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Phosphoenolpyruvate Carboxytransphosphorylase. III. Comparison of the Fixation of Carbon Dioxide and the Conversion of Phosphoenolpyruvate and Phosphate into Pyruvate and Pyrophosphate*

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ABSTRACT: Carboxytransphosphorylase from propionibacteria catalyzes the conversion of phosphoenolpyruvate, P_i, and CO_2 into oxalacetate and PP_i and when CO_2 is excluded the products are pyruvate and PP_i. The latter reaction has not been studied previously. The stoichiometry of the pyruvate reaction has been determined and evidence is presented that both reactions are catalyzed by the same enzyme. A comparison of the characteristics of the two reactions is presented. The CO_2 fixation reaction is reversible but the pyruvate reaction is experimentally irreversible. Both reactions have about the same pH optimum. A divalent metal is required for either reaction and Mg^{2+}, Co^{2+}, or Mn^{2+} meets this requirement. The apparent K_m values for the pyruvate reaction are Mg^{2+}, 6.3×10^{-4} M; Co^{2+}, 2.3×10^{-4} M; Mn^{2+}, 4×10^{-5} M, phosphoenolpyruvate, 3.6×10^{-5} M; and P_i, 6.6×10^{-4} M. These values are quite similar to those previously observed for the CO_2 fixation reaction except for that of phosphoenolpyruvate and Mn^{2+} which are about tenfold lower for the pyruvate reaction. It has been shown that the presence of bicarbonate alters the apparent K_m for phosphoenolpyruvate in the pyruvate reaction to a value approaching that found for the fixation of CO_2. Both the pyruvate reaction and the CO_2 fixation reaction are inhibited by 10^{-5} M EDTA even in the presence of 12 mM Mg^{2+} and also by other metal chelators. It is postulated that two types of metals are required: type I which is dissociable (*i.e.*, Mg^{2+}, Co^{2+}, or Mn^{2+}) and an unidentified metal, type II, which is firmly bound to the carboxytransphosphorylase and whose function is blocked by the chelators. Co^{2+} removes the EDTA inhibition of the oxalacetate reaction and Cu^{2+} of the pyruvate reaction. This difference in the two reactions is believed to arise because in addition to type I and type II metals the pyruvate reaction requires a heavy metal. The Co^{2+} complexes the EDTA and thus removes it from the type II metal-restoring activity for CO_2 fixation. The Cu^{2+} removes the EDTA from the type II metal and also serves as the heavy metal thus restoring the pyruvate reaction. Cu^{2+} inhibits the CO_2 fixation reaction. Thiols such as mercaptoethanol stimulate the rate of the oxalacetate reaction but inhibit the pyruvate reaction. The thiols may act by complexing the heavy metals and thereby stimulate the CO_2 fixation reaction but inhibit the pyruvate reaction. Further evidence of this role of thiols is the fact that the EDTA-treated enzyme is fully active in the oxalacetate reaction in the presence of Co^{2+} but without addition of thiol. It thus appears that different forms of the enzyme catalyze the two reactions. The pyruvate reaction apparently is not required for the conversion of phosphoenolpyruvate into pyruvate in propionibacteria since they contain pyruvate kinase. It seems likely that the physiological role of carboxytransphosphorylase is for fixation of CO_2 to yield oxalacetate rather than for formation of pyruvate.

Phosphoenolpyruvate carboxytransphosphorylase (pyrophosphate:oxalacetate carboxylyase (phosphorylating), EC 4.1.1.38) catalyzes reactions 1 and 2. The enzyme occurs in propionic acid bacteria and has been obtained in crystalline form by Lochmüller *et al.* (1966). The catalysis of reaction 1 both in the forward reaction to form oxalacetate from phosphoenolpyruvate and in the back-reaction to form phosphoenolpyruvate from oxalacetate and inorganic pyrophosphate has been

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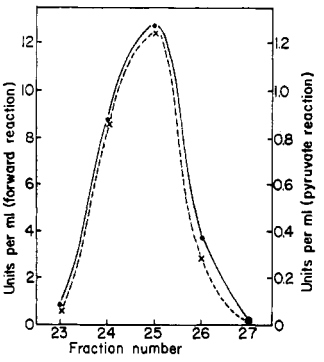

FIGURE 1: Correlation of activity in the forward and pyruvate reaction of fractions obtained from a cellulose phosphate column. Broken line is for the pyruvate reaction, solid line for the oxalacetate reaction. Conditions for the oxalacetate and pyruvate reactions are described in Materials and Methods.

$$\text{phosphoenolpyruvate} + CO_2 + P_i \rightleftharpoons \text{oxalacetate} + PP_i \quad (1)$$

$$\text{phosphoenolpyruvate} + P_i \longrightarrow \text{pyruvate} + PP_i \quad (2)$$

described previously (Siu and Wood, 1962; Lochmüller *et al.*, 1966; Wood *et al.*, 1966; Wood *et al.*, 1969a). The present paper primarily deals with reaction 2, which we will refer to as the pyruvate reaction and comparison of its properties with that of the CO_2 fixation or oxalacetate reaction. Papers dealing with the role of metals and the mechanism of the two reactions are also presented (Willard *et al.*, 1969; Wood *et al.*, 1969b).

Materials and Methods

Phosphoenolpyruvate and β-NADH were purchased from Sigma Chemical Co.; ADP and NADP from P-L Biochemicals Inc.; β-mercaptoethanol from Eastman Kodak Co.; pyruvate and oxalacetate from California Corp. for Biochemical Research; lactate dehydrogenase, pyruvate kinase, hexokinase, and glucose 6-phosphate dehydrogenase were Boehringer products obtained from California Corp. for Biochemical Research. Chelex 100 (analytical grade, 50–100 mesh) as the sodium form was from Bio-Rad Laboratories. $^{32}P_i$ was a gift from Dr. Cecil Cooper and had been chromatographed on Dowex 1-Cl. The other chemicals were reagent grade obtained from commercial sources. Malate dehydrogenase was purified from propionic acid bacteria (Allen *et al.*, 1964) and had a specific activity of 500.

Protein determinations were carried out by ultraviolet absorption (Warburg and Christian, 1941) using the relationship: mg of protein/ml = $1.45\ A_{280} - 0.74\ A_{260}$. A unit of enzyme is expressed as micromoles of product formed per minute at 25°, and the specific activity as units per milligram of protein. Most of the carboxytransphosphorylase used in these studies was crystalline material isolated as described by Lochmüller *et al.* (1966) and Wood *et al.* (1969a). It had a specific activity of about 18 but had lost activity during prolonged storage.

Assays of Carboxytransphosphorylase. Catalysis of the forward and back-reactions by carboxytransphosphorylase (reaction 1) is stimulated by mercaptoethanol but the pyruvate reaction (reaction 2) is inhibited. All specific activities of carboxytransphosphorylase, unless otherwise stated, are given in terms of the optimum forward reaction, which is the most rapid of the three.

Assay of the Pyruvate Reaction. The pyruvate reaction is determined by linking carboxytransphosphorylase with lactate dehydrogenase and measuring the disappearance of NADH. The reaction mixture contains in micromoles per milliliter: phosphoenolpyruvate, 0.6; potassium phosphate (pH 6.8), 10; $MgCl_2$, 12; and NADH, 0.12. A mixture containing these components 1.32 times more concentrated than the final concentration to be used in the assay is prepared and N_2 gas is bubbled through the solution for 30 min to remove any dissolved CO_2. CO_2 has been shown to inhibit the pyruvate reaction (Wood *et al.*, 1969b); 0.25 ml of this mixture, water, lactate dehydrogenase (0.2 unit), and carboxytransphosphorylase are added to a 0.2 × 1.0 cm cuvet to give a final volume of 0.33 ml. The carboxytransphosphorylase is diluted in 50 mM potassium phosphate buffer (pH 6.8) (without thiols). The enzyme is quite stable at 0° in this buffer; if less concentrated phosphate buffer is used, *e.g.*, 2 mM, it is less stable. The measurement is made at 25° and the rate is linear with time for 4 or more min and with enzyme concentration.

Assay of Reaction 1. Optimum conditions for assay of the forward reaction and back-reaction, *i.e.*, formation of oxalacetate or phosphoenolpyruvate, have been described previously (Lochmüller *et al.*, 1966; Wood *et al.*, 1969a). The carboxytransphosphorylase was diluted in 50 mM phosphate buffer (pH 6.8) containing 1 mM β-mercaptoethanol, for the forward oxalacetate reaction unless stated otherwise. The diluted enzyme was held for at least 15 min before use. The treatment of the enzyme for the oxalacetate back-reaction was the same except the buffer was 2 mM phosphate instead of 50 mM.

The forward oxalacetate reaction mixture (saturated with CO_2) contained in micromoles per milliliter: phosphoenolpyruvate, 2.0; $KHCO_3$, 30.0; K_2HPO_4 (pH 6.8), 10; $MgCl_2$, 12.0; NADH, 0.125; and in units per milliliter, malate dehydrogenase, 1.8; and the carboxytransphosphorylase. Frequently 0.1 mM $CoCl_2$ was included in assay to obtain optimal activity. The oxalacetate back-reaction contained in micromoles per milliliter:oxalacetate, 3.0; PP_i, 0.5; $MgCl_2$, 4.0; Tris-HCl (pH 7.4), 15; ADP, 0.5; glucose, 1.0; NADP, 0.3; KCl, 20; and in units per milliliter, pyruvate kinase, 1.6; hexokinase, 2.5; glucose 6-phosphate dehydrogenase, 1.1; and carboxytransphosphorylase.

Results

Evidence That the Enzyme Catalyzing the Pyruvate Reaction Is Identical with That Catalyzing the Forward and Back-Reactions. It has been considered that the same enzyme catalyzes reactions 1 and 2 (Lochmüller *et al.*, 1966). If these reactions are catalyzed by the same enzyme the activity in the various reactions should correlate during the purification of the enzyme. Such a correlation is shown in Figure 1. Carboxytransphosphorylase was purified according to procedure 2 of Lochmüller *et al.* (1966) on a cellulose phosphate column as described for step III. The fractions in the eluate with 0.08 M phosphate were assayed for carboxytransphosphorylase activity in the forward direction and are indicated by the solid line of Figure 1, and for activity in the pyruvate reaction as shown by the broken line. The activity of the two reactions was found to coincide. The activity in the pyruvate reaction was about one-tenth that of the forward reaction. Mercapto-

TABLE I: Stoichiometry of the Pyruvate Reaction Catalyzed by Carboxytransphosphorylase in the Absence of CO_2.[a]

phosphoenolpyruvate + $^{32}P_i$ ⟶ pyruvate + $^{32}PP_i$

	Zero Time		40 min		Δ
	cpm × 10^{-3}/ml	μmoles/ml	cpm × 10^{-3}/ml	μmoles/ml	(μmoles/ml)
Phosphoenolpyruvate		2.00	0.00	1.27	0.73
P_i	1.725	3.40	1370	2.70[b]	0.70
PP_i		0.00	354[c]	0.68[b]	0.68
Pyruvate		0.00		0.67	0.67

[a] The mixture contained in addition to the phosphoenolpyruvate and phosphate, $MgCl_2$ (4.0 μmole), β-mercaptoethanol (1.5 μmoles), and 8.6 μg of carboxytransphosphorylase per ml. The orthophosphate contained 507 × 10^3 cpm/μmole. The mixture was gassed with N_2 and incubation was under N_2 at 25°. After 40 min the reaction was stopped by addition of acid and the oxalacetate, pyruvate, and phosphoenolpyruvate were determined in duplicate samples as described by Wood *et al.* (1966), using malate dehydrogenase, lactate dehydrogenase, and pyruvate kinase. There was no oxalacetate present. A second portion (0.2 ml) was treated with ammonium molybdate and extracted with isobutyl alcohol–benzene as described by Walters and Cooper (1965) to remove the orthophosphate. The radioactivity was determined in the aqueous layer. A third portion (0.5 ml) was placed on a Dowex 1-Cl column (0.8 × 11 cm). The orthophosphate was eluted with 0.01 N HCl (260 ml) and the pyrophosphate with 0.01 N HCl in 0.05 M KCl (240 ml). [b] The specific activity of the orthophosphate was 507 × 10^3; 1370 × 10^3/507 × 10^3 = 2.70 μmole of P_i, 354 × 10^3/507 × 10^3 = 0.68 μmole of PP_i. [c] The radioactivity in the aqueous phase after reaction with molybdate and extraction with isobutyl alcohol–benzene was equivalent to 357 × 10^3 cpm.

ethanol was present in the buffers during the purification and inhibits the pyruvate reaction.

Stoichiometry of the Pyruvate Reaction. The stoichiometry of the reaction is shown in Table I. The orthophosphate was labeled with ^{32}P and the yield of pyrophosphate was estimated from the radioactivity found in this product. Pyruvate and phosphoenolpyruvate were determined spectrophotometrically at zero time and after 40 min using lactate dehydrogenase and pyruvate kinase. The orthophosphate and pyrophosphate were separated on a Dowex 1-Cl column. Both compounds were found to be labeled and the sum of their radioactivities equaled the initial radioactivity of the $^{32}P_i$. The orthophosphate from another portion was removed as the phosphomolybdate complex by isobutyl alcohol–benzene extraction (Walters and Cooper, 1965). The same number of counts per minute were found in the residue of extraction as were accounted for as PP_i from the Dowex 1-Cl column. The micromoles of PP_i formed and the P_i utilized could be calculated since the specific activity of the $^{32}P_i$ was known. The reaction is seen to be stoichiometric. There was no conversion of $^{32}P_i$ into phosphoenolpyruvate since the reaction is irreversible (Wood *et al.*, 1969b). A portion was treated with pyrophosphatase to convert the PP_i into P_i and the phosphate was then removed as the phosphomolybdate. No significant radioactivity was found in the aqueous phase which contains the phosphoenolpyruvate.

pH Optimum of the Pyruvate Reaction. Like the forward and back-reactions of carboxytransphosphorylase (Lochmüller *et al.*, 1966), the pH optimum of the pyruvate reaction is fairly broad, being between 6.4 and 7.2, as shown in Figure 2. The enzymic activity declines sharply below 6.2 and above 7.2. The optimum is not as broad as that of the forward reaction but is similar to that of the back-reaction.

Substrate Affinity Constants. The K_m values for the substrates of the pyruvate reaction were determined as described under Materials and Methods except that the substrates being studied were omitted from the combined mixture and were varied and added separately to the reaction mixture. Initial rates were measured at 25° and expressed as millimicromoles of pyruvate formed per minute per milliliter and the concentration of substrate as millimolar. When phosphoenolpyruvate was the variable substrate and nonsaturating concentrations of orthophosphate were the fixed variable substrate, the reciprocal plots were as shown in Figure 3. Replotting the intercepts against the reciprocal of the phosphate concentration gives a K_m for orthophosphate of 6.6 × 10^{-4} M (insert of Figure 3). Data are shown in Figure 4 in which phosphate was the variable substrate and phosphoenolpyruvate at nonsaturating concentrations was the fixed substrate. A replot of the intercepts against the reciprocal of the phosphoenolpyruvate

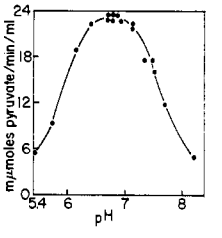

FIGURE 2: Effect of pH on the pyruvate reaction of carboxytransphosphorylase. A mixture containing all the components of the reaction except lactate dehydrogenase and carboxytransphosphorylase as described in Materials and Methods was gassed with N_2 for 30 min and the solutions were stored under N_2. A portion was adjusted with either KOH or HCl solution to the desired pH. The determinations were done in triplicate and a control with pyruvate as the substrate was done at each pH to determine that the lactate dehydrogenase was not limiting; 0.9 unit of lactate dehydrogenase and 7.2 μg of carboxytransphosphorylase were used per ml of the assay mixture.

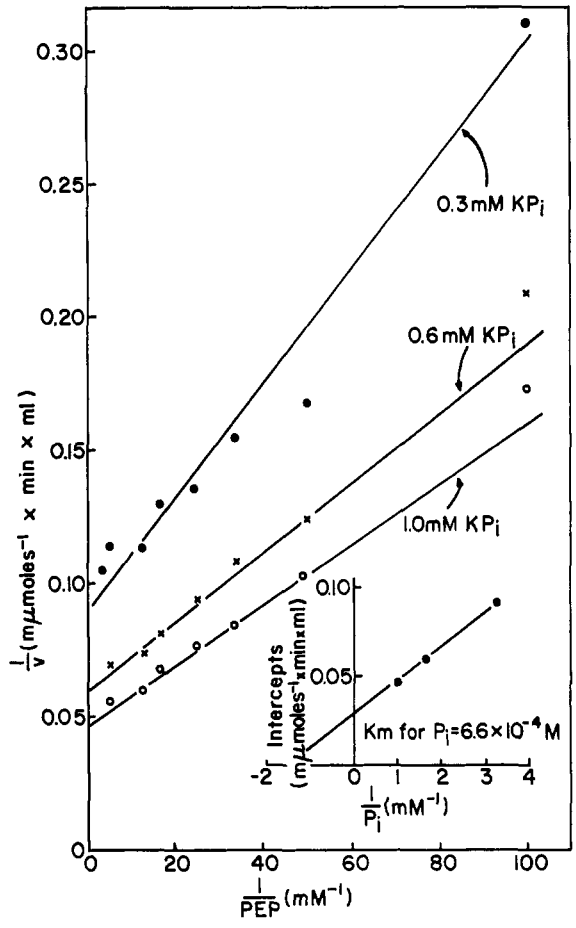

FIGURE 3: Reciprocal plots of the initial velocity of the pyruvate reaction with phosphoenolpyruvate as the varied substrate and orthophosphate as the changing fixed substrate. The mixture contained in millimolar concentrations: $MgCl_2$, 12.0; β-NADH, 0.12; phosphoenolpyruvate and potassium orthophosphate as indicated; and per milliliter, 0.6 unit of lactate dehydrogenase (dialyzed 3 hr against 5 mM Tris-HCl, pH 7.4) and 16.2 μg of carboxytransphosphorylase (specific activity 13.0) which had been diluted in 50 mM phosphate (pH 6.9). All solutions except that containing the lactate dehydrogenase and carboxytransphosphorylase were gassed for 30 min with N_2 and stored under N_2. In the insert the vertical intercepts are plotted against reciprocal concentrations of orthophosphate to obtain the K_m for orthophosphate at an infinitephos phoenolpyruvate concentration.

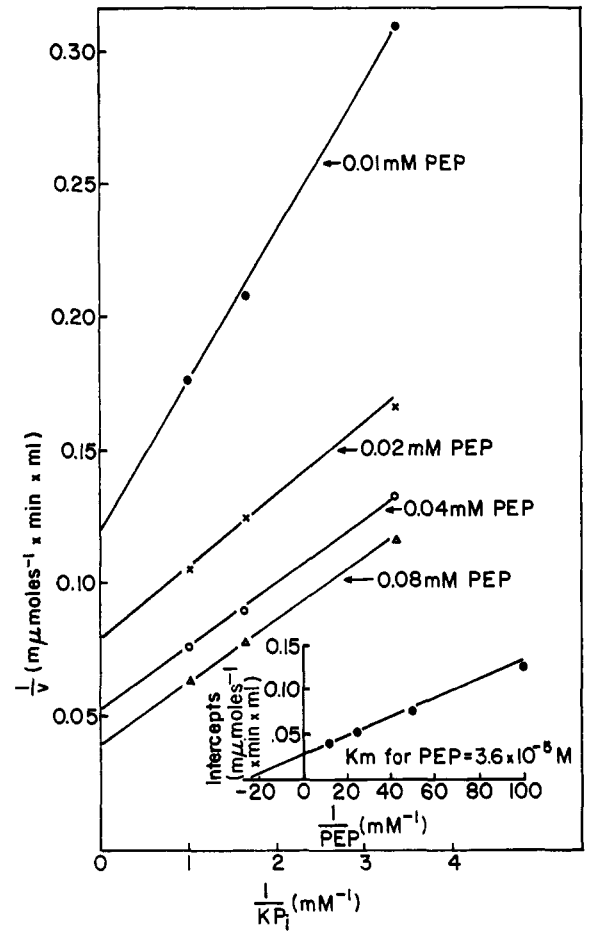

FIGURE 4: Reciprocal plots of the initial velocity of the pyruvate reaction with orthophosphate as the varied substrate and phosphoenolpyruvate as the changing fixed substrate. The reaction conditions are those as given in Figure 3. The insert shows a plot of the vertical intercepts against the reciprocal of the phosphoenolpyruvate concentration to obtain the K_m for phosphoenolpyruvate at infinite orthophosphate concentration.

concentration gives a K_m for phosphoenolpyruvate of 3.6×10^{-5} (insert of Figure 4).

It has been found that orthophosphate is inhibitory if the phosphoenolpyruvate is nonsaturating. Figure 5 shows results with variable phosphoenolpyruvate and fixed amounts of saturating concentrations of orthophosphate. It is seen that the V_{max} values are nearly the same for the different phosphate concentrations but $1/v$ is greater at low concentrations of phosphoenolpyruvate and high concentrations of orthophosphate. It has been shown previously (Lochmüller *et al.*, 1966) that there is some inhibition of carboxytransphosphorylase by salts and this inhibition appears in part to be due to increase in ionic strength. Since the V_{max} values are nearly the same at high concentration of phosphoenolpyruvate, it is evident that the inhibition by phosphate is not due to increased ionic strength. We have no definite explanation of these data but in general the results are in accord with the mechanism we have proposed (Wood *et al.*, 1969b), *i.e.*, the orthophosphate may compete with phosphoenolpyruvate by complexing with the tightly bound metal.

The reverse situation, *i.e.*, inhibition by phosphoenolpyruvate with nonsaturating concentration of phosphate does not occur to a significant extent. For example, with 0.3 mM orthophosphate there was no inhibition by 2, 4, or 6 mM phosphoenolpyruvate.

The apparent K_m values for Mg^{2+}, Co^{2+}, and Mn^{2+} are given in Figure 6. The values are: Mg^{2+}, 6.3×10^{-4} M; Co^{2+}, 2.3×10^{-4} M; and Mn^{2+}, 4.0×10^{-5} M. Mg^{2+} was slightly inhibitory at 18 mM (Figure 6A). Co^{2+} and Mn^{2+} cannot be increased much higher than 1 mM in the pyruvate reaction because each is precipitated by orthophosphate.

The K_m values for orthophosphate, Mg^{2+}, and Co^{2+} are approximately the same for the forward and pyruvate reaction (phosphate, 1.2×10^{-3} M; Mg^{2+}, 1.2×10^{-3} M; and Co^{2+}, 0.5×10^{-3} M for the forward reaction (Lochmüller *et al.*, 1966)). However, the K_m values for phosphoenolpyruvate and Mn^{2+} are each about one-tenth that observed for the forward

TABLE II: Comparison of Effect of Mercaptoethanol on the Forward and Pyruvate Reactions (Reactions 1 and 2).

Treatment of Enzyme	Concn of Thiol in Assay (M)	Rate of Forward Reaction[a] μmoles of Oxalacetate/min per mg of Enzyme	Rate of Pyruvate Reaction[a] μmoles of Pyruvate/min per mg of Enzyme
Diluted in thiol[b]	6×10^{-5}	12.1	1.5
Diluted in absence of thiol[c]	10^{-3}	11.2	1.8
Diluted in absence of thiol[c]	0	2.6	2.9

[a] The forward reaction was measured with malic dehydrogenase in the presence of bicarbonate (Lochmüller *et al.*, 1966). The conditions are given in Materials and Methods. The pyruvate reaction was assayed with lactate dehydrogenase in the absence of bicarbonate as described in the Materials and Methods section. [b] The carboxytransphosphorylase was diluted at 0° in 50 mM phosphate (pH 6.9) and 1 mM mercaptoethanol and contained 30 μg of enzyme/ml. After 15 min a portion of the solution was used in the assay. [c] The conditions were as described in *b* above except the dilution contained no thiol.

reaction (Mn^{2+}, 0.5×10^{-3} M; and phosphoenolpyruvate, 0.5×10^{-3} M; Lochmüller *et al.*, 1966). The K_m values for the forward reactions were measured with enzyme which had been treated with 1 mM mercaptoethanol and with 30 mM bicarbonate in the assay. It seemed possible that the thiol or bicarbonate might alter the carboxytransphosphorylase in such a manner as to affect the K_m for phosphoenolpyruvate. Therefore the K_m value for phosphoenolpyruvate was tested in the forward reaction with enzyme which had not been treated with thiol and at different concentrations of bicarbonate (3.0–30 mM). No significant influence of bicarbonate on the K_m was observed and the value was nearly the same (0.2–0.4 mM) as that reported previously for thiol-treated enzyme. However, when the apparent K_m for phosphoenolpyruvate was determined for the pyruvate reaction in the presence of variable fixed concentrations of bicarbonate, the situation was quite different. It is seen in Figure 7 that the slopes of the lines increase with bicarbonate concentration and the apparent K_m for phosphoenolpyruvate increased with the bicarbonate concentration from 0.8×10^{-4} to 2.4×10^{-4}. The significance of the effect of CO_2 on the apparent K_m for phosphoenolpyruvate is uncertain at present.

Comparison of the Effect of Mercaptoethanol on the Pyruvate and Forward Reactions. It has been observed previously (Lochmüller *et al.*, 1966) that catalysis by carboxytransphosphorylase of the forward and back-reactions is greatly stimulated by the presence of thiols such as cysteine, homocysteine, glutathione, β-mercaptoethanol, or dithioerythritol. It has been found that thiols inhibit the pyruvate reaction rather than stimulate it. A comparison of the effect of thiols is shown in Table II. Three conditions were tested: one with enzyme which had been treated with thiol, a second with enzyme not treated with thiol but with thiol added to the assay mixture, and a third with no thiol present. When the carboxytransphosphorylase is diluted in phosphate containing 1 mM mercaptoethanol, the rate of the forward reaction is maximal immediately and no additional thiol is required in the assay. If the enzyme is diluted without addition of thiol and no thiol is added to the assay, the rate is about one-fourth that observed in the presence of thiol (12.1 compared with 2.6, Table II). If 1 mM thiol is added to the assay mixture and none to the enzyme, the rate increases during the first 2–3 min and then becomes constant at a rate quite similar to that observed with the enzyme diluted in thiol (11.2 compared with 12.1). The situation is reversed in the pyruvate reaction; here the rate is most rapid in the absence of thiol and if thiol is present in the assay, the rate decreases during the first 2 or 3 min and then becomes constant approaching that observed when the enzyme is diluted in thiol (1.8 compared with 1.5). It is seen that the pyruvate reaction and the forward reaction proceed at about the same rate in the absence of thiol (2.9 compared with 2.6) but under optimum conditions for both the forward reaction is about 4 times faster than the pyruvate reaction (12.1 compared with 2.9).

The effect of mercaptoethanol is reversible. Dialysis of carboxytransphosphorylase to remove the mercaptoethanol lowers the activity of the enzyme in the forward reaction about fourfold and raises the activity in the pyruvate reaction about twofold. Thus the removal of thiol gives results just the opposite of the addition of thiol.

Inhibition of Carboxytransphosphorylase by EDTA. It has been noted previously (Lochmüller *et al.*, 1966) that 10^{-5} M EDTA inhibits the forward reaction. We therefore studied the effect of EDTA on all three reactions catalyzed by carboxy-

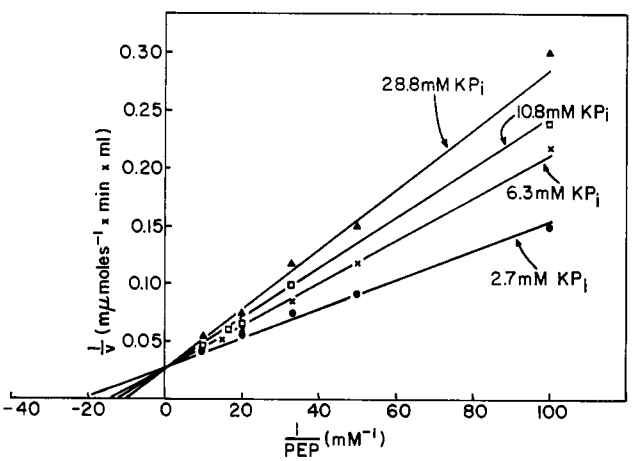

FIGURE 5: Inhibition of pyruvate reaction by high concentrations of orthophosphate with phosphoenolpyruvate as the variable substrate. The reaction conditions are given in Figure 3.

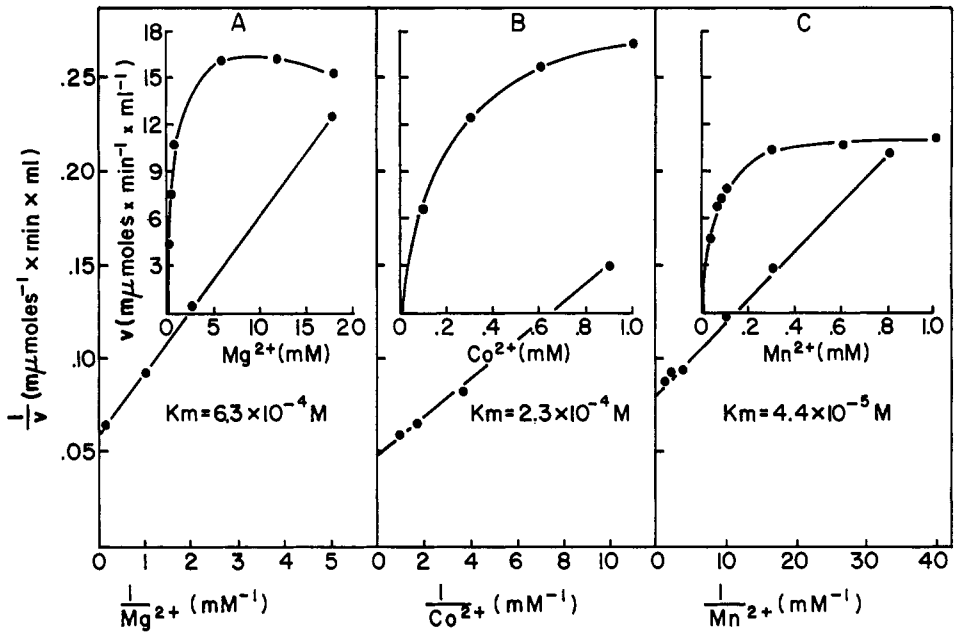

FIGURE 6: Effect of $MgCl_2$, $CoCl_2$, or $MnCl_2$ as the varied type I metal on the pyruvate reaction. The conditions were as described under Materials and Methods except for variation of the concentration of $MgCl_2$ or of the $CoCl_2$ or $MnCl_2$ which were substituted for $MgCl_2$; 7.2 μg of carboxytransphosphorylase (specific activity 6.5) diluted in 50 mM phosphate buffer (pH 6.9) was used per ml. The inset is velocity in millimicromoles of pyruvate formed per milliliter of assay plotted against millimolar concentration of Mg^{2+}, Co^{2+}, or Mn^{2+}. Part 6A is for $MgCl_2$, part 6B for $CoCl_2$, and part 6C for $MnCl_2$.

transphosphorylase. The results are shown in Figure 8. The rate of the reaction is plotted as a function of the concentration of EDTA. Rates are reported for enzyme which had been diluted in either the presence or absence of 1 mM β-mercaptoethanol. Inhibition was not great of either the forward or pyruvate reactions even at 10^{-3} M EDTA if the enzyme was not treated with mercaptoethanol. In the back-reaction the effect of EDTA is not shown at 10^{-4} and 10^{-3} M in Figure 8 because EDTA at 10^{-4} M or higher inhibits the linking enzymes.

The thiol-treated enzyme behaves quite differently toward EDTA. There was almost complete inhibition of all three reactions by 10^{-5} M EDTA. Clearly this inhibition could not be due solely to the binding of Mg^{2+} by EDTA because Mg^{2+} is present in a 1200-fold excess of the EDTA in the forward and pyruvate reactions and a 400-fold excess in the back-reaction. It appears that the inhibition is due to complexing of a metal firmly bound to the enzyme. The evidence for this conclusion will be considered in detail by Willard *et al.* (1969). It is possible that the mercaptoethanol aids in the chelation of the metal bound to the enzyme by inducing a conformational change in the protein, thus making the metal more accessible to the chelator, EDTA.

Inhibition of Carboxytransphosphorylase by Sulfhydryl Reagents. It has been shown (Table II) that thiol groups stimulate the forward reaction but inhibit the pyruvate reaction. This raised the question of whether a sulfhydryl group of the enzyme is required for the forward reaction whereas for the pyruvate reaction its presence is inhibitory. If this were the case the sensitivity of the enzyme to sulfhydryl reagents might differ in the two reactions. Tests, therefore, were done with *p*-hydroxymercuribenzoate and with *N*-ethylmaleimide. The results will not be presented in detail since no significant difference was observed in the sensitivity of the two reactions. For example, in one set of tests, the enzyme was treated at 25° with 10^{-3} or 10^{-4} M of the sulfhydryl reagent for 10 min.[1] The treated enzyme was then diluted 25-fold in buffer containing 1 mM mercaptoethanol and also in buffer without mercaptoethanol. After further incubation at 25° for 30 min the activity of the enzyme was assayed. With 10^{-3} M *p*-hydroxymercuribenzoate there was approximately 50% inhibition of both the forward oxalacetate reaction and of the pyruvate reaction, whether or not the *p*-hydroxymercuribenzoate-treated enzyme was diluted in mercaptoethanol. Tests were also made with enzymes held 24 hr at 0° in the presence of the *p*-hydroxymercuribenzoate and then diluted in buffer with and without addition of thiol. In this case the inhibition, in both reactions was nearly 100% with 10^{-3} M *p*-hydroxymercuribenzoate and 25% with 10^{-4} M *p*-hydroxymercuribenzoate with or without thiol. Thus the inhibition by *p*-hydroxymercuribenzoate was not reversed by mercaptoethanol.

With 10^{-3} M *N*-ethylmaleimide there was no significant inhibition of either reaction following treatment of the enzyme for 10 min or 24 hr. With 5×10^{-3} M *N*-ethylmaleimide after 10 min or 24 hr there was about 25% inhibition of both the pyruvate and oxalacetate reactions when the *N*-ethylmaleimide-treated enzyme was not incubated with mercaptoethanol. But

[1] Enzyme (180 μg) of specific activity 14 was placed in 0.5 ml of 50 mM phosphate buffer (pH 7) containing 10^{-3} or 10^{-4} M of the sulfhydryl reagent and held at 25° for 10 min or 0° for 24 hr; 0.02-ml portions of the solution were then diluted in 0.5 ml of 50 mM phosphate buffer (pH 7) with or without 1 mM mercaptoethanol and were held for 30 min at 25° before assay. Suitable quantities, 0.04 ml or less, were then used in 0.33 ml for assay of the activity of the enzyme in the pyruvate and oxalacetate reactions. For controls, enzyme which was not treated with sulfhydryl reagent was carried through the same dilution. The control values were used in calculations of the per cent inhibition.

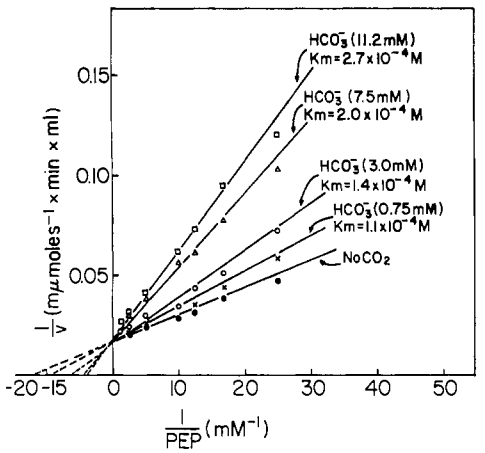

FIGURE 7: Effect of fixed bicarbonate concentrations on the initial velocity of the pyruvate reaction with phosphoenolpyruvate as the varied substrate. Reaction conditions were as described in the Materials and Methods section for the pyruvate assay except for the inclusion of freshly prepared bicarbonate and the mixture was not gassed with N_2; 12.3 μg of carboxytransphosphorylase (specific activity 16.6) diluted in the 50 mM phosphate buffer (pH 6.9) was used per ml of assay.

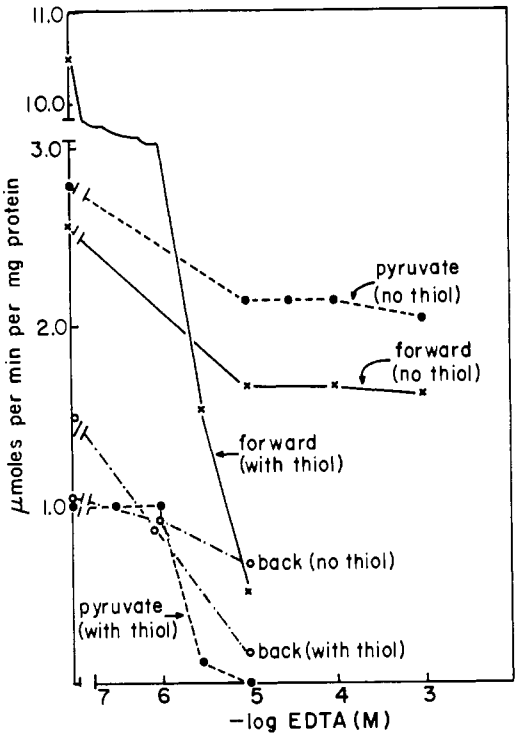

FIGURE 8: Requirement of thiol for inhibition of the three reactions of carboxytransphosphorylase by EDTA. Conditions for the respective reactions are as described under Materials and Methods and Mg^{2+} is present in all reactions. EDTA was included in the initial mixture in each assay and the reaction was started by addition of the enzyme. Five dilutions of carboxytransphosphorylase (specific activity 10.0) were made: (A) 52 μg/ml in 50 mM phosphate buffer (pH 6.9), (B) 52 μg/ml in same buffer containing 1 mM β-mercaptoethanol, (C) 13 μg/ml in same buffer containing 1 mM mercaptoethanol, (D) 91 μg/ml in 2.5 mM phosphate buffer, and (E) 91 μg in the same buffer containing 1 mM mercaptoethanol. After 20-min incubation at 0°, 6.3 μg of A and B was used per ml of assay to test the inhibition of the pyruvate reaction by EDTA. Inhibition of the forward reaction by EDTA was measured using 6.24 μg of A and 1.56 μg of C per assay. For EDTA inhibition of the back-reaction, 10.8 μg of D and E was used per ml of assay. Tests of the linking enzyme by addition of pyruvate, oxalacetate, or phosphoenolpyruvate showed that they were not inhibited by the EDTA except at 10^{-3} and 10^{-4} M in the back-reaction. Note the scale of the ordinate is changed at 3.0 μmoles. (-----) Pyruvate reaction, (———) forward reaction, and (—·—·—) back-reaction.

the inhibition was removed by incubation with mercaptoethanol.

Tests were also done with carboxytransphosphorylase which was incubated with 10^{-4} M mercaptoethanol and then treated with 5×10^{-3} M *N*-ethylmaleimide. The prior treatment with mercaptoethanol should convert the enzyme into the SH form and thus make it susceptible to the SH reagent. Subsequently, the enzyme was diluted in buffer with and without 1 mM mercaptoethanol, incubated at 25°, and assayed. Again the inhibition by *N*-ethylmaleimide was reversed with mercaptoethanol. If the inhibition by *N*-ethylmaleimide occurred by reaction with a thiol it would not be expected to be reversed by mercaptoethanol; on the other hand, that by *p*-hydroxymercuribenzoate might be expected to be reversed since such inhibition is reversible in the case of most SH enzymes. It thus seems unlikely that carboxytransphosphorylase is inhibited by *p*-hydroxymercuribenzoate and *N*-ethylmaleimide through reaction with an SH group at an active site. The expansion of how they act is not obvious at present.

Comparison of Effects of Co^{2+} and Cu^{2+} on the Pyruvate and Forward Reactions. It has been shown that the presence of thiol is required for inhibition by EDTA of the pyruvate and forward reactions as tested in Figure 8. Inhibition is observed, however, in the absence of thiol, on prolonged treatment of the enzyme with EDTA. The EDTA-treated enzyme can be activated for the forward reaction by the addition of excess Co^{2+} (Zn^{2+} or Ni^{2+}) or by Cu^{2+} for the pyruvate reaction. Table III shows the effect of Co^{2+} and Cu^{2+} with three preparations: the first in which the carboxytransphosphorylase was diluted in 50 mM phosphate buffer (pH 6.8) and 1 mM EDTA and held for 48 hr at 0°, the second in which the dilution was made in 50 mM phosphate (pH 6.8) and 1 mM mercaptoethanol, and the third in the phosphate buffer only. It is seen that in the absence of Co^{2+} or Cu^{2+}, the EDTA-treated enzyme had a low activity in either the forward or pyruvate reactions. In the presence of Co^{2+} the activity was 17.3 in the forward reaction and in the presence of Cu^{2+} 3.9 in the pyruvate reaction. These values are comparable with the activity of the enzyme under optimum conditions for each, *i.e.*, diluted in thiol for the forward reaction and in the absence of thiol for the pyruvate reaction. It is seen that 10^{-3} M Co^{2+} stimulates the activity of the enzyme in the forward reaction even when it has not been treated with EDTA but 10^{-5} M Cu^{2+} has no effect (higher concentrations of Cu^{2+} are inhibitory). Neither Co^{2+} nor Cu^{2+} has much effect on the catalysis of the pyruvate reaction with enzyme that has not been treated with EDTA.

These results will be considered in the discussion. The EDTA preparation is of particular interest since addition of thiol groups is no longer required to obtain strong catalysis of the forward reaction and in addition the enzyme responds differently to Co^{2+} and Cu^{2+} in the two reactions.

Inhibition of the Carboxytransphosphorylase by Malate and Pyrophosphate. It has previously been observed (Lochmüller *et al.*, 1966) that malate inhibits the forward reaction rather

TABLE III: Comparison of Effect of Co^{2+} and Cu^{2+} on Pyruvate and Forward Reactions by Carboxytransphosphorylase.[a]

Treatment of Enzyme	Concn of Co^{2+} in Assay (M)	Concn of Cu^{2+} in Assay (M)	Forward Reaction: μmoles of Oxalacetate/min per mg of Protein	Pyruvate Reaction: mμmoles of Pyruvate/min per mg of Protein
Held 48 hr at 0° in 50 mM phosphate and 1 mM EDTA	0	0	1.0	0.3
	10^{-3}	0	17.3	0.3
	0	10^{-5}	1.6	3.9
Diluted in 50 mM phosphate and 1 mM thiol	0	0	12.9	1.3
	10^{-3}	0	17.5	1.5
	0	10^{-5}	12.8	1.3
Diluted in 50 mM phosphate (no thiol)	0	0	2.8	3.5
	10^{-3}	0	4.1	3.5
	0	10^{-5}	2.8	3.5

[a] Crystalline carboxytransphosphorylase (51 μg/ml) (specific activity 17.5) was incubated 0.5 hr in 50 mM phosphate (pH 6.8); a second 51 μg/ml was incubated similarly in phosphate (pH 6.8) and 1 mM mercaptoethanol, a third 51 μg/ml was incubated 48 hr at 0° in phosphate (pH 6.8) and 1 mM EDTA. For the assays 1.02 μg of enzyme in the phosphate buffer alone was used per cuvet for both the forward and pyruvate reactions; 0.5 μg in phosphate and thiol for the forward reaction and 2.04 μg for the pyruvate reaction; 1.02 μg in phosphate and EDTA for the forward reaction and 2.04 μg for the pyruvate reaction.

effectively, 10 mM giving about 50% inhibition and 25 mM 90% inhibition. Likewise, inorganic pyrophosphate was a very effective inhibitor, 1 mM giving 30% inhibition and 2 mM 86% inhibition. It was of interest to determine if similar inhibition occurred in the pyruvate reaction. Experiments presented in Figure 9 show that the inhibition by these two reagents was very similar to that observed in the forward reaction. Evidence to be presented elsewhere has been obtained that pyrophosphate is a competitive inhibitor of phosphoenolpyruvate and that the K_I of pyrophosphate is the same for both reactions. Thus the results are compatible with the interpretation (Wood *et al.*, 1969b) that both the pyruvate and forward reaction in part involve a common intermediate.

Discussion

The results presented in this and the accompanying papers (Willard *et al.*, 1969; Wood *et al.*, 1969b) show that carboxytransphosphorylase catalyzes the conversion of phosphoenolpyruvate and phosphate into oxalacetate and pyrophosphate in the presence of CO_2 (reaction 1) while in the absence of CO_2 pyruvate and pyrophosphate are the products (reaction 2). Evidence is presented that the same enzyme catalyzes both reactions and that the enzyme has similar properties for both reactions such as pH optimum, K_m values, and response to inhibitors such as PP_i and malate. The reactions are believed to involve pathways which are in part the same (Wood *et al.*, 1969b), the pyruvate reaction occurring by protonation of an intermediate to pyrophosphate and pyruvate and the CO_2 fixation reaction involving carboxylation of the intermediate with accompanying cleavage to pyrophosphate and oxalacetate.

Although the reactions are catalyzed by the same enzyme, it is proposed that different forms of the enzyme exist which preferentially catalyze the two reactions. The relationships of the forms are presented in Figure 10 for purposes of discussion although it is recognized that the evidence is as yet inconclusive. It is proposed that the enzyme contains a tightly bound metal designated type II which is essential for the catalysis of both reactions. Evidence supporting this conclusion is presented by Willard *et al.* (1969). A type I dissociable metal (Mg^{2+}, Co^{2+}, or Mn^{2+}) also is required for these reactions. In addition to these two metals it is suggested that a heavy metal is involved in the pyruvate reaction. We propose that the enzyme when combined with a heavy metal assumes the A form (Figure 10) and this is the predominant form of the enzyme as usually isolated. When the enzyme is treated with thiols such as β-mercaptoethanol, it is proposed that the thiols react with the heavy metal so that it is nonfunctional and thus shifts the equilibrium of the two forms toward the B form which catalyzes the CO_2 fixation reaction. At the same time the amount of the A form is diminished so that catalysis of the pyruvate reaction is decreased. Thus thiol groups stimulate the CO_2 fixation reaction and inhibit the pyruvate reaction (Table II). The stimulation by thiol is reversible since dialysis reverses the thiol effect. Presumably the thiol is removed from the heavy metal so that it is now functional and the enzyme therefore assumes the A form.

On prolonged incubation of the enzyme with EDTA in the absence of thiols there is inactivation of both reactions (Table III) (conversion of A into C, Figure 10). The inactivation by EDTA is much more effective in the presence of thiols (Figure 8). This latter inactivation is proposed to occur *via* A, B, and C (Figure 10), the B form being more susceptible to chelation of the type II metal than the A form. The C form is inactive for both reactions because the type II and heavy metals are blocked by EDTA but it can be converted into the B form by treatment with Co^{2+} which complexes the EDTA and removes it from the type II metal of the enzyme. In this case the heavy metal is nonfunctional or has been removed by the EDTA

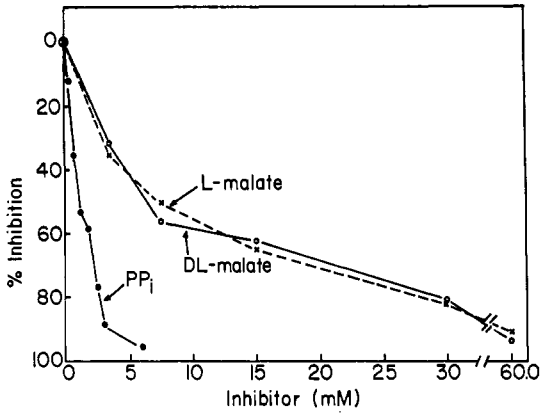

FIGURE 9: Inhibition of the pyruvate reaction with pyrophosphate and L- and DL-malate. Conditions were as described under Materials and Methods with all reagents including pyrophosphate and malate gassed with and stored under N_2. Before use both the pyrophosphate and malate were neutralized to a bromothymol blue end point with HCl and KOH solutions, respectively; 8.1 μg of carboxytransphosphorylase (specific activity 13.0) diluted in 50 mM phosphate buffer (pH 6.9) was used per ml of assay.

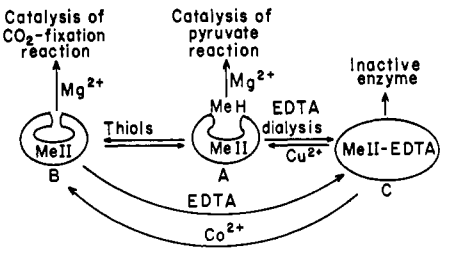

FIGURE 10: Schematic representation of the different forms of carboxytransphosphorylase. A dissociable type I metal (Mg^{2+}) is required for both the oxalacetate and pyruvate reactions. The A form catalyzes the pyruvate reaction. It is proposed to contain a heavy metal (Me H) as well as the tightly bound type II metal (ME II). Treatment with thiols converts the heavy metal into a nonfunctional form to yield form B which catalyzes the fixation of CO_2. Treatment of A with EDTA yields the inactive form C. Treatment of C with Cu^{2+} removes the EDTA from the type II metal and from the heavy metal or substitutes for it yielding the A form. Treatment of C with Co^{2+} removes the EDTA from the type II but since there is no functioning heavy metal it gives rise to the B form in the absence of thiols. The EDTA and dialysis treatment is considered by Willard *et al.* (1969).

treatment and the enzyme therefore assumes the B form. Thus conversion of A into B *via* C can occur without the intervention of thiols (Table III).

If the EDTA–enzyme, C, is treated with Cu^{2+}, it is converted into A. It is proposed that this occurs by removal of the EDTA from the type II metal. The Cu^{2+} also either removes EDTA from the heavy metal or can replace it causing conversion into the A form. This form preferentially catalyzes the pyruvate reaction (Table III). It is proposed that the enzyme as isolated may have combined with heavy metals which may be present in the $(NH_4)_2SO_4$ or other reagents used for fractionation.

Mills (1966) has found that Hg^{2+} activates an exopolygalacturonase from molds. He states that is unlikely that Hg^{2+} acts *in vivo*. He proposes that *in vivo* the enzyme may be held in a given configuration and extraction may destroy this configuration and thus its activity. The conformation may be restored by combining with the Hg^{2+}, *e.g.*, by forming a sulfhydryl–Hg–sulfhydryl bridge. In contrast the pyruvate reaction may be minimal *in vivo* and Cu^{2+} or the heavy metal may convert carboxytransphosphorylase into a form which does not occur *in vivo*. Hg^{2+} does not replace Cu^{2+} in activating the EDTA-treated carboxytransphosphorylase for the pyruvate reaction.

Although most of the K_m values are similar for either the pyruvate or CO_2 fixation reactions, the K_m for phosphoenolpyruvate is about tenfold lower for the pyruvate reaction than for the forward reaction. However, when the apparent K_m for phosphoenolpyruvate was determined by varying phosphoenolpyruvate in the presence of different fixed concentrations of bicarbonate, the reciprocal plots gave intersecting lines on the ordinate and an apparent increase in K_m with increasing bicarbonate concentration (Figure 7). The interpretation of these results is difficult, because alternative reaction sequences are involved in which CO_2 is a substrate of one sequence. Procedures have been developed to handle kinetics which involve single pathways (Cleland, 1963) but thus far not for those involving alternative pathways. It appears from the results of Figure 7 that inhibition of the pyruvate reaction by CO_2 is partly overcome by increasing concentrations of phosphoenolpyruvate. The same difficulty of interpretation applies to the inhibition by phosphate with variable phosphoenolpyruvate as shown in Figure 5. Here too one is dealing with alternative pathways.

The question arises whether the pyruvate reaction has any physiological function in the propionic acid bacteria *in vivo*. Fermentation by these bacteria normally yields CO_2 from carbohydrates and at least under anaerobic conditions one would expect that the enzyme would be kept in a reduced state. In the presence of thiols bicarbonate almost completely inhibits the pyruvate reaction (Wood *et al.*, 1969b). Furthermore the propionic acid bacteria, when grown on glycerol or glucose, contain large quantities of pyruvate kinase (unpublished observations of H. Evans) and this enzyme no doubt provides the means for conversion of the phosphoenolpyruvate into pyruvate. It seems likely that the normal role of the enzyme is to form oxalacetate from phosphoenolpyruvate.

It is of interest that Evans and Wood (1968a,b) have found the propionic acid bacteria also contain a second enzyme (pyruvate, phosphate dikinase) which generates inorganic pyrophosphate as shown in reaction 3. The enzyme is induced

$$\text{pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{phosphoenolpyruvate} + \text{AMP} + PP_i \quad (3)$$

by growth on lactate and it seems likely that *in vivo* the enzyme catalyzes the conversion of pyruvate into phosphoenolpyruvate rather than the reverse. Enzymes catalyzing similar reactions have been identified by Hatch and Slack (1968) from tropical grass and by Reeves (1968) from a parasitic amoeba, *Entamoeba hystolytica*. Unlike in propionibacteria Reeves considers the enzyme replaces the function of pyruvate kinase in the amoeba since the latter was not found in this organism. Cooper and Kornberg (1965, 1967) previously had found an enzyme in *Escherichia coli* which catalyzes a similar reaction as shown in reaction 4. Phosphate is not required as an acceptor

$$\text{pyruvate} + \text{ATP} \rightleftharpoons \text{phosphoenolpyruvate} + \text{AMP} + P_i \quad (4)$$

and pyrophosphate is not a product.

Pyruvate phosphate dikinase from propionibacteria can be linked with carboxytransphosphorylase with formation of oxalacetate and pyrophosphate.

$$\text{pyruvate} + \text{ATP} + 2P_i + CO_2 \rightleftharpoons \text{oxalacetate} + \text{AMP} + 2PP_i \quad (5)$$

Pyrophosphate is a strong inhibitor of carboxytransphosphorylase (Figure 9) (Lochmüller *et al.*, 1966) and it may serve as part of the control mechanism which regulates the interplay of these enzymes as well of pyruvate kinase during the interconversions of pyruvate, oxalacetate, and phosphoenolpyruvate (Wood, 1968).

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