Glyoxylate Carboligase of Escherichia coli

IDENTIFICATION OF CARBON DIOXIDE AS THE PRIMARY REACTION PRODUCT*

(Received for publication, February 24, 1969)

ROBERT L. HALL,‡ BIRGIT VENNESLAND,§ AND FERENC J. KÉZDY

From the Department of Biochemistry, University of Chicago, Chicago, Illinois 60637

SUMMARY

An acidimetric method is proposed to distinguish between carbon dioxide and carbonic acid as the primary product of enzymic decarboxylation reactions. Application of this method to the thiamine pyrophosphate-requiring Escherichia coli glyoxylate carboligase reaction (the decarboxylating condensation of glyoxylate to yield hydroxymalonic semialdehyde) at pH 8 shows a transient disappearance of protons. Kinetic analysis of this proton uptake shows that the reaction occurs according to the pathway

\[ 2 \text{CHO-COO}^- + H^+ \xrightleftharpoons{k_1} \text{CHO-CHOH-COO}^- + CO_2 \]

\[ \text{CO}_2 + \text{H}_2\text{O} \xrightarrow{k_2} \text{HCO}_2^- + H^+ \]

The value of \( k_1 \) measured during the enzyme-catalyzed reaction is identical to the independently determined rate constant of carbon dioxide hydration. Thus, the intermediate is carbon dioxide, and the enzyme does not catalyze its hydration. The identity of the intermediate is further confirmed by the fact that addition of carbonic anhydrase to the reaction mixture suppresses the pH changes.

Biochemical decarboxylation and CO₂ fixation play an important role in intermediary metabolism and photosynthesis. Because of the intimate relationship existing between the nature of the coenzyme and the mechanism of the reaction, the many different enzymes catalyzing the formation of CO₂ are usually classified according to their cofactor requirements. A grouping by this criterion would include enzymes requiring no cofactors (e.g. aconitate decarboxylase); biotin-requiring enzymes (e.g. propionyl-CoA carboxylase); metal ion-requiring enzymes (e.g. oxalacetate decarboxylase); pyridoxal phosphate-requiring enzymes (e.g. amino acid decarboxylase); and enzymes requiring thiamine pyrophosphate (e.g. yeast pyruvate decarboxylase). The mechanism of the last group of enzymes is one of the best understood, mainly on the basis of extensive studies on thiamine and thiazole model systems (1).

The function of thiamine-PP-requiring enzymes is not necessarily restricted to decarboxylation. The same enzyme often catalyzes further transformation of the substrate, e.g. by carbon-carbon bond formation. One such enzyme is E. coli glyoxylate carboligase (2-4), which catalyzes Reaction 1.

\[ 2 \text{CHO-COO}^- + H^+ \xrightarrow{\text{thiamine-PP}} \text{CHO-CHOH-COO}^- + \text{CO}_2 \]

The identity of the 3-carbon product, hydroxymalonic semialdehyde, has been established (5, 6), but the 1-carbon product has only been determined as acid-released CO₂. The identification of the hydration state of this primary product is essential to the establishment of the mechanism of enzyme-catalyzed thiamine-PP-mediated decarboxylation reactions. For this reason, we have undertaken to determine whether the primary product of the glyoxylate carboligase-catalyzed reaction is CO₂ or H₂CO₃.

In doing so, we hoped also to shed some light on the function of the FAD moiety in the enzyme (2, 3).

Several methods have been devised for discerning between CO₂ and H₂CO₃ as the primary product of enzyme-catalyzed decarboxylations (7-10). All are dependent or based on the physical process of diffusion of CO₂ between the gas and liquid phases—a process too difficult to handle experimentally to permit mathematical description of the phenomena observed. Recently, spectrophotometric and radiochemical methods have been introduced for determining the hydration state of CO₂ fixed in carboxylation reactions (11-13) and thus, by the principle of microscopic reversibility, the form released in the reverse reaction. However, these methods cannot be applied to all systems because of either the requirement for an NADH/NAD⁺ or NADPH/ NADP⁺-linked enzyme which acts on the non-CO₂ product, or the necessity for measuring the fixation of CO₂ and not its release.

In view of this, we devised an acidimetric method, independent of diffusion considerations and applicable to most enzyme decarboxylations. It is based on the stoichiometry of decarboxylation reactions, as shown in Equations 2 and 3 of Scheme 1.

\[ \text{CHO-CHOH-COO}^- + \text{CO}_2 \]
At pH values where the substrate acid is dissociated and where 
H₂CO₃ exists mainly in the form of HCO₃⁻ (5 < pH < 9), there 
should be a net proton uptake commensurate with the enzymatic 
reaction rate if CO₂ is the product, but none if HCO₃⁻ is the 
product. Taking into account the nonenzymic interconversion 
catalyzed reaction can then be written as in Scheme 2.

athi + HC03⁻ → 2 H⁺ + CO₂⁻

At intermediate pH values, the experimental curves would lie 
below these limiting curves. Thus, by monitoring the time 
dependency of the hydrogen ion concentration during 
the reaction will be determined by both the pathway and the 
Scheme 1. Qualitatively, the following limiting kinetic behaviors can 
be visualized. In Case I, at a pH where the final product is CO₂ 
(pH ≤ 5), Pathway 4 predicts a proton uptake from the solution at 
the rate of the enzyme reaction; in Case II, at pH ≤ 5, Pathway 
5 would give no proton uptake initially, followed by proton 
uptake at an increasing rate as the k₅-limiting dehydration of 
H₂CO₃ became significant; in Case III, at a pH where HCO₃⁻ is 
the final product (pH ≥ 8), Pathway 4 would give a k₄-dependent 
proton uptake followed by the subsequent production of HCO₃⁻ 
and protons through the hydration of CO₂; in Case IV, at pH ≥ 8, 
Pathway 5 would give no pH change during the course of the 
experiment. 

At intermediate pH values, the experimental curves would lie 
below these limiting curves. Thus, by monitoring the pH changes 
of the reaction mixture as a function of time at each pH, it should be possible to distinguish between the two possible pathways.

The experimental conditions necessary for being able to dis-
with a glass stirring rod and starting the recorder after mixing (usually 5 to 7 sec). The CO₂ released by this amount of substrate diffused out of solution at a negligible rate in the time scale of the experiment. Absorbance of the indicator dye at its \( \lambda_{\text{max}} \) was recorded with a Cary model 15 spectrophotometer, with the 0.0 to 0.1 absorbance unit slidewire. The indicator dye used was creosol red (\( \rho \text{K} 8.3, \lambda_{\text{max}} = 570 \text{ mp} \)) at pH 8. Under our conditions, absorbance increased with increasing pH. Preliminary experiments indicated that (a) the indicator did not affect the enzyme reaction, and (b) absorbance changes were nearly linear with pH changes in the very narrow pH range used (approximately 0.1 pH unit). At the end of the reaction (after at least six half-lives), a known amount of NaOH was added to the cuvette, and the \( \Delta A \) was recorded. This value was used to convert absorbance units to micromoles of H⁺. In a similar manner, the rate constants for dehydration of H₂CO₃ (addition of NaHCO₃ solution to the cuvette) and hydration of CO₂ (addition of saturated CO₂ solution) were determined in the reaction mixture without substrate. Controls containing no enzyme showed a moderate increase followed by a downward drift in absorbance upon addition of substrate, and further experiments showed that this was due to some interaction of cysteine, substrate, and indicator dye. This drift was significant in the experiments with lower enzyme concentrations and caused inaccuracies in the \( k_4 \) values obtained.

**Potentiometric pH Measurement**—Although the spectrophotometric data clearly indicated a transient proton uptake during the reaction, the inherent inaccuracy of the measurements prevented us from obtaining conclusive quantitative results. For this reason, we measured the pH change directly with a recording pH meter. A Beckman Expandomatic pH meter (precision, ±0.003 pH) with glass and saturated calomel electrodes was used in conjunction with a Houston Omnigraphic T-Y recorder, model HR-80-2. The output voltage from the pH meter was read across a 100,000-ohm resistor, so that the current drawn by the recorder was negligible and did not affect the pH readings. Mixing was done with a low speed electric motor and glass stirrer. With low stirring, no CO₂ was lost from the solution by diffusion during the enzyme reaction, as shown by control experiments in which stirring was discontinued shortly after addition of the substrate (Table II, below).

The reaction was carried out with 10 ml of solution in a 50-ml beaker. The recorder was first calibrated with two buffers, the pH values of which were about 0.2 pH unit apart. Then the pH of the reaction mixture was adjusted to the lower end of the pH range with NaOH or HCl, the recorder was started, 15 CO₂ peq were converted to micromoles of H⁺ by using a calibration curve obtained by adding known amounts of OH⁻ to an identical reaction mixture. Measurements of the CO₂ hydration rate (saturated CO₂ solution added) and controls containing no enzyme were performed in a similar manner. In some experiments, 0.1 ml of bovine carbonic anhydrase (10 mg per ml) was added at the peak of the pH curve.

**THEORY**

If the substrate concentration (\( S \)) is much lower than its \( K_m \) in the enzyme-catalyzed reaction, if the enzyme concentration is much lower than the initial substrate concentration (\( S_0 \) (turnover conditions)), and if the enzyme does not catalyze the interconversion of H₂CO₃ and CO₂, then the pathways represented in Scheme 2 can each be treated kinetically as simple systems of two consecutive first order reactions. The solutions of the kinetic equations for these systems are known (16). Their application to the above mentioned limiting cases yields the following expressions.

**Case I: pH ≤ 5, CO₂ Primary Product**—Under these conditions, the second step of Pathway 4 does not occur to a significant extent, and the reaction can be considered as a first order production of OH⁻. Then the change in proton concentration with time can be written as

\[
-\Delta H_t = \left[ H^+ \right]_0 - \left[ H^+ \right] = S_0 \left(1 - e^{-k_4 t} - k_4 e^{-k_3 t} \right)
\]

**Case II: pH ≤ 5, HCO₃⁻ Primary Product**—Pathway 5 of Scheme 2 shows that the sequence will consist of an enzyme-catalyzed production of HCO₃⁻ causing no change in proton concentration, followed by a stoichiometric proton uptake and dehydration of H₂CO₃ governed by the rate constant \( k_4 \). The changes in proton concentration with time are described by the equation

\[
-\Delta H_t = \left[ H^+ \right]_0 - \left[ H^+ \right] = S_0 \left(1 + \frac{1}{(k_4 - k_3)}(k_3 e^{-k_3 t} - k_4 e^{-k_4 t}) \right)
\]

**Case III: pH ≥ 8, CO₂ Primary Product**—At this pH HCO₃⁻ is the final product, and both first order reactions in Pathway 4 are operative. The OH⁻ produced in the enzyme reaction will subsequently be neutralized as CO₂ is converted into HCO₃⁻ with the simultaneous release of a proton. The changes in proton concentration with time are described by Equation 8.

\[
-\Delta H_t = \left[ H^+ \right]_0 - \left[ H^+ \right] = \left[ k_3 S_0 / (k_4 - k_3) \right](e^{-k_3 t} - e^{-k_4 t})
\]

**Case IV: pH ≥ 8, HCO₃⁻ Primary Product**—Since the primary product is also the final product, Pathway 5 predicts no pH change during the reaction.

Comparison of the results predicted on the basis of the two pathways shows that the experiments at pH 8 should give a clearer qualitative distinction than those at pH 5 between CO₂ and HCO₃⁻ as the primary product; the mere presence of a transient OH⁻ formation at pH 8 would rule out Pathway 5. However, in order to confirm the identity of the intermediate as CO₂ and to determine the amount formed by Pathway 4, a quantitative analysis of the curves of H⁺ concentration with respect to time is desirable.

The evaluation of the two rate constants from an experimental curve described by Equation 8 can be carried out in the following way. Analysis of the first derivative of Equation 8 shows that the time at which the H⁺ concentration is a minimum is given by Equation 9.

\[
-t_{\text{min}} = \frac{1}{(k_4 - k_3)} \ln \left( \frac{k_4}{k_3} \right)
\]

Substituting this value into Equation 8 in order to obtain an expression for \( -\Delta H_{\text{min}} \) and rearranging terms yields Equation 10.

\[
\left(1/t_{\text{min}}\right) \ln \left( -\Delta H_{\text{min}} / S_0 \right) = -k_3
\]

Thus the value of \( k_3 \) can be determined from the position and height of the peak of the curve of \( -\Delta H_t \) versus time. Once \( k_3 \) is known, then \( k_4 \) can be obtained graphically from the rate equation

\[
d(-\Delta H_t) / dt = k_3 S_0 e^{-k_3 t} - k_4 (-\Delta H_t)
\]

which can be transformed into Equation 11.
**Table I**

**Effect of glyoxylate carboligase on rate constant for CO₂ hydration**

The reaction mixtures contained 50 mM phosphate, pH 7.3; 0.2 mM thiamine-PP; 1.0 mM MgCl₂; 1.0 mM dithiothreitol; 0.025 mg of phenol red per ml; and enzyme as indicated. Temperature was 25°C.

<table>
<thead>
<tr>
<th>Carboxylic anhydride</th>
<th>Glyoxylate carboligase</th>
<th>10⁻⁶ × kh</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td>µg/ml</td>
<td>sec⁻¹</td>
</tr>
<tr>
<td>0.33</td>
<td>21</td>
<td>8.8</td>
</tr>
<tr>
<td>1.33</td>
<td>263</td>
<td>8.0</td>
</tr>
<tr>
<td>1.38</td>
<td>221</td>
<td>48.7</td>
</tr>
</tbody>
</table>

**Table II**

**Effect of stirring and aerobiosis on kₚ and kₚ**

Manometric reaction mixtures contained 17 mM phosphate, pH 7.9; 0.11 mM thiamine-PP; 0.6 mM MgCl₂; 1 mM cysteine; 0.8 enzyme unit per ml; and 5.0 CO₂ eq of sodium glyoxylate per ml. Potentiometric reaction mixtures contained 10 mM phosphate, pH 7.8; 0.12 mM thiamine-PP; 0.6 mM MgCl₂; 1 mM cysteine; 0.7 enzyme unit per ml; and 1.5 CO₂ eq of sodium glyoxylate per ml.

<table>
<thead>
<tr>
<th>Stirring</th>
<th>Aerobiosis</th>
<th>Relative kₚ</th>
<th>Relative kₚ, potentiometric*</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>0.92</td>
<td>0.04</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>1.14</td>
<td>1.11</td>
</tr>
</tbody>
</table>

* kₚ values calculated from Equation 10.

The correctness of the above kinetic analysis can be judged by two criteria. (a) A theoretical curve of (−ΔHᵢ) against time can be constructed with the obtained values of kₚ and kₚ, and Equation 11. A plot of ln [(d(−ΔHᵢ/S₀)/dt) + [kₚ(−ΔHᵢ/S₀)]] = ln kₚ − kₚt (11) with respect to time should yield a straight line with a slope of (−kₚ) and an intercept of ln (kₚ).

The production of H⁺ in the substrate control is due to CO₂ uptake from the air, but is due instead to a spontaneous reaction involving substrate, cysteine, and indicator dye. Approximate rate constants for the curves in Fig. 1, calculated as described under

**Results**

Preliminary experiments using the stopped flow spectrophotometric method (17) confirmed that glyoxylate carboligase has no measurable carbonic anhydrase activity; i.e. it does not affect the rate constant of CO₂ hydration. In these experiments, CO₂ solution and the appropriate reaction mixture containing enzyme and phenol red (pK 7.9, λ_max = 560 nm) were rapidly mixed in a Durrum-Gibson stopped flow spectrophotometer, and the absorbance decrease at 560 nm was followed. First order rate constants were calculated for the different enzyme concentrations employed (Table I). Manometric assay of the carboligase under these conditions showed complete activity for CO₂ release from glyoxylate. From the results in Table I, we conclude that the specific carbonic anhydrase activity of the glyoxylate carboligase is at least 10⁴ times smaller than that of the bovine carbonic anhydrase used.

A second set of control experiments showed the effects of stirring and aerobiosis on the kₚ and kₚ values obtained from the recording pH meter experiments. No significant changes in the values of the rate constants were observed (Table II). In other words, the amount of CO₂ diffusing out of solution in the time scale of the experiment is negligible compared with the total amount of CO₂ produced.

**Spectrophotometric Method, pH 8**—Typical curves of (−ΔHᵢ) with respect to time (obtained from the spectrophotometric data after graphical correction for a linear drift in the base-line) are shown in Fig. 1. An easily observable maximum occurs in each curve, indicating strongly that CO₂ is the product. The production of H⁺ in the substrate control is not due to CO₂ uptake from the air, but is due instead to a spontaneous reaction involving substrate, cysteine, and indicator dye. Approximate rate constants for the curves in Fig. 1, calculated as described under

**Fig. 1.** Proton uptake during the glyoxylate carboligase reaction as a function of time, by the spectrophotometric method pH 7.8, 25°C. Reaction mixtures contained 25 mM phosphate, pH 7.8; 0.4 mM thiamine-PP; 2 mM MgCl₂; 3.3 mM cysteine; 0.1 ml of 0.01% cresol red; enzyme; and 5.0 CO₂ eq of sodium glyoxylate per ml. Enzyme concentrations: Curve 1, none; Curve 2, 5.4 unit per ml; Curve 3, 10.8 units per ml; Curve 4, 21.6 units per ml.
"Theory," are summarized in Table III. The agreement is reasonable between the values for $k_a$ obtained by the manometric and spectrophotometric methods. Good agreement is also obtained between the rate constant for disappearance of OH$^-$ in the enzyme-catalyzed reaction and the rate constant for hydration of added CO$_2$ when the rate of the enzyme reaction is large relative to the rate of the spontaneous decomposition of the substrate. At lower enzyme concentrations the agreement is less satisfactory, presumably owing to the larger uncertainties in correcting for the blank reaction.

Experiments have also been carried out at pH 6, with the spectrophotometric method. The qualitative results are compatible with Pathway 4, but quantitative evaluation of the data is difficult because of the decreased stability of the substrate in the presence of cysteine and indicator dye, and the fact that it was impossible to satisfy fully the condition $S_b \ll K_m$.

**Potentiometric Method, pH 8**—When the potentiometric method is used to monitor the pH of the reaction mixture, curves similar in shape to the spectrophotometric curves are obtained. Because the rate constant calculations are not trivial, we will present sample calculations for a typical experiment. The experimental pH versus time curve is first corrected graphically for a linear drift in the base-line. Then, by use of the calibration curve (see "Experimental Procedures"), the data are converted into a curve of $(-\Delta H_t)$ with respect to time (Fig. 2). From this curve, the value of $k_b$ is calculated first, by use of Equation 10 and the data from the position and height of the peak. We obtain $k_b = 4.7 \times 10^{-2}$ sec$^{-1}$. Next, values of $[\Delta (-\Delta H_t/S_0)/\Delta t + [k_a(-\Delta H_t/S_0)]]$ are calculated, by dividing the curve into small equal time intervals and calculating the slope at the middle of each interval from the chord. The sum $[\Delta (-\Delta H_t/S_0)/\Delta t + [k_a(-\Delta H_t/S_0)]]$ is

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Plot of log $[\Delta (-\Delta H_t/S_0)/\Delta t + [k_a(-\Delta H_t/S_0)]]$ against time for the experiment presented in Fig. 2. The characteristics of the least squares line are: slope, $-2.19 \times 10^{-4}$ sec$^{-1}$; intercept, $-1.632$.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Hydration of CO$_2$ added to the reaction mixture of Fig. 2. The first order rate constant obtained was $k_a = 5.4 \times 10^{-2}$ sec$^{-1}$.

### Table III

<table>
<thead>
<tr>
<th>Enzyme*units/ml</th>
<th>$10^6 \times k_a$</th>
<th>$10^5 \times k_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spectrophotometric</td>
<td>Manometric</td>
</tr>
<tr>
<td>5.4</td>
<td>1.6</td>
<td>2.1</td>
</tr>
<tr>
<td>10.8</td>
<td>4.8</td>
<td>6</td>
</tr>
<tr>
<td>21.6</td>
<td>6.4</td>
<td>6</td>
</tr>
</tbody>
</table>

$^a$ Specific activity, 7.5 enzyme units per mg of protein.

$^b$ Determined by addition of saturated CO$_2$ solution to the standard reaction mixture without substrate.

$^c$ Enzyme reaction, with $k_a$ calculated from Equation 10.

---

**Fig. 2.** Proton uptake during the glyoxylate carboligase reaction as a function of time, by the potentiometric method. pH 7.8, 25°C. The reaction mixture contained 10 mM phosphate, pH 7.8; 1.12 mM thiamine-PP; 0.6 mM MgCl$_2$; 1 mM cysteine; 8.1 enzyme units per ml; and 1.5 CO$_2$ eq, sodium glyoxylate per ml.
then calculated for each point. A plot of the logarithm of this sum with respect to time should yield $k_a$, both from the slope ($-k_a$) and from the intercept ($k_a$) of the line. Such a plot is shown in Fig. 3. The values obtained from the slope and intercept are, respectively, $k_a = 2.2 \times 10^{-2}$ sec$^{-1}$ and $k_a = 2.3 \times 10^{-2}$ sec$^{-1}$. For comparison with the value of $k_a$ obtained from Eq. 2, the rate constant for CO$_2$ hydration was also measured in the same mixture by adding an aliquot of saturated CO$_2$ solution and monitoring the decrease in pH. Conversion of the change in pH to the change in micromoles of H$^+$ as before, followed by plotting the logarithm of ([H$^+$]$_t$ - [H$^+$]$_0$) against time yields $k_a = 5.4 \times 10^{-5}$ sec$^{-1}$ (Fig. 4). This value is in excellent agreement with the value obtained from Eq. 10. Finally, the values for $k_a$ and $k_b$ obtained in the enzyme-catalyzed reaction can be used with Equation 8 to synthesize the experimental curve (Fig. 5).

![Fig. 5. Comparison of theoretical and experimental curves for the experiment presented in Fig. 2. ---, theoretical curve obtained from Equation 8, with $k_a = 2.26 \times 10^{-2}$ sec$^{-1}$ and $k_b = 4.7 \times 10^{-2}$ sec$^{-1}$; O, experimental points.](image)

**TABLE IV**

Rate constants $k_a$ and $k_b$ determined by potentiometric method at pH 8

Reaction mixtures were the same as in Fig. 2 except that enzyme concentration was varied as shown. Values of $k_a$ were corrected to 25°, when necessary, by using a value of 21,600 cal per mole for E* experimental (3).

<table>
<thead>
<tr>
<th>Substrate concentration</th>
<th>Thiamine-PP concentration</th>
<th>$10^5 \times k_a$</th>
<th>$10^5 \times k_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$ concentration</td>
<td>mM</td>
<td>sec$^{-1}$</td>
<td>sec$^{-1}$</td>
</tr>
<tr>
<td>0.75</td>
<td>0.12</td>
<td>3.1</td>
<td>7.2</td>
</tr>
<tr>
<td>1.5</td>
<td>0.12</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>3.0</td>
<td>0.12</td>
<td>2.6</td>
<td>6.5</td>
</tr>
<tr>
<td>4.2</td>
<td>0.12</td>
<td>2.8</td>
<td>4.8</td>
</tr>
<tr>
<td>1.5</td>
<td>0.24</td>
<td>2.7</td>
<td>5.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CO$_2$ added &amp; Substrate added</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$ added</td>
</tr>
<tr>
<td>0.75</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>3.0</td>
</tr>
<tr>
<td>4.2</td>
</tr>
<tr>
<td>1.5</td>
</tr>
</tbody>
</table>

a The average of values obtained from the slope and intercept of the plot.

b Saturated CO$_2$ solution added to the complete reaction mixture.

c Calculated from Equation 10.

d Determined before adding substrate.

e Determined after enzyme reaction completed.

5). The good agreement between the theoretical curve and experimental points indicates that Equation 8 indeed correctly describes the phenomenon, and that the CO$_2$ pathway accounts for more than 90% of the reaction product.

Table IV summarizes our pertinent experiments with varying enzyme concentrations. The following points should be noted. First, the agreement between the manometric and potentiometric value for $k_a$ is good, considering that the calculations to obtain the potentiometric value are quite involved and that the two values are measured by different experimental techniques. Satisfactory agreement has also been observed between the potentiometrically determined value of $k_a$ and the rate constant obtained by measuring colorimetrically the disappearance of glyoxylate (as its semicarbazone derivative). Secondly, that the $k_a$ value is actually a measure of the enzyme-catalyzed reaction is shown by the dependence of $k_a$ on the enzyme concentration. Finally, the value of $k_b$ (8 added) is calculated from only one experimental point and therefore is inherently less accurate than the $k_b$ value obtained from the first order plots with CO$_2$ added. In view of this, we feel that the agreement between the two values is quite satisfactory.

The values of the rate constants $k_a$ and $k_b$ obtained at the enzyme concentration of 15.9 units per ml, with varying substrate and cofactor concentrations, are shown in Table V. $k_a$ is nearly independent of substrate concentration over a 4-fold range, thus indicating that the $K_m$ of the enzyme-catalyzed reaction is larger than the substrate concentrations used. However, a small trend in the value of $k_a$ as a function of $S_0$ indicates that $S_0$ is not much larger than $K_m$, and thus the reaction might deviate from first order kinetics in the early stages. The independence of $k_a$ from the thiamine-PP concentration suggests that the enzyme is saturated with respect to this cofactor.

TABLE V

Effect of substrate and thiamine-PP concentrations on $k_a$ and $k_b$ determined by potentiometric method at pH 8

Experimental conditions were the same as in Fig. 2, except that enzyme concentration was constant at 15.9 units per ml, and substrate and cofactor concentrations were varied as shown. Values of $k_a$ were corrected to 25°, when necessary, by using a value of 21,600 cal per mole for E* experimental (3).

<table>
<thead>
<tr>
<th>Substrate concentration</th>
<th>Thiamine-PP concentration</th>
<th>$10^5 \times k_a$</th>
<th>$10^5 \times k_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$ added &amp; Substrate added</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO$_2$ added</td>
<td>Substrate added</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>0.12</td>
<td>3.1</td>
<td>7.2</td>
</tr>
<tr>
<td>1.5</td>
<td>0.12</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>3.0</td>
<td>0.12</td>
<td>2.6</td>
<td>6.5</td>
</tr>
<tr>
<td>4.2</td>
<td>0.12</td>
<td>2.8</td>
<td>4.8</td>
</tr>
<tr>
<td>1.5</td>
<td>0.24</td>
<td>2.7</td>
<td>5.9</td>
</tr>
</tbody>
</table>

a The average of values obtained from the slope and intercept of the plot.

b Saturated CO$_2$ solution added to the complete reaction mixture.

c Calculated from Equation 10.

d Determined before adding substrate.

e Determined after enzyme reaction completed.

3 R. L. Hall, unpublished data.
Effects of Carbonic Anhydrase—The effects of carbonic anhydrase on the reaction systems are shown in Figs. 6 and 7. In the spectrophotometric method, when carbonic anhydrase is added to the substrate, the pH changes observed during the enzyme reaction are reduced to the low level of those observed in the course of the nonenzymic decomposition of the substrate (Fig. 6). Moreover, addition of carbonic anhydrase to the potentiometric reaction mixture at the peak of the pH change rapidly restores the initial pH (Fig. 7). These results give good qualitative evidence that CO₂ is the reaction product, and that the changes in pH are actually due to the build-up of CO₂ and the removal of H⁺. The final pH of the mixture is slightly lower than the starting pH, probably because of some decomposition of substrate or product, or both.

DISCUSSION

The results presented support consistently the hypothesis that CO₂ is the primary 1-carbon product of the glyoxylate carboligase reaction. A similar conclusion has been reached from studies on another thiamine-P₃-requiring enzyme, yeast pyruvate decarboxylase (9). According to the proposed mechanism for thiamine catalysis (1), an addition product (Structure I) is formed between substrate and cofactor at C-2 of the thiazole ring. The adduct then decarboxylates and condenses with a second substrate molecule. If HCO₃⁻ were to be the primary product, Intermediate I should be hydrated to give Structure II before or during further condensation. Hydration of Structure I to yield the ortho-carboxylate is energetically unfavorable, however, and bicarbonate should not be a better leaving group than carbon dioxide. Also, nonenzymic decarboxylations always yield CO₂ as the primary product (18, 19). The occurrence of the ortho-carboxylate is therefore unlikely to be mechanistically significant. Thus, the identification of CO₂ as the reaction product in the glyoxylate carboligase reaction agrees with the predictions from model studies. Furthermore, it indicates that the presence of the FAD moiety on the enzyme does not modify the mechanism of the decarboxylation step to the point of changing the nature of the reaction product. The number of moles of CO₂ produced per mole of substrate in the enzyme-catalyzed reaction is, within experimental error, equal to the value of 0.5 predicted from the stoichiometry of the condensation reaction. It appears, then, that the enzyme is unable to catalyze decarboxylation without coupling it to the condensation.

The method described for the identification of the hydration state of the decarboxylation product of an enzyme reaction has several advantages over previous methods. (a) No special apparatus or glassware is required. Instrumentation is simple and readily available. (b) The method is generally applicable to any decarboxylation reaction occurring in physiological pH ranges. (c) With proper precautions, the kinetics can be analyzed to give rate constants, and these can be compared with independently obtained values. Also, the method permits the quantitative estimate of the fraction of product appearing in each hydration state by comparing the theoretical and experimental curves.

The limitations of the method are as follows. (a) The substrate and product must not enter into H⁺-consuming or -producing side reactions. If this condition is not fully met—as appears to be the case with glyoxylate carboligase—then the enzyme...
reaction rate must be made large relative to the rates of the side reactions, necessitating larger amounts of enzyme. (b) The pH change during the reaction should be less than 0.2 pH unit, especially in pH regions where the enzyme-catalyzed reaction is pH-dependent. (c) Quantitative analysis of the kinetic data requires that the enzyme-catalyzed reaction be first order with respect to the substrate; i.e. $S_0 \ll K_m$. However, even if this condition is not fully satisfied, the presence or absence of a transient OH$^-$/formation at pH 8 still would distinguish qualitatively between Pathways 4 and 5.

Preliminary manometric studies (15) on the glyoxylate carboligase reaction under turnover conditions gave seemingly good evidence that HCO$_3^-$, and not CO$_2$, was the primary reaction product. These experiments showed that active carbonic anhydrase stimulated the rate of CO$_2$ release from the reaction mixture into the gas phase. Further kinetic and analogue computer studies of this system have shown, however, that the acceleration of CO$_2$ release by carbonic anhydrase is far from being a simple phenomenon: the diffusion across the interface, the failure of the system to reach a true steady state of release of CO$_2$, and the nondecarboxylating interaction of the substrate with carbonic anhydrase render the manometric results mechanistically inconclusive. We believe that the methods presented in this paper are simpler in design and more straightforward to interpret.

**CONCLUSION**

The predominant, and probably the only, 1-carbon product of the glyoxylate carboligase reaction is CO$_2$. This is in agreement with thiazole model studies and gives evidence that the FAD moiety of glyoxylate carboligase does not drastically alter the mechanism of the decarboxylation step. The method presented gives not only qualitative, but also quantitative, evaluation of the hydration state of the primary reaction product of an enzymic decarboxylation.

Acknowledgments—We wish to thank Professors John H. Law and John Westley for helpful discussions during the preparation of the manuscript.

**REFERENCES**

Glyoxylate Carboligase of *Escherichia coli*: IDENTIFICATION OF CARBON DIOXIDE AS THE PRIMARY REACTION PRODUCT

Robert L. Hall, Birgit Vennesland and Ferenc J. Kézdy


Access the most updated version of this article at http://www.jbc.org/content/244/15/3991

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/244/15/3991.full.html#ref-list-1