The Carboxylation of Phosphoenolpyruvate and Pyruvate

I. THE ACTIVE SPECIES OF "CO₂" UTILIZED BY PHOSPHOENOLPYRUVATE CARBOXYKINASE, CARBOXYTRANSPHOSPHORYLASE, AND PYRUVATE CARBOXYLASE*

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SUMMARY

Previous studies with propionyl coenzyme A carboxylase and with phosphoenolpyruvate carboxylase using ¹⁸O-labeled bicarbonate have indicated that bicarbonate is the reactive species in these fixations of "CO₂." We have investigated the species of CO₂ in reactions catalyzed by pyruvate carboxylase, P-enolpyruvate carboxykinase, and P-enolpyruvate carboxytransphosphorylase. Since propionyl-CoA carboxylase and pyruvate carboxylase are biotin enzymes, they would be expected to have similar mechanisms. Likewise, the reactions catalyzed by P-enolpyruvate carboxykinase and carboxy transphosphorylase are, in some respects, similar to that of P-enolpyruvate carboxylase, and it has been suggested that bicarbonate might be the reactant in each case. By means of radiochemical and spectrophotometric techniques, we have obtained evidence that the active species in the carboxykinase and carboxytransphosphorylase reactions is CO₂ and not bicarbonate. Bicarbonate appears to be the active species in the pyruvate carboxylase reaction, in conformity with the results obtained with propionyl-CoA carboxylase.

It is important in considerations of the mechanism of CO₂ fixation or decarboxylation to know whether or not the reactant is CO₂ or HCO₃⁻ (or H₂CO₃). Two methods have been used to obtain such information. The first involves the use of carbonic anhydrase as exemplified by the studies of Krebs and Roughton (1). They presented evidence that CO₂ is the product of decarboxylation of pyruvate, as catalyzed by pyruvate decarboxylase (EC 4.1.1.1). The evidence consisted of the demonstration, by manometric methods, of an "overshoot" in CO₂ pressure during the decarboxylation, which was eliminated in the presence of carbonic anhydrase. This observation could be explained if CO₂ is the product and if the rate of hydration of CO₂ is limiting. The CO₂ under these conditions escapes into the gas phase, but as the substrate becomes limiting and the rate of the reaction decreases, the CO₂ is reabsorbed to attain the equilibrium shown below.

\[
\text{CO}_2 (\text{gaseous}) \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \quad (1)
\]

In the presence of carbonic anhydrase the hydration of CO₂ is so rapid that equilibrium is maintained throughout the decarboxylation. In contrast, if bicarbonate were the initial product, the evolution of CO₂ into the gas phase would be faster in the presence of carbonic anhydrase than in its absence, and there would be no overshoot of CO₂ pressure. Hansl and Waygood (2), by use of this method and with a heavy suspension of disintegrated cells of Chlorella, or extracts from plants, presented evidence that CO₂ is the primary product of decarboxylation of pyruvate, oxaloacetate, glutamate, and α-ketoglutarate.

The second method of determining CO₂ species has involved the use of ¹⁸O-bicarbonate. Kaziro et al. (3) and Maruyama et al. (4) have presented evidence that HCO₃⁻ (or H₂CO₃) is the reactant in certain CO₂ fixations. They showed that all 3 oxygens of HCO₃⁻ were incorporated into the products, organic acids and phosphate, whereas if CO₂ were the reactant only 2 would be expected to be utilized. Kaziro et al. (3) used the biotin enzyme, propionyl-CoA carboxylase (EC 6.4.1.3), and found the equivalent of 1 ¹⁸O atom in the orthophosphate and 2 in the free carboxyl of the methylmalonyl-CoA. The results are illustrated in Reaction 2.

\[
\text{CH}_3\text{CH}^-\text{CO}^-\text{SCoA} + \text{HCO}_3^- + \text{ATP} \rightleftharpoons \text{CH}_3\text{CH}^-(\text{CH}_2\text{O}^-\text{O}^-\text{O}^-)^\text{SCoA} + \text{P}_i \text{(containing 1 ¹⁸O atom)} + \text{ADP (containing no excess ¹⁸O)} \quad (2)
\]

Maruyama et al. (4) used P-enolpyruvate carboxylase (EC 4.1.1.31) and observed that ¹⁸O was incorporated into the orthophosphate and oxaloacetate in a ratio of 1:2, which is in accord with Reaction 3.
P-Enolpyruvate + HC\textsuperscript{18}O\textsubscript{2} \rightarrow \text{Pi} \rightarrow \text{Pi} + CO\textsubscript{2} + Pi \quad (3)

Although the \textsuperscript{18}O results appear quite unequivocal, the method does involve one basic assumption. It is that OH\textsuperscript{-} or water at the active site of the enzyme is in equilibrium with the solvent water. The reaction could occur as illustrated in Reaction 4 where \textit{E} is the enzyme, P-Pyr is P-enolpyruvate, OA is oxalacetate, and P-OA is P-enoloxalacetate.

$$E + OH^- \rightarrow E + \text{CO}_2$$

The reactions were conducted at pH 8.0, and, therefore, practically all the CO\textsubscript{2} was in the form of bicarbonate. Conceivably, on reaching the site, HC\textsuperscript{18}O\textsubscript{3} could be converted to CO\textsubscript{2} and \textsuperscript{18}OH\textsuperscript{-}, and then the CO\textsubscript{2} could react to form P-enoloxalacetate, which is subsequently cleaved to oxalacetate by the reaction involving the OH\textsuperscript{-}. If this occurred and the OH\textsuperscript{-} at the site were protein-bound and equilibrated slowly with the solvent water, the bound OH\textsuperscript{-} by turnover would acquire \textsuperscript{18}O equal to that of the bicarbonate. Possibly, if the reaction were conducted at pH 6 or lower, not as much \textsuperscript{18}O would have been converted to the phosphate. Kaziro \textit{et al.} (3) did conduct a control with H\textsubscript{2}\textsuperscript{18}O and HC\textsuperscript{18}O\textsubscript{3}\textsuperscript{-}. They found incorporation of \textsuperscript{18}O into the orthophosphate, but there was exchange of \textsuperscript{18}O from the H\textsubscript{2}\textsuperscript{18}O with the HC\textsuperscript{18}O\textsubscript{3}\textsuperscript{-}, and they estimated that all the \textsuperscript{18}O entered the phosphate via the resulting HC\textsuperscript{18}O\textsubscript{2} and none via H\textsubscript{2}\textsuperscript{18}O. However, as noted above, if the \textsuperscript{18}O at the site did not equilibrate with the solvent water, a hydrolytic or hydroxyl cleavage would not necessarily reflect the \textsuperscript{18}O of the solvent H\textsubscript{2}\textsuperscript{18}O as was assumed by them.

In view of these considerations and since we had available three highly purified enzymes which catalyzed CO\textsubscript{2} fixations, it seemed worthwhile to investigate further the problem of CO\textsubscript{2} species. The enzymes studied were P-enolpyruvate carboxykinase (EC 4.1.1.32) which catalyzed Reaction 5, P-enolpyruvate carboxytransphosphorylase (EC 4.1.1.38) which catalyzed Reaction 6, and pyruvate carboxylase (EC 6.4.1.1) which catalyzed Reaction 7.

P-Enolpyruvate + CO\textsubscript{2} + GDP ➙ \text{Mg}^2+ \text{oxalacetate} + GTP \quad (5)

P-Enolpyruvate + CO\textsubscript{2} + Pi ➙ \text{Mg}^2+ \text{oxalacetate} + PP\textsubscript{i} \quad (6)

Pyruvate + HCO\textsubscript{3} + ATP ➙ \text{Mg}^2+ \text{oxalacetate} + ADP + Pi \quad (7)

Reactions 5 and 6 resemble Reaction 3 except that, instead of cleavage of the phosphate group of P-enolpyruvate to inorganic phosphate, the high energy phosphoryl group is preserved in GTP or PP\textsubscript{i}. Because of this similarity, Chang \textit{et al.} (5) and Maruyama \textit{et al.} (4) have considered that HCO\textsubscript{3} may be the functional species in Reactions 5 and 6 as well as in Reaction 3. Likewise, following the studies by Kaziro \textit{et al.} (3) it has generally been considered that all fixations of CO\textsubscript{2} by biotin enzymes occur with HCO\textsubscript{3}-. It therefore was of interest to investigate a second biotin enzyme, pyruvate carboxylase.

This report presents two types of experiments which were used to determine the species of CO\textsubscript{2} utilized in the reactions. The results indicate that CO\textsubscript{2} is the reactive species in Reactions 5 and 6 and HCO\textsubscript{3} (or HCO\textsubscript{2}) is the reactive species in Reaction 7. A preliminary communication describing part of this work has appeared (6).
labeled with $^{14}$C, and the accompanying member of the pair was unlabeled.

Following initiation of the reaction, the mixture was sampled at approximately 20-sec intervals. The 0.1-ml samples were quickly transferred to pieces of filter paper which had been previously saturated with formic acid. These slips of paper were then sprayed with formic acid and allowed to stand 15 min before being dried in a 90° oven. The dried samples were sprayed with formic acid a second time and allowed to again stand for 15 min. After being dried the samples were placed in scintillation vials, and the total radioactivity was determined in a Packard Tri-Carb scintillation spectrometer. Deviation from this treatment resulted in highly erroneous amounts of radioactivity being retained upon the filter paper, perhaps in the form of carbamates (13). In order to determine the validity of the assumption that all of the acid-stable radioactivity was malic acid, the reaction products were co-chromatographed with authentic $r_{4}$C-malic acid in a solvent system of butanol, formic acid, and water (5:1:4, v/v/v). Only one radioactive species was found, and its $R_{F}$ value of 0.41 coincided exactly with that of the malic acid standard.

**THEORETICAL AND EXPERIMENTAL RESULTS**

**Spectrophotometric Assays**—The equilibrium between $CO_{2}$, $HCO_{3}^{-}$, and $H_{2}CO_{3}$ may be represented as follows.

\[
\begin{align*}
K_{11} & : HCO_{3}^{-} + H^{+} \\
K_{12} & : H_{2}CO_{3} \\
K_{13} & : CO_{2}
\end{align*}
\]

where $K_{11} = K_{12} + K_{13}, K_{12} = K_{13}/K_{HCO_{3}^{-}}$. Using the values for these constants ($K_{11} = 0.0375$ sec$^{-1}, K_{13} = 5.5 \times 10^{-4}$ M$^{-1}$ sec$^{-1}$) that have been accurately determined by Gibbons and Edsall (12) and Ho and Sturtevant (14), one can calculate the rate of hydration of $2.5 \times 10^{-3}$ M $CO_{2}$ or the rate of dehydration of $2.5 \times 10^{-3}$ M $HCO_{3}^{-}$ with respect to time (Fig. 1, A and C). From these rates, one can determine the variations in the concentrations of $CO_{2}$ and $HCO_{3}^{-}$, and these are shown in Fig. 1, B and D.

When these considerations are applied to the enzymatic reactions, the theoretical rates of formation of oxalacetate may be calculated by assuming that the rate of fixation is directly proportional to the concentration of the active species of $CO_{2}$. This assumption is valid if the concentrations of the $CO_{2}$ species are below the $K_{m}$ values. The $K_{m}$ for $HCO_{3}^{-}$ plus $CO_{2}$ with carboxytransphosphorylase is 9.5 mm at pH 7.8 and 4.0 mm at pH 8.5 (8), that of P-enolpyruvate carboxykinase is 20 mm at pH 7.5 (7), and that of pyruvate carboxylase is 1 mm at pH 7.4 (15). Thus, if the $K_{m}$ is calculated on the basis of either species it is $3$ mm or above, except for pyruvate carboxylase. The fact that the concentration of the added species was in fact greater than the $K_{m}$ with pyruvate carboxylase does not invalidate the procedure. The results will, in general, be the same, but the inflection of the curves will be somewhat less and thus the sensitivity of the method.

The rate of hydration of $CO_{2}$ may be written

\[
\frac{-d(CO_{2})}{dt} = K_{11}(CO_{2})K_{12}(HCO_{3}^{-})(HCO_{3}^{-})
\]

FIG. 1. A, estimated rate of change of concentration of $CO_{2}$ after addition of $2.5 \times 10^{-3}$ M $CO_{2}$; B, the variation of the concentrations of $CO_{2}$ and $HCO_{3}^{-}$ with time following addition of $2.5 \times 10^{-3}$ M $CO_{2}$; C, the change of concentration of $HCO_{3}^{-}$ after addition of $2.5 \times 10^{-3}$ M $HCO_{3}^{-}$; D, variation of the concentrations of $HCO_{3}^{-}$ and $CO_{2}$ after addition of $2.5 \times 10^{-3}$ M $HCO_{3}^{-}$. Calculations were made with the constants of Gibbons and Edsall (12) for a system at 25°, pH 7.0, and low ionic strength.

The curves of Fig. 2A have been derived for the situation when $CO_{2}$ is the species utilized; those of Fig. 2B depict an instance when $HCO_{3}^{-}$ (or $H_{2}CO_{3}$) is the active species. In each figure the curve designated $CO_{2}$ or $HCO_{3}^{-}$ is for the case when the reaction is initiated with this species; that labeled C.A. indicates that carbonic anhydrase is present. In the absence of carbonic anhydrase, if $CO_{2}$ is the active species, the reaction proceeds with a high initial rate when $CO_{2}$ is added and at a low

FIG. 2. Estimated theoretical formation of oxalacetate for the spectrophotometric assay: A, if the active species used in the fixation is $CO_{2}$ and B, if it is $HCO_{3}^{-}$. The lines designated $CO_{2}$ are for the situation when $CO_{2}$ is the initially added species, the $HCO_{3}^{-}$ lines indicate that $HCO_{3}^{-}$ is the initially added species, and those designated C.A. indicate the situation when either $CO_{2}$ or $HCO_{3}^{-}$ is added in the presence of carbonic anhydrase. The calculations have been made on the basis that the rate of fixation is directly proportional to the concentration of the active species with the use of the values shown in Fig. 1. OAA, oxalacetic acid. Absolute values are not given for the concentration of OAA, since the amount is proportional, but not necessarily equal, to the concentration of the active species.
initial rate when \( \text{HCO}_3^- \) is added (Fig. 2A). After approximately 50 sec, when equilibrium among the three species of \( \text{CO}_2 \) is reached (Reaction 8), the rate becomes linear and identical, regardless of the species of \( \text{CO}_2 \) added. At that time the rate is the same as that observed in the presence of carbonic anhydrase, with which equilibrium is reached almost instantaneously.

When \( \text{HCO}_3^- \) is the active species, the situation is reversed (Fig. 2B); the initial rate is more rapid when \( \text{HCO}_3^- \) is added and slower when \( \text{CO}_2 \) is added. In Fig. 2, A and B, the difference from the rate observed when carbonic anhydrase is present is greater when the reaction is initiated with \( \text{CO}_2 \) than when initiated with \( \text{HCO}_3^- \). This occurs because there is a much greater change in concentration of both the \( \text{CO}_2 \) and \( \text{HCO}_3^- \) when \( \text{CO}_2 \) is added (Fig. 1B) than when \( \text{HCO}_3^- \) is added (Fig. 1D). Thus, initiation of the reaction with \( \text{CO}_2 \) provides the best indicator, no matter whether \( \text{CO}_2 \) or \( \text{HCO}_3^- \) is the reactive species.

It should be mentioned that the curves of Fig. 1 were obtained with rate constants for 25° and low ionic strength and assume a direct proportionality between the rate of oxalacetate formation and the concentration of the active species of \( \text{CO}_2 \). Actually the experiments were performed at 10°, which would slow down the attainment of equilibrium between \( \text{CO}_2 \) and \( \text{HCO}_3^- \), and at higher ionic strength, which would speed up the attainment of this equilibrium. However, qualitatively, one should obtain curves that are similar in shape to those depicted in the theoretical curves.

The experimental results obtained with \( \text{P-enolpyruvate carboxykinase} \) and \( \text{P-enolpyruvate carboxytransphosphorylase} \) by the spectrophotometric method are shown in Figs. 3 and 4. This method was not used with \( \text{pyruvate carboxylase} \). It is a biotin enzyme and it seemed likely that the active species would be \( \text{HCO}_3^- \). The radiochemical assay to be described later was considered to be the most reliable for the \( \text{HCO}_3^- \) species. Fig. 3 shows the results obtained with \( \text{P-enolpyruvate carboxykinase} \). It is seen that a constant and identical rate was obtained when \( \text{CO}_2 \) was the initiating species is not known. The concentration of the species added was \( 1.8 \times 10^{-3} \) M instead of the \( 2.5 \times 10^{-3} \) M used for the calculations, but this does not alter the relative shapes of the expected curves.

The experimental results with \( \text{P-enolpyruvate carboxytransphosphorylase} \) are shown in Fig. 4. Here again, the initial rate was high and then decreased when \( \text{CO}_2 \) was the species used to

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**Fig. 3.** Spectrophotometric assay of \( \text{P-enolpyruvate carboxykinase} \) activity when either \( \text{CO}_2 \) (■—■) or \( \text{HCO}_3^- \) (O—O) is added initially. The complete mixture contained, in a volume of 1.39 ml: 2.4 \( \times 10^{-4} \) M Tris buffer, pH 7.60; 3.6 \( \times 10^{-4} \) M GSH; 5.0 \( \times 10^{-4} \) M GDP; 5.9 \( \times 10^{-4} \) M \( \text{P-enolpyruvate} \); 2.5 \( \times 10^{-4} \) M \( \text{MgCl}_2 \); 1.2 \( \times 10^{-4} \) M phosphate buffer, pH 7.60; 4.0 \( \times 10^{-4} \) M \( \text{P-enolpyruvate} \); 2.0 \( \times 10^{-4} \) M \( \text{HCO}_3^- \) or 2.0 \( \times 10^{-4} \) \( \text{HCO}_2^- \) plus 2.0 \( \times 10^{-4} \) M \( \text{HCl} \); (when \( \text{CO}_2 \) was the desired species); 2.0 \( \times 10^{-4} \) M \( \text{NADH} \); 75 \( \mu \)g of malate dehydrogenase; and 0.10 mg of carboxytransphosphorylase preparation. Temperature, 10°. The same experiments were done in the presence of 100 \( \mu \)g of \( \text{carbonic anhydrase} \), \( \text{CO}_2 \) plus 100 \( \mu \)g of \( \text{carbonic anhydrase} \) and 0.10 mg of \( \text{carboxytransphosphorylase} \) preparation. Temperature, 10°.

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**Fig. 4.** Spectrophotometric assay of \( \text{P-enolpyruvate carboxytransphosphorylase} \) activity when either \( \text{CO}_2 \) (■—■) or \( \text{HCO}_3^- \) (O—O) is added initially. The complete mixture contained, in a volume of 1.25 ml: 2.4 \( \times 10^{-4} \) M Tris buffer, pH 7.60; 1.6 \( \times 10^{-4} \) M \( \text{MgCl}_2 \); 1.2 \( \times 10^{-4} \) M phosphate buffer, pH 7.60; 4.0 \( \times 10^{-4} \) M \( \text{P-enolpyruvate} \); 2.0 \( \times 10^{-4} \) M \( \text{HCO}_3^- \) or 2.0 \( \times 10^{-4} \) \( \text{HCO}_2^- \) plus 2.0 \( \times 10^{-4} \) M \( \text{HCl} \); (when \( \text{CO}_2 \) was the desired species); 2.0 \( \times 10^{-4} \) M \( \text{NADH} \); 75 \( \mu \)g of malate dehydrogenase; and 0.10 mg of carboxytransphosphorylase preparation. Temperature, 10°.
initiate the reaction. With HCO$_3^-$ there was a slight indication that the initial reaction was slower and then increased, but the results were the same whether carbonic anhydrase was present or not. Again the results are clearly more similar to the theoretical curves of Fig. 2A than of Fig. 2B and indicate that CO$_2$ is the active species for carboxytransphosphorylase.

Radiochemical Assay—Another approach to determine the active species is to use a mixture of CO$_2$ and HCO$_3^-$ with only one of the pair containing $^{14}$C. The addition of the nonradioactive species of CO$_2$ assures that prior to isotopic equilibrium one member of the pair will have a higher specific activity than the other. The $^{14}$C incorporated into oxalacetate is converted to malate with the use of malate dehydrogenase.

The theoretical results are shown in Figs. 5 and 6. The changes in the concentration of CO$_2$ and HCO$_3^-$ are shown in Fig. 5A and have been calculated with the constants of Gibbons and Edsall (12) and Equation 10. In addition, by assuming the initial specific activity of one of the species to be 1 and the other to be 0, and by use of Equation 10, the amount of $^{14}$C transferred from one pool to the other in a given interval has been estimated. The change in specific activity of the CO$_2$ and HCO$_3^-$ with time was then calculated. The change when CO$_2$ is the initially labeled species is shown in Fig. 5B, and that when it is HCO$_3^-$ is shown in Fig. 5C.

When these values are applied to the enzymatic reactions, the theoretical incorporation of $^{14}$C into oxalacetate (malate) may be calculated by assuming CO$_2$ to be the active species as shown in Fig. 6A and by assuming that HCO$_3^-$ is the active species in Fig. 6B. For the calculations of Fig. 6A it was assumed that the fixation is directly proportional to the CO$_2$ concentration shown in Fig. 5A and that the CO$_2$ at any given time had the specific activity shown in Fig. 5B or C. The CO$_2$ line of Fig. 6A depicts the situation when CO$_2$ is the source of the label and the HCO$_3^-$ line illustrates that when HCO$_3^-$ is the source. The specific activities of CO$_2$ of Fig. 5B were used for the former calculations, and those of the CO$_2$ of Fig. 5C for the latter. Line C.A. of Fig. 6A is for the case when carbonic anhydrase is present and either species is labeled. The results are the same with either labeled species since isotopic equilibrium is attained almost instantly with carbonic anhydrase present.

Similar calculations were made for Fig. 6B, in which HCO$_3^-$ is the active species, by use of the HCO$_3^-$ concentrations of Fig. 5A and the specific activities of the HCO$_3^-$ of Fig. 5, B and C, respectively.

It is seen in Fig. 6A, when CO$_2$ is the active species and CO$_2$ is the source of $^{14}$C, that the incorporation of $^{14}$C into the oxalacetate is rapid during the first 50 sec and then levels off to a rate similar to that when carbonic anhydrase is present (Line C.A. of Fig. 6A). When HCO$_3^-$ is added (HCO$_3^-$ of Fig. 6A), the rate of $^{14}$C incorporation is slower for about 30 sec than that with

![Fig. 5](image-url)

**Fig. 5.** A, estimated change in the concentration of CO$_2$ and HCO$_3^-$ when $5 \times 10^{-3}$ M of each is added at zero time; B, estimated change in radioactivity of CO$_2$ and HCO$_3^-$ when the initial CO$_2$ is radioactive with a specific activity of 1.0 and the HCO$_3^-$ is unlabeled; C, estimated change in radioactivity of CO$_2$ and HCO$_3^-$ when the initial HCO$_3^-$ is radioactive with a specific activity of 1.0 and the CO$_2$ is unlabeled. Calculations were made with the constants of Gibbons and Edsall (12) for a system at 25°, pH 7.0, and low ionic strength.
carbonic anhydrase present, but the difference is not as great as when CO₂ is the source of the label. Fig. 6B shows the results when HCO₃⁻ is the active species. The rate of ¹⁴C incorporation is faster if H⁺HCO₃ is the initial labeled species than it is in the presence of carbonic anhydrase, and when the initial labeled species is CO₂ it is slower. In Fig. 6, A and B, the difference from that with carbonic anhydrase is greater with CO₂ as the labeled species than with HCO₃⁻; thus, labeled CO₂ theoretically is the best indicator, no matter which species is utilized in the reaction.

Experimentally, the three enzymes, P-enolpyruvate carboxykinase, carboxytransphosphorylase, and pyruvate carboxylase, behaved quite differently. With the first two enzymes (Figs. 7 and 8), a high initial rate of formation of oxalacetate-¹⁴C was observed when H¹⁴CO₃⁻ and ¹⁴CO₂ were added. In contrast, with pyruvate carboxylase (Fig. 9), a high initial rate of formation of oxalacetate-¹⁴C was observed when H¹⁴CO₃⁻ and ¹⁴CO₂ were added. The latter results indicate that HCO₃⁻ is the reactant (compare Figs. 9 and 6B). These results are in agreement with the conclusions of Kaziro et al. (3) from their studies with ¹⁴O and the biotin-containing enzyme, propionyl-CoA carboxylase. Thus, there are two lines of evidence that HCO₃⁻ is the active species for the enzymes of CO₂ fixation which contain biotin. It will be noted that the equilibration time of the pyruvate carboxylase system was approximately 4.5 times shorter than that observed in the case of the P-enolpyruvate carboxykinase and carboxytransphosphorylase. This is very close to the predicted behavior, since the cold lability of the avian enzyme preparation necessitated that the analysis be carried out at 25° rather than at 10°. Experiments with carbonic anhydrase were not done with pyruvate carboxylase, owing to the limited amount of pyruvate carboxylase which was available to us at that time.

With P-enolpyruvate carboxykinase (Fig. 7) and carboxytransphosphorylase (Fig. 8) the high initial rate of incorporation of ¹⁴C into oxalacetate occurred when H¹⁴CO₃⁻ was the labeled species, an indication that CO₂ is the active species for these two enzymes. The results are in agreement with those of the spectrophotometric assay described in the preceding section. However, there was some discrepancy from the theoretical curves of Fig. 6A. With P-enolpyruvate carboxykinase (Fig. 7), the rate of ¹⁴C incorporation with H¹⁴CO₃⁻ was linear from time zero, whereas it was expected to be slow at first and then increase to a constant rate.
However, with carboxytransphosphorylase (Fig. 8), the slow initial rate was observed in accordance with expectations. A partial explanation of the discrepancy may be that HCO₃⁻ was always added prior to the CO₂ (see "Materials and Methods"). This might mask the expected slow rate with H¹⁴CO₃⁻, since highly active H¹⁴CO₃⁻ would be formed from the H²⁰CO₃⁻ prior to the addition of the H¹⁴CO₃⁻ (time, about 6 sec). A further discrepancy is noted with carbonic anhydrase. The initial value in both Figs. 7 and 8 was somewhat greater than the subsequent constant rate. Again, the reason for this divergence from the theoretical curves of Fig. 6A is not known.

**DISCUSSION**

The present results indicate that CO₂ rather than HCO₃⁻ (or H₂CO₃) is the active species of the reactions catalyzed by P-enolpyruvate carboxykinase and by P-enolpyruvate carboxytransphosphorylase. Apparently, the mechanisms of these fixation reactions differ significantly from that catalyzed by P-enolpyruvate carboxylase which, according to the ^¹⁸O labeling observed by Maruyama et al. (4), involves HCO₃⁻ (or H₂CO₃) as a reactant. It would be interesting to check the carboxykinase and carboxytransphosphorylase reactions by the ^¹⁸O method and the carboxylase by the spectrophotometric and radiochemical procedures. It is conceivable that the latter two methods are not a reflection of the mechanism per se, but rather a reflection of the rate of binding at the active site. For example, the charged HCO₃⁻ might be hindered from approaching the active site and CO₂ might more readily approach the site. At the active site CO₂ might react with H₂O to form H₂CO₃⁻ and in this form undergo the actual chemical reaction. In this case only 2 ^¹⁸O atoms would enter the products from HCO₃⁻ but ^¹⁸O from H₂¹⁸O should enter the product very effectively. Thus, neither the ^¹⁸O studies (see the Introduction) nor the spectrophotometric and radiochemical assays provide conclusive evidence that the species involved in the actual chemical mechanism is CO₂ or HCO₃⁻. However, with carboxytransphosphorylase there was an indication prior to the present studies that bicarbonate per se need not be involved in a nucleophilic attack on the enolphosphorylphosphorous atom, which would lead to cleavage of the P—O bond as proposed by Maruyama et al. (4) and Chang et al. (5). Lochmüller et al. (8) have shown that carboxytransphosphorylase catalyzes cleavage of the enolphosphate bond in the absence of CO₂, as indicated in Reaction 11.

\[
P\text{-enolpyruvate} + P + H^+ \rightarrow \text{pyruvate} + PP_i \quad (11)
\]

Thus, CO₂ is not required for the cleavage of the bond for the combination of the phosphate with the orthophosphate to form inorganic pyrophosphate.

The results from these experiments are in agreement with results of Kaziro et al. (3) and indicate that HCO₃⁻ (or H₂CO₃) is the reactant in CO₂ fixation by biotin enzymes. This confirmation is significant because our methods did not involve the same assumption as those of the experiments of Kaziro et al. (3) with ^¹⁸O. Although we have noted that the methods cannot be considered conclusive, it seems reasonable to assume that agreement between the methods is fairly strong evidence that HCO₃⁻ (or H₂CO₃) is the reactant with the biotin enzymes. It is to be noted that the manometric method of Krebs and Roughton (1) only gives an "overshoot" if CO₂ is the reactant. If HCO₃⁻ (or H₂CO₃) is the reactant, carbonic anhydrase has little effect in the manometric measurement, whereas in the spectrophotometric or radiochemical assay, carbonic anhydrase has an effect no matter which species is involved.

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**REFERENCES**

The Carboxylation of Phosphoenolpyruvate and Pyruvate: I. THE ACTIVE SPECIES OF "CO2" UTILIZED BY PHOSPHOENOLPYRUVATE CARBOXYKINASE, CARBOXYTRANSPHOSPHORYLASE, AND PYRUVATE CARBOXYLASE

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