutralized to pH 6.3 with Na\textsubscript{2}PO\textsubscript{4}), 29 \textmu M of ATP, 24 \textmu M of MnCl\textsubscript{2}, and 1.2 mM of NaH\textsubscript{14}CO\textsubscript{3} containing \textasciitilde 4 \times 10\textsuperscript{5} counts. After 4 minutes incubation, the pH was adjusted to 5.4 with 1 n HCl and tank CO\textsubscript{2} bubbled through the solution. After evacuation to remove the CO\textsubscript{2}, the mixture was placed in a large Warburg vessel and permitted to decarboxylate at 38° in the presence of KOH in the center well. At the stated intervals duplicate aliquots were withdrawn, deproteinized, and the \textit{β}-COOH of the oxalacetate obtained by Al\textsuperscript{+++} decarboxylation as described under "Methods." The phenomenon may be interpreted as a demonstration that the "oxalacetate" contains at least two fractions, differing in specific activity and in rate of decarboxylation. The fraction with the higher activity apparently decarboxylates more slowly.

The inhomogeneity of the "oxalacetate" is shown also in Table VII, in which C\textsuperscript{14}-oxalacetate prepared as described above was allowed to decarboxylate spontaneously after deproteinization. The CO\textsubscript{2} produced during the 1st, 2nd, and 3rd hours of the process was collected and analyzed for C\textsuperscript{14} content. The specific activity of the CO\textsubscript{2} increased markedly with time, and Al\textsuperscript{+++} decarboxylation of the oxalacetate remaining after 3 hours showed an even higher activity. Calculation of the average specific
activity of these four fractions gave a value fairly comparable to a control run at the start of the decarboxylation by the usual Al+++ method.

Thus, the following information is available: (a) the fraction called "oxalacetate" is not homogenous and contains a higher activity fraction which decarboxylates more slowly; (b) after the handling procedures preparatory to and incidental to chromatography, oxalacetate attains homogeneity, suggesting that all of the fractions are converted to oxalacetate by mild procedures.

Yankwich and Calvin (25) have reported that decarboxylation rates of

<table>
<thead>
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<th>Table VII</th>
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<tr>
<td>Inhomogeneity of Oxalacetate As Demonstrated by Changing Specific Activity of CO₂ Produced by Spontaneous Decarboxylation</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Type of decarboxylation</th>
<th>Time of treatment</th>
<th>CO₂ from oxalacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
<td>Weight as BaCO₃ mg.</td>
</tr>
<tr>
<td>Spontaneous decarboxylation</td>
<td>0-60</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>60-120</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>120-180</td>
<td>7.9</td>
</tr>
<tr>
<td>Al+++</td>
<td>Residual after 180</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>Average, calculated</td>
<td></td>
</tr>
<tr>
<td>Al+++</td>
<td>0 time</td>
<td>17.6</td>
</tr>
</tbody>
</table>

C¹⁴-Oxalacetate prepared as described in Table VI, except that the volume was 30.0 ml. instead of 24.0 ml. and all components were increased proportionately. After 4 minutes incubation, the mixture was deproteinated by the addition of 1.5 ml. of 10 N H₂SO₄. After centrifuging, the supernatant was gassed with tank CO₂ and CO₂-free air successively. After removal of an aliquot for the Al+++ determination at zero time, the remainder of the supernatant was placed in a large Warburg vessel containing 2.5 N NaOH in the center well and was shaken at 38°. At the specified intervals the alkali was rinsed out of the center well and the collected CO₂ plated as BaCO₃; fresh NaOH was placed in the center well.

C¹⁴ compounds may be slower than those of the comparable C¹² compounds. They found that the C¹²—C¹² bond of malonic acid and bromomalonic acid ruptured 1.12 and 1.4 times as fast, respectively, as their C¹⁴—C¹² counterparts. The observed differences in rates of decarboxylation of the two components of oxalacetate appear to be considerably higher than these values. Also, in other experiments in which the C¹⁴-oxalacetate was treated in other ways, inhomogeneity has been scarcely detectable. The existence of the negative experiments and the higher rates observed in the experiments reported in Tables VI and VII make it unlikely that the difference in rates of decarboxylation of the C¹⁴—C¹² and C¹²—C¹² forms of oxalacetate is the sole cause of the apparent inhomogeneity.
The origin of the fraction of higher activity is not shown by the above experiments and may be either enzymatic or non-enzymatic. The possibility exists that the various fractions represent different isomers of oxalacetate, keto, cis-enol, and trans-enol. Regardless of origin, however, the fraction of high activity must be regarded as potentially important in CO₂ fixation by virtue of its high activity.

It is considered that this fraction may be the common precursor in Scheme 1 or at least a precursor of oxalacetate, but additional study will be necessary to verify this possibility. The nature and origin of the fraction are under investigation.

The author is indebted to Mrs. Ellen Wolfe for her invaluable technical assistance and to Dr. H. G. Wood for his constant and stimulating interest in this work.

SUMMARY

The relationship between oxalacetate and L-malate in CO₂ fixation as catalyzed by ATP (oxalacetate carboxylase) and TPN ("malic enzyme") has been studied by comparison of the specific activities of the two acids incubated together in the presence of C¹⁴O₂ under various conditions.

With ATP, oxalacetate has a much higher specific activity than malate at early stages of the reaction. Assuming that an intermediate formed metabolically mixes with the general pool of the substance, these experiments demonstrate that malate is not a precursor of oxalacetate. With longer incubation the specific activities of the two acids approach equality, presumably through the mediation of malic dehydrogenase. Pretreatment with a brain preparation capable of destroying DPN markedly decreases the C¹⁴O₂ content of the malate but not of the oxalacetate.

When TPN is substituted for ATP, CO₂ is fixed principally in malate during the early stages of the reaction, indicating that oxalacetate is not a precursor of malate under these circumstances.

When both ATP and TPN are added, the distribution of radioactivity between oxalacetate and malate is dependent on the relative concentrations of the two cofactors. The total number of counts fixed seems to be relatively constant, suggesting initial fixation in a common precursor.

Balance studies showed that no product other than oxalacetate and malate contained a significant amount of C¹⁴ after short time CO₂ fixation experiments.

By purification the fixation products have been identified as (a) oxalacetate or a substance easily converted to oxalacetate non-enzymatically, and (b) malate. Oxalacetate as analyzed by decarboxylation has been shown to be inhomogenous with a high activity fraction present.
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INTERRELATIONSHIPS OF OXALACETIC AND L-MALIC ACIDS IN CARBON DIOXIDE FIXATION

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