Phosphoenolpyruvate Carboxytransphosphorylase. V. Mechanism of the Reaction and the Role of Metal Ions*

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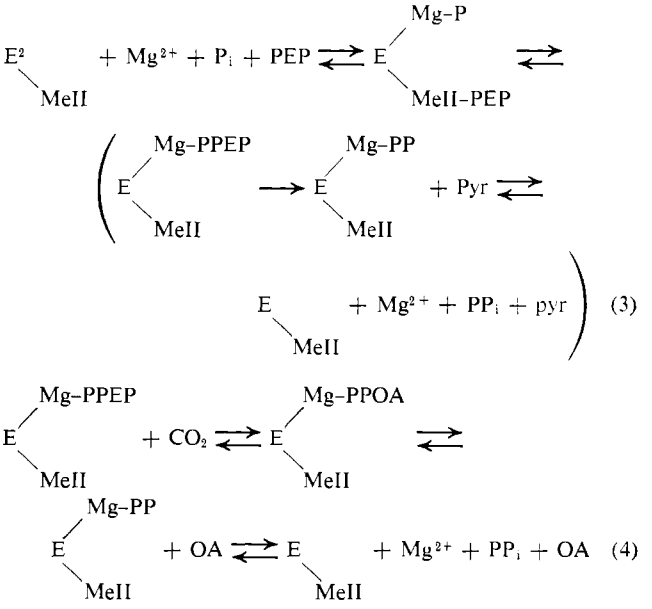
ABSTRACT: The roles of type I metals (dissociable) and of the type II metal (tightly bound) have been investigated in the carboxytransphosphorylase reaction using $^{32}P_i$, $^{32}PP_i$, $^{14}CO_2$, and pyruvate-1-^{14}C. The type II metal is blocked by EDTA. By use of EDTA the functions of type I and type II metals have been investigated independently in the oxalacetate reaction (reaction 1) and in the pyruvate reaction (reactions 2). It has been possible to exclude a number of mechanisms and as a working hypothesis reactions 3 and 4 have been postulated to explain the role of these metals, where Mg^{2+} is the type I metal and MeII represents the unidentified type II metal. Both the type I metal, Mg^{2+}, and type II metal are required for reaction 3 and EDTA inhibits this reaction in the presence of Mg^{2+} in excess of the EDTA. Reaction 4 through which $^{14}CO_2$ may exchange into oxalacetate requires Mg^{2+} and PP_i but does not require a functioning type II metal and is not inhibited by EDTA. It is proposed in reaction 4 that the Mg^{2+} forms a ligand between the enzyme and pyrophosphate with which oxalacetate combines to form pyrophosphoenoloxalacetate. The latter is reversibly decarboxylated to enzyme-bound pyrophosphoenolpyruvate thus explaining the requirements of both Mg^{2+} and PP_i for the exchange of CO_2 into oxalacetate. The pyruvate reaction is proposed to occur *via* reaction 3. The Mg^{2+} forms a ligand between the enzyme and the P_i and the type II metal a ligand with phosphoenolpyruvate. An enzyme–Mg–pyrophosphoenolpyruvate complex is suggested as an intermediate which is common to both the oxalacetate and pyruvate reactions. In the presence of CO_2 this intermediate is converted into oxalacetate *via* reaction 4. In the absence of CO_2 the intermediate is converted into PP_i and pyruvate in an irreversible step, shown in parenthesis in reaction 3. The exchange of $^{32}P_i$ into phosphoenolpyruvate requires type I and type II metals and CO_2. This conversion occurs *via* the reversible oxalacetate reaction (reaction 1). The CO_2 is required to form the oxalacetate and labeled $^{32}PP_i$ is formed at the same time from the $^{32}P_i$. The incorporation of $^{32}P_i$ into phosphoenolpyruvate then occurs by reversal of reaction 4 and the reversible portion of reaction 3. Pyruvate apparently is not an intermediate of the oxalacetate reaction. Pyruvate-1-^{14}C is not incorporated into oxalacetate or phosphoenolpyruvate under any condition tested and the enzyme does not decarboxylate oxalacetate to pyruvate and CO_2. The intermediate compounds are therefore proposed to be covalent enolpyrophosphate esters of oxalacetate and pyruvate, or alternatively the reactions occur by concerted mechanisms. The proposed detailed mechanism of the reaction is indicated in Figure 2.

The CO_2-fixing enzyme, phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38), may be obtained in crystalline form and has a molecular weight of 430,000 ± 30,000, a sedimentation constant $s^0_{20,w}$ = 15.2 S,[1] and a specific activity of about 20 μmoles of oxalacetate formed per min per mg of protein (Lochmüller *et al.*, 1966). An active form of the enzyme with a sedimentation constant of 7.4 S has also been observed, having a specific activity of about 7 (Wood *et al.*, 1969).[1] Both forms of the active enzyme catalyze reactions 1 and 2 and are inhibited strongly by low concentrations of EDTA in the presence of 12 mM Mg^{2+} (Davis *et al.*, 1969; Lochmüller *et al.*, 1966). Studies with a variety of chelators (Willard *et al.*, 1969)

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

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



indicate that carboxytransphosphorylase requires two different metal ions which have been designated as type I and type II. Type I metals are freely dissociable and have K_m values of about 1 mM (Lochmüller *et al.*, 1966). Mg^{2+}, Mn^{2+}, and Co^{2+}

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[1] Unpublished observations by M. Haberl,and M. J. Willard, and H. G. Wood.

TABLE I: Effect of the Concentration of Bicarbonate on the Conversion of $^{32}P_i$ into PP_i and Phosphoenolpyruvate.[a]

Expt	$KHCO_3$ Added (μmole/ml)	Net Change: Phosphoenolpyruvate Utilized (μmole/ml)	Net Change: Pyruvate Formed (μmole/ml)	Net Change: Oxalacetate Formed (μmole/ml)	Radioactivity: Phosphoenolpyruvate (cpm/μmole $\times 10^{-3}$)	Radioactivity: PP_i (cpm/μmole $\times 10^{-3}$)	Exchange of PP_i with Phosphoenolpyruvate[b] (%)
1	0.00	0.55	0.55	0.005	18.6	1,550	**2.4**
2	0.05	0.60	0.55	0.016	37.3	1,200	**6.2**
3	0.20	0.58	0.56	0.022	90.4	1,420	**13**
4	1.00	0.73	0.61	0.065	249.0	1,000	**50**

[a] The initial mixture contained in micromoles per milliliter: phosphoenolpyruvate, 1.79; pyruvate, 0.97; P_i, 0.75 ($\sim 2.0 \times 10^6$ cpm/μmole); $MgCl_2$, 3.5; mercaptoethanol, 0.8; bicarbonate as indicated; and 0.15 unit of carboxytransphosphorylase (not treated with EDTA). The mixture with the exception of the bicarbonate (0.04 ml) and enzyme (0.015 ml) was placed in cuvets which were closed with rubber stoppers bearing syringe needles. The mixture was gassed through the syringe needles for 0.5 hr with CO_2-free N_2 at 25° and the pH was about 6.5. The gassing was then terminated and the $KHCO_3$ solution and carboxytransphosphorylase were injected through the syringe needles which were then withdrawn from the stoppers. Final volume was 1 ml in a cuvet of 1.5-ml capacity. Incubation was at 25° for 2.75 hr without shaking. [b] % Exchange = 100 × specific activity of phosphoenolpyruvate/0.5 specific activity of PP_i.

meet the type I requirements. The type II metal is firmly bound to the enzyme and has not been identified as yet. In addition to the requirement for type I and type II metals a heavy metal appears to stimulate the pyruvate reaction (Davis *et al.*, 1969; Willard *et al.*, 1969). We report here a study of the role of the type I and type II metals in the carboxytransphosphorylase reaction as determined by use of $^{32}P_i$, $^{32}PP_i$, $^{14}CO_2$, and pyruvate-1-^{14}C. We propose that these metals (type I and type II) function as illustrated in reactions 3 and 4. That part of reaction 3 in parenthesis represents the irreversible portion of the pyruvate reaction. The enzyme–Mg–pyrophosphate complex is common to both reactions.

Experimental Procedure

Materials and Methods

The sources of reagents, preparations, and assay methods are those described previously (Davis *et al.*, 1969). $Ba^{14}CO_3$, orthophosphoric-^{32}P acid, and pyruvate-1-^{14}C were obtained from New England Nuclear Corp. Inorganic pyrophosphatase was from Nutritional Biochemical Corp.

Preparation of Inorganic-^{32}P Pyrophosphate. The $^{32}PP_i$ was synthesized from $^{32}P_i$ and phosphoenolpyruvate using carboxytransphosphorylase (reaction 1). This reaction was carried out at 25° for 25 min with 1.5 ml of a mixture containing the following in micromoles: $KHCO_3$, 45.0; $MgCl_2$, 6.0; $CoCl_2$, 0.015; NADH, 0.4; β-mercaptoethanol, 1.5; phosphoenolpyruvate, 0.5; $KH_2{}^{32}PO_4$, 0.75 (200×10^6 cpm); and in units: malate dehydrogenase, 5; and carboxytransphosphorylase, 0.2. It was estimated from the change in absorbancy at 340 mμ that 0.41 μmole of $^{32}PP_i$ was formed. The reaction mixture was placed on a 0.8×10 cm Dowex 1-Cl^- column and the $^{32}P_i$ was eluted with 10 mM HCl, then the $^{32}PP_i$ with 10 mM HCl in 200 mM KCl. Fractions of $^{32}PP_i$ containing 50×10^6 cpm were pooled and neutralized in a final volume of 8.4 ml. Treatment with phosphomolybdate (Walters and Cooper, 1965) showed that the product was free of $^{32}P_i$ and on hydrolysis with inorganic pyrophosphatase there was complete conversion into $^{32}P_i$.

Treatment of the Reagents with Chelex. All reagents except the enzymes and the metals were treated with Chelex to remove metals (Willard *et al.*, 1969) and the water was triple glass distilled.

Preparation of Carboxytransphosphorylase for Study of the Function of Type I and Type II Metals. Two types of the enzyme were used: EDTA-treated enzyme and untreated enzyme. The EDTA-treated enzyme was prepared by incubation in 10 mM EDTA and 1 mM mercaptoethanol for 48 hr at 0° and then the excess EDTA was removed from the enzyme by dialysis for 8 hr as described by Willard *et al.* (1969). Part of the EDTA remains bound to the enzyme and inhibits the type II function (Willard *et al.*, 1969). The enzyme therefore does not catalyze reactions 1 and 2 in the presence of Mg^{2+} alone. If Co^{2+} at 10^{-5} M is added to complex the bound EDTA and thus liberate the type II metal, reaction 1 is catalyzed. Cu at 10^{-5} M is more effective for reaction 2 (Davis *et al.*, 1969; Willard *et al.*, 1969).

The untreated carboxytransphosphorylase catalyzes the complete reaction in the presence of Mg^{2+} alone. If it was desired to block the type II function of untreated enzyme, it was incubated for 12 min at 25° in 0.1 mM EDTA in the presence of all constituents of the assay except for the $MgCl_2$ and PP_i. The reaction then was started by addition of the $MgCl_2$ and PP_i. The inhibition of reaction 1 by the EDTA was removed by addition of 1 mM Co^{2+}.

Determination of Compounds. The experiments were set up as described in the tables and portions of the mixture were removed at the indicated times for determination of the products. The reaction mixture (usually 0.65 ml) was placed in a

[2] Abbreviations used are: E, enzyme; OA, oxalacetate; PEP, phosphoenolpyruvate; PPEP, pyrophosphoenolpyruvate; PPOA, pyrophosphoenoloxalacetate; Pyr, pyruvate.

TABLE II: Effect of CO_2 and the Removal of Oxalacetate with Malate Dehydrogenase on the Conversion of $^{32}P_i$ into PP_i and Phosphoenolpyruvate.[a]

Expt	Malate Dehydrogenase (units/ml)	$KHCO_3$ Added (μmole/ml)	Net Change: Phosphoenolpyruvate Utilized (μmole/ml)	Net Change: Pyruvate Formed (μmole/ml)	Net Change: NADH Formed (μmole/ml)	Radioactivity: Phosphoenolpyruvate (cpm/μmole $\times 10^{-3}$)	Radioactivity: PP_i (cpm/μmole $\times 10^{-3}$)	Exchange of PP_i with Phosphoenolpyruvate[b] (%)
5	6.2	0.00	0.56	0.59	0.05	4.4	1870	**0.47**
6	0.0	0.00	0.66	0.57	0.02	10.8	1480	**1.5**
7	6.2	0.20	0.63	0.50	0.14	5.7	1750	**0.65**
8	0.0	0.20	0.66	0.50	0.08	35.5	1220	**5.8**
9	6.2	0.00	0.64	0.62	0.03	6.5	1290	**1.0**
10	0.0	0.00	0.63	0.56	0.04	53.0	1295	**8.2**
11	6.2	0.20	0.61	0.54	0.15	6.4	1330	**0.96**
12	0.0	0.20	0.66	0.65	0.05	125.5	1240	**20.2**

[a] The initial mixture in expt 5, 6, 7, and 8 contained in micromoles per milliliter: phosphoenolpyruvate, 1.79; pyruvate, 0.95; P_i ($\sim 2.0 \times 10^6$ cpm/μmole), 0.80; $MgCl_2$, 3.5; mercaptoethanol, 0.8, and NADH, 0.25. The initial mixture in expt 9, 10, 11, and 12 in which mercaptoethanol was omitted contained in micromoles per milliliter: phosphoenolpyruvate, 0.86; pyruvate, 1.08; P_i, 0.60 ($\sim 1.5 \times 10^6$ cpm/μmole); $MgCl_2$, 3.5; and NADH, 0.25. The mixture was gassed for 0.5 hr with CO_2-free nitrogen, then the bicarbonate (0.01 ml), malate dehydrogenase (0.01 ml) where indicated, and the carboxytransphosphorylase (0.15 unit in expt 5–8 and 0.9 unit in expt 9–12 in 0.01 ml) were added. The reaction was for 2.75 hr and the conditions were as given in Table I. It was found necessary to omit the malate dehydrogenase from the mixture during the gassing since it was inactivated during this process. [b] % Exchange = 100 $\times$ specific activity of phosphoenolpyruvate/0.5 specific activity of PP_i.

small stoppered test tube unless CO_2 was to be excluded (Tables I and II). If the conversion or exchange of both $^{14}CO_2$ into oxalacetate and of $^{32}P_i$ into PP_i and phosphoenolpyruvate was to be determined as in the experiments of Table V, the reactions were set up in duplicate, one containing $KH^{14}CO_3$ and the other $^{32}P_i$.

The oxalacetate, pyruvate, and phosphoenolpyruvate were determined using successively malate dehydrogenase, lactate dehydrogenase, and pyruvate kinase as described below. The amount of PP_i was estimated to be equal to the amount of PP_i added plus the decrease in phosphoenolpyruvate (reaction 1). For these determinations 0.15 ml of the reaction mixture was added to 0.9 ml of 30 mM HCl in a cuvet. After 3 min 0.5 ml of 0.5 M Tris-HCl buffer (pH 7.4) and 0.05 ml of 0.5 M Tris base were added, and then 0.5 ml of a mixture containing in micromoles, KCl, 50; $MgCl_2$, 10; ADP, 3.2; and NADH, 0.6. The change in absorbance at 340 mμ was determined after addition of 2.2 units of malate dehydrogenase, and again after addition of 4 units of lactate dehydrogenase and finally after addition of 1 unit of pyruvate kinase each in 0.02 ml of solution. After completion of these measurements the resulting mixture was used for determination of the fixed $^{14}CO_2$ in oxalacetate (as malate) as described below. If the ^{14}C in oxalacetate was not to be determined, the assay was done in a smaller cuvet using 0.02 ml of the reaction mixture. The compounds were determined at zero time by the same procedure.

The activity of the carboxytransphosphorylase was determined at each time interval using 0.01 ml of the reaction mixture and it was found that there was little loss of activity during the incubation at 25°.

Determination of Fixed $^{14}CO_2$ in Oxalacetate. The fixed $^{14}CO_2$ in the oxalacetate was determined by converting it into malate as described above; 0.5 ml of 1 M acetic acid was added to the mixture after conclusion of the spectrophotometric measurements and 0.6 ml of the mixture (0.3 ml successively) was plated in triplicate on planchets which were ringed with silicon grease. Acetone was added until the solution was spread over the planchet. The solution was evaporated under a heat lamp and warm air was blown over the planchet with a hair dryer. The radioactivity was measured using a low-background gas-flow counter.

The specific activity of the original $KH^{14}CO_3$ was determined by converting it into malate using carboxytransphosphorylase, phosphoenolpyruvate, $KH^{14}CO_3$, P_i, NADH, and malate dehydrogenase, and determining the specific activity of the malate.

Determination of ^{32}P in PP and in Phosphoenolpyruvate. This determination was usually done by removing the orthophosphate by treatment of a part of the mixture with molybdate and then extracting the phosphomolybdate complex with isobutyl alcohol–benzene as described by Walters and Cooper (1965). The $^{32}PP_i$ plus phosphoenolpyruvate-^{32}P remains in the aqueous phase and its radioactivity was determined. A second part of the mixture was treated with inorganic pyrophosphatase to hydrolyze the PP_i to P_i and then the P_i was removed by treatment with molybdate and extraction with isobutyl alcohol–benzene. The phosphoenolpyruvate-^{32}P remains in the aqueous phase and its radioactivity was determined. The radioactivity of the PP_i plus the phosphoenolpyruvate in the aqueous layer before pyrophosphatase treatment minus that of the phosphoenolpyruvate in the aqueous layer after pyrophosphatase equals that of the PP_i. The reliability of this procedure was confirmed in some cases using a third part of the mixture. The PP_i was converted into P_i with pyrophosphatase

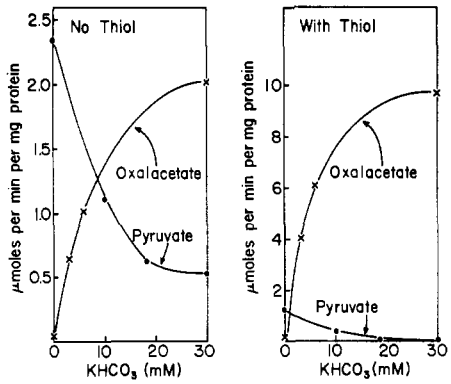


FIGURE 1: Effect of bicarbonate and mercaptoethanol on the rates of formation of pyruvate and oxalacetate by carboxytransphosphorylase. The reaction mixtures contained in micromoles per milliliter: phosphoenolpyruvate, 2.0 for measurement of oxalacetate formation and 0.6 for the pyruvate formation; orthophosphate (pH 7.0), 10; $MgCl_2$, 12; and NADH, 0.125. These mixtures were made more concentrated than in the reaction and gassed with N_2 to remove dissolved CO_2. The bicarbonate and enzymes were then added to the mixture in the cuvet. The rates were measured at each bicarbonate concentration in three cuvets, the first containing malate dehydrogenase (5 units), the second lactate dehydrogenase (1.2 units), and the third both the lactate dehydrogenase and malate dehydrogenase. The rate of the pyruvate reaction was measured by the difference in rates between the third cuvet and the first cuvet (see text). The lactate dehydrogenase was dialyzed against Tris buffer (pH 7.4) to remove sulfate which inhibits carboxytransphosphorylase (Lochmüller *et al.*, 1966). For the reaction in the presence of thiol, the carboxytransphosphorylase was diluted in 50 mM phosphate buffer containing 1 mM mercaptoethanol (the thiol was 0.06 mM in the final reaction mixture). For the reactions in the absence of thiol, the enzyme was diluted in the buffer only. The bicarbonate (0.1 or 0.033 M) was gassed with CO_2 to obtain a pH of 6.8 prior to addition to the reaction mixture.

and then the phosphoenolpyruvate-^{32}P was converted into ATP-^{32}P using pyruvate kinase. The resulting ATP-^{32}P was separated from the $^{32}P_i$ by chromatography on Dowex 1-Cl^- using successively 10 mM HCl, 10 mM HCl in 50 mM KCl, and finally 10 mM HCl in 100 mM KCl to obtain the ATP-^{32}P. There was good agreement between the radioactivity of the ATP-^{32}P and that found by the procedure with molybdate as outlined above.

For the above determinations 0.25 ml of the reaction mixture was added to 1.15 ml of 32 mM HCl and after a few minutes it was neutralized with KOH to the bromothymol blue end point and made up to 2.1 ml. Duplicate samples (0.2 ml) were treated with molybdate and extracted (Walters and Cooper, 1965). Duplicate samples (1 ml) of the aqueous phase were added to planchets and the radioactivity of the solution was determined. The radioactivity of the PP_i plus phosphoenolpyruvate was calculated from this value. For determination of the radioactivity of the phosphoenolpyruvate 1.2 ml of the 2.1 ml of solution was incubated with pyrophosphatase (1.2 units) for 20 min at 25°. Duplicate samples (0.5 ml) were treated with molybdate and extracted. The radioactivity was measured on duplicate samples (1 ml) of the aqueous phase. The specific activity of the PP_i and phosphoenolpyruvate was calculated as counts per minute per micromole.

Determination of ^{14}C Incorporated in Oxalacetate and Phosphoenolpyruvate from Pyruvate-^{14}C. The pyruvate-1-^{14}C was purified prior to use as described by Scrutton *et al.* (1965) by chromatography on a Dowex 1-Cl^- column to remove the parapyruvate.

For determination of the ^{14}C of the oxalacetate it was converted into aspartate by transamination with glutamate (Scrutton *et al.*, 1965). The aspartate was then purified by passage over Dowex 50-H^+ and finally by paper chromatography on Whatman No. 1 paper in a *n*-propyl alcohol-NH_3 (70:30, v/v) solvent system. The amount of aspartate in the eluate was determined using transaminase and malate dehydrogenase and the radioactivity using a low-background counter. The ^{14}C specific activity was calculated as counts per minute per micromole.

Prior to determination of the ^{14}C in the phosphoenolpyruvate it was purified on a Dowex 1-Cl^- column (McQuate and Utter, 1959). Contaminating parapyruvate or pyruvate-^{14}C was removed by oxidation with ceric sulfate using a modification of the method of Fromageot and Desnuelle (1935). To 0.5 ml of the eluate containing 0.1–0.5 μmole of phosphoenolpyruvate were added 0.1 ml of 0.2 N H_2SO_4 and 0.14 ml of 0.1 N $Ce(SO_4)_2$ in 0.2 N H_2SO_4. The mixture was stirred by vigorously gassing with N_2 for 10 min at room temperature and then centrifuged. The supernatant fluid was decanted and the ceric precipitate containing phosphoenolpyruvate was washed by suspension in 0.5 ml of H_2O and centrifugation. The phosphoenolpyruvate in the precipitate was extracted by suspending it in 0.5 ml of H_2O which was made alkaline to bromothymol blue by adding 1 drop of 1 N KOH. After 5 min at room temperature, the phosphoenolpyruvate in the solution was recovered by centrifugation. It was found using unlabeled phosphoenolpyruvate mixed with pyruvate-1-^{14}C that about 25% of the phosphoenolpyruvate was recovered by this method with little or no contamination from the pyruvate-1-^{14}C. The phosphoenolpyruvate concentration was determined with pyruvate kinase and lactate dehydrogenase and the radioactivity was determined using a low-background counter.

Results

The Effect of the Concentration of CO_2 on the Relative Rates of Formation of Oxalacetate and Pyruvate. Carboxytransphosphorylase catalyzes the two conversions described in reactions 1 and 2. Experiments were undertaken to ascertain whether these two reactions involve a common intermediate which is alternatively carboxylated to form oxalacetate or protonated to pyruvate. If so, as the concentration of CO_2 is increased the rate of formation of oxalacetate should increase and that of pyruvate should decrease. It has been shown previously (Davis *et al.*, 1969) that the addition of thiol groups stimulates the formation of oxalacetate (reaction 1) and partially inhibits the formation of pyruvate (reaction 2). Therefore the effect of $KHCO_3$ concentration on the rates of formation of oxalacetate and pyruvate was determined both in the presence and absence of mercaptoethanol (Figure 1). Assays of carboxytransphosphorylase were set up in triplicate at each concentration of bicarbonate. To one of these was added malate dehydrogenase, to another was added lactate dehydrogenase, and to the third was added malate dehydrogenase plus lactate dehydrogenase. The first test provided a measure of the rate of oxalacetate formation, and the third of the combined rates of the two reactions catalyzed by carboxytransphosphorylase. The second test does not give a true measure of the pyruvate reaction since there is some nonenzymic decarboxylation of

the oxalacetate which is formed *via* reaction 1. Therefore the lactate dehydrogenase measures not only pyruvate formed by the pyruvate reaction but also pyruvate formed by the nonspecific decarboxylation of oxalacetate. Thus the sum of the rates measured in the first and second tests was somewhat greater than that of the third. The rate of the pyruvate reaction *per se* was obtained by subtracting the rate of the oxalacetate reaction measured in the first test from that of the sum of the combined reactions measured in the third test. The spontaneous decarboxylation of oxalacetate is eliminated in the third test since the oxalacetate is converted into malate as rapidly as it is formed.

It is evident from Figure 1 that with increasing concentration of bicarbonate there is a reciprocal relationship between the rates of the oxalacetate and the pyruvate reactions both without and with addition of thiols. In the absence of added mercaptoethanol (Figure 1A) and bicarbonate the rate of the pyruvate reaction was as rapid as the oxalacetate reaction with the bicarbonate at 30 mM. In the presence of free thiol groups (Figure 1B) the rate of the pyruvate reaction is reduced but the oxalacetate reaction is almost five times faster in 30 mM bicarbonate than it is in the absence of thiols. In the presence of thiols (Figure 1B) addition of bicarbonate almost completely checked the pyruvate reaction, but in the absence of thiols (Figure 1A) considerable pyruvate formation occurred even in the presence of 30 mM bicarbonate.

The results indicate that pyruvate is formed from phosphoenolpyruvate and does not arise by the decarboxylation of oxalacetate formed in the oxalacetate reaction. If the latter occurred the rate of formation of pyruvate would have increased along with the formation of oxalacetate. However, there was a decrease in pyruvate formation with increase in CO_2 concentration, even when the oxalacetate was not removed and pyruvate was measured with lactate dehydrogenase. The observed reciprocal relationship indicates that the oxalacetate and pyruvate may arise from a common intermediate and that pyruvate rather than oxalacetate is formed from this intermediate in the absence of CO_2.

Tests of the Reversibility of the Pyruvate Reaction and of the Effect of the Concentration of CO_2 on the Conversion or Exchange of $^{32}PP_i$ into Phosphoenolpyruvate. Reaction 2 is in some respects similar to the pyruvate kinase reaction, P_i replacing ADP as an acceptor of phosphate in the formation of pyruvate from the phosphoenolpyruvate. McQuate and Utter (1959) have shown that the pyruvate kinase reaction is reversible and have demonstrated formation of phosphoenolpyruvate from pyruvate and ATP. Similar attempts to form phosphoenlpyruvate from PP_i and pyruvate with carboxytransphosphorylase have not been successful.[3]

The conversion or exchange of $^{32}PP_i$ into phosphoenolpyruvate should provide a more sensitive test of reversal of the reaction. For this purpose $^{32}PP_i$ was generated from $^{32}P_i$ using carboxytransphosphorylase (reaction 2). The amount of $^{32}P_i$ added to the reaction mixture was less than the phosphoenolpyruvate so that some phosphoenolpyruvate remained present after the $^{32}PP_i$ had been generated. The incubation was then continued for sometime so that there was ample opportunity for the exchange reactions to occur. The concentration of phosphoenolpyruvate, pyruvate, and oxalacetate and the radioactivity in the phosphoenolpyruvate and PP_i were determined. The per cent conversion was calculated from the relative specific activities of the phosphoenolpyruvate and PP_i. The effect of CO_2 concentration on the conversion was also determined. This was done because it is extremely difficult to remove the last traces of CO_2 from a solution. Therefore the residual CO_2 might be utilized forming oxalacetate by reaction 1 and the exchange of $^{32}PP_i$ into phosphoenolpyruvate might occur *via* reaction 1 rather than reaction 2 even though precautions were taken to remove the CO_2. The results with low concentrations of CO_2 would permit some evaluation of the likelihood of exchange occurring *via* the use of residual CO_2.

It is seen (Table I) that phosphoenolpyruvate is utilized and $^{32}P_i$ is converted into PP_i whether or not bicarbonate is added to the reaction mixture. The addition of bicarbonate caused an increase in the formation of oxalacetate and incorporation of ^{32}P into the phosphoenolpyruvate. The results in the last column show that only 2% of the phosphorous of the phosphoenolpyruvate was from the $^{32}PP_i$ when no bicarbonate was added; when 0.05 mM bicarbonate was present the value increased to 6% and at 1 mM to 50%. The K_m for HCO_3^- plus CO_2 in the oxalacetate reaction has been found to be 4 mM at pH 6.5 and 9.5 mM at pH 7.8 (Lochmüller *et al.*, 1966).

It is apparent that the conversion of $^{32}PP_i$ into phosphoenolpyruvate which occurred in the absence of added bicarbonate may not have been due to reversal of the pyruvate reaction (reaction 2) but rather to formation of oxalacetate from residual CO_2 and then reversal of reaction 1. The experiments of Table II were carried out using malate dehydrogenase and NADH to remove the oxalacetate. If the observed conversion was due to reversal of the pyruvate reaction and not to reversal *via* reaction 1, then inclusion of NADH and malate dehydrogenase in the reaction should have little effect on the conversion. On the other hand, if it was due to the formation of oxalacetate from residual CO_2, the removal of oxalacetate by conversion into malate should reduce the conversion. The results of Table II show that this was the case. The conversion of $^{32}PP_i$ into phosphoenolpyruvate was measured both in the presence of mercaptoethanol (expt 5–8) and in its absence (expt 9–12). In the absence of added bicarbonate with mercaptoethanol present the exchange was 1.5% (expt 6) and with malate dehydrogenase added it was reduced to 0.5% (expt 5). When 0.2 mM bicarbonate was added, the exchange was reduced from 6 to 0.7% by the addition of malate dehydrogenase. Similar results were obtained when conditions favored the pyruvate reaction, *i.e.*, no thiol was added (expt 9–12). In these experiments more enzyme was used and there was more exchange in the absence of malate dehydrogenase but the exchange was small in the presence of malate dehydrogenase. It is to be noted that the rate of oxalacetate formation is very slow in the absence of added bicarbonate (Figure 1) as compared with the pyruvate reaction. It therefore is not likely that the removal of oxalacetate by malate dehydrogenase would alter significantly the concentration of the common intermediate of the oxalacetate and pyruvate reactions (see reactions 3 and 4) and in this way retard exchange by the pyruvate reaction.

[3] PP_i (0.5 μmole), pyruvate (2.0 μmoles), and $MgCl_2$ (12 μmoles) per ml were incubated 2.75 hr at 25° with 1.9 units of carboxytransphosphorylase (0.6 unit when measured by the pyruvate reaction). No phosphoenolpyruvate was detected in the mixture using pyruvate kinase and lactate dehydrogenase. McQuate and Utter (1959) used a high concentration of ATP but similar concentrations of PP_i could not be used with carboxytransphosphorylase because PP_i is a strong inhibitor (Davis *et al.*, 1969; Lochmüller *et al.*, 1966).

TABLE III: Effect of EDTA on the Exchange of $^{14}CO_2$ into Oxalacetate by Enzyme in the Presence of Mg^{2+} and PP_i.[a]

Expt	EDTA (μmoles/ml)	Time (hr)	Pyruvate (μmoles/ml)	Oxalacetate		Exchange[b] (%)
				μmoles/ml	cpm/μmole	
13	0.00	0.5	0.78	1.69	3650	**22**
		1.0	1.22	1.12	4880	**35**
14	0.10	0.5	0.62	1.91	2670	**18**
		1.0	1.01	1.48	3760	**27**

[a] The initial mixture contained 0.15 unit of carboxytransphosphorylase/ml and the following in μmoles/ml: oxalacetate, 2.48; pyruvate, 0.10; PP_i, 0.25; $KH^{14}CO_3$ (22,000 cpm/μmole), 3.0; P_i, 1.8; $MgCl_2$, 4.0; KCl, 25; and the EDTA where indicated. The mixture was incubated for 12 min at 25° to allow combination of the EDTA with the enzyme before the reaction was started by addition of the $MgCl_2$ and PP_i. Incubation was at 25° in small stoppered test tubes. [b] The $^{14}CO_2$ is diluted by decarboxylation of the oxalacetate to pyruvate and by exchange. The approximate specific activity of the final CO_2 was estimated to be ~16,000 cpm/μmole at 0.5 hr and ~14,000 at 1 hr and the % exchange = 100 × specific activity of oxalacetate/specific activity of CO_2.

It appears from these results that the exchange of $^{32}PP_i$ into phosphoenolpyruvate is dependent upon formation of oxalacetate and does not occur to a significant extent in the absence of CO_2. Thus the pyruvate reaction is experimentally irreversible.

Requirements of Phosphoenolpyruvate for the Conversion or Exchange of $^{32}P_i$ with PP_i. It is evident from Tables I and II that $^{32}P_i$ is rapidly converted into $^{32}PP_i$ in the presence of phosphoenolpyruvate whether or not CO_2 is present. The question of whether phosphoenolpyruvate is required for the conversion or exchange of $^{32}P_i$ with PP_i is important in terms of mechanisms. One possible mechanism for the oxalacetate reaction is as follows.

$$E + PEP + CO_2 \rightleftharpoons \overset{PEP}{\underset{CO_2}{E}} \rightleftharpoons \overset{P}{\underset{OA}{E}} \rightleftharpoons \overset{P}{E} + OA \quad (5)$$

$$\overset{P}{E} + P_i \rightleftharpoons \overset{PP}{E} \rightleftharpoons E + PP_i \quad (6)$$

This mechanism involves a phosphorylated enzyme as an intermediate. If this were the mechanism of the oxalacetate reaction, $^{32}P_i$ would exchange into PP_i *via* reaction 6 in the absence of phosphoenolpyruvate. It has been found that carboxytransphosphorylase does not catalyze this exchange even when pyruvate and CO_2 are present. A mixture containing in micromoles per milliliter: $MgCl_2$, 3.5; β-mercaptoethanol, 1.0; $KHCO_3$, 12.5; pyruvate, 1.8; PP_i, 0.3; $^{32}P_i$ (8×10^5 cpm/μmole), 1.0; and in units: carboxytransphosphorylase, 0.68, was incubated for 2.5 hr at 25° under CO_2 gas. The reaction mixture was treated with molybdate and extracted with isobutyl alcohol–benzene (Walters and Cooper, 1965); 3040 cpm was found in the aqueous phase with the enzyme present and 2400 cpm when the enzyme was omitted. Thus 640 cpm out of a total of 800,000 cpm in the $^{32}P_i$ was fixed, which is within experimental error.

It also has been determined that phosphoenolpyruvate is required for the formation of oxalacetate. Pyruvate carboxylase (Scrutton *et al.*, 1965) catalyzes the conversion of pyruvate, ATP, and CO_2 into oxalacetate and ADP. A similar reaction is not catalyzed by carboxytransphosphorylase, *i.e.*, oxalacetate is not formed from pyruvate, PP_i, and CO_2.

Evidence That Type II Metal Is Not Required for the Exchange of CO_2 into Oxalacetate. EDTA at 0.1 mM inhibits the over-all oxalacetate reaction (Davis *et al.*, 1969; Willard *et al.*, 1969) completely and EDTA is believed to block the function of the type II enzyme-bound metal. Therefore, studies were undertaken with EDTA to determine whether partial reactions are catalyzed by carboxytransphosphorylase which require only type I metals. Mg^{2+}, PP_i, and bicarbonate were used at concentrations favorable for the back-reaction; at elevated concentrations they are inhibitory (Lochmüller *et al.*, 1966). It is seen (Table III) that 0.1 mM EDTA had very little effect on the exchange of $^{14}CO_2$ into oxalacetate although this concentration of EDTA almost completely inhibits the over-all back-reaction under similar conditions (Davis *et al.*, 1969).

The pyruvate (0.1 μmole) present in the original mixture of zero time arose by spontaneous decarboxylation of the oxalacetate. The increase during the incubation arises largely if not exclusively by spontaneous decarboxylation of the oxalacetate formed during the prolonged incubation. Practically the same amount of pyruvate was formed whether or not carboxytransphosphorylase was added to the mixture.

Experiments are shown in Table IV in which an EDTA-treated enzyme was used. This preparation is convenient for studies because it retains sufficient bound EDTA to inhibit the type II function but can be reactivated for the over-all oxalacetate reaction by addition of 0.01 mM Co^{2+} when Mg^{2+} is present as the type I metal (Davis *et al.*, 1969). It is seen (Table IV) that there was approximately the same amount of exchange of $^{14}CO_2$ with the oxalacetate whether Co^{2+} was added or not (expt 15 and 16). This shows that exchange takes place under conditions in which the over-all reaction does not occur and that the type II function of metals is not required for the CO_2–oxalacetate exchange. There was no exchange if the Mg^{2+} was omitted and Co^{2+} was present at 0.01 mM (expt 17). The type I metal (Mg^{2+}) is required for the exchange. Co^{2+} (or Mn^{2+}) will serve as a type I metal (with $K_m \sim 1$ mM) but at 0.01 mM they are not effective. It is seen that PP_i is required for the exchange; little or no exchange occurred when PP_i was omitted (expt 18). This requirement for PP_i can also be shown using

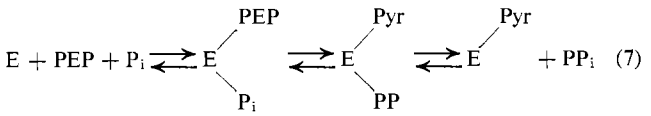
TABLE IV: Effect of PP_i, Type I (Mg^{2+}), and Type II Metal on the Exchange of $^{14}CO_2$ into Oxalacetate by EDTA-Treated Enzyme.[a]

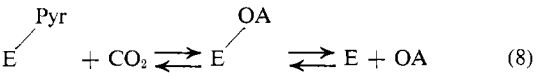
Expt	Omission	Time (hr)	Pyruvate (μmoles/ml)	Oxalacetate		Exchange[b] (%)
				μmoles/ml	cpm/μmole	
15	None	0.5	0.77	1.71	4830	**30**
		1.0	1.11	1.31	5720	**44**
16	Co^{2+}	0.5	0.72	1.89	4550	**28**
		1.0	1.08	1.48	5060	**39**
17	Mg^{2+}	0.5	0.20	2.46	0	**0**
		1.0	0.24	2.32	0	**0**
18	PP_i	0.5	0.75	1.85	174	**1**
		1.0	1.07	1.48	140	**1**

[a] The initial complete mixture contained 0.27 unit of EDTA-treated carboxytransphosphorylase/ml and in the complete mixture the following in μmoles/ml: oxalacetate, 2.84; pyruvate, 0.10; PP_i, 0.25; $KH^{14}CO_3$ (22,000 cpm/μmole), 3.0; P_i, 1.8; KCl, 25; $MgCl_2$, 4.0; and $CoCl_2$, 0.01. Incubation was at 25°. [b] The specific activity of the CO_2 was estimated to be ~16,000 cpm/μmole at 0.5 hr and ~13,000 at 1 hr. The % exchange = 100 × specific activity of oxalacetate/specific activity of CO_2.

enzyme which has not been treated with EDTA. Thus PP_i is required for the exchange of CO_2 into oxalacetate even though the type II metal is not blocked and Mg^{2+} is present.

The requirement of PP_i excludes the following over-all mechanism for the oxalacetate reaction which involves an intermediate complex of enzyme and pyruvate.





According to this mechanism CO_2 would exchange by reaction 8 into oxalacetate in the absence of PP_i. Krampitz *et al.* (1943) have shown that an exchange such as shown in reaction 8 does occur with oxalacetate decarboxylase from *Micrococcus lysodeikticus*. Although they observed CO_2 exchange, they found there was no exchange or conversion of pyruvate into oxalacetate.

It is noted that less pyruvate is formed when Mg^{2+} is omitted (expt 17) than when it is present (expt 15, 16, and 18), thereby reflecting the spontaneous decarboxylation of oxalacetate by Mg^{2+}.

Requirement of Both Type I and Type II Metals for the Conversion of Or Exchange of $^{32}P_i$ into Phosphoenolpyruvate. It has been shown that the type I, but not type II, metal is required for the exchange of CO_2 in oxalacetate, whereas both are required for the over-all reaction. It, therefore, appeared that type II metal and probably type I would be required for the conversion or exchange of $^{32}P_i$ into PP_i and phosphoenolpyruvate. It has been shown in Tables I and II that conversion or exchange of $^{32}P_i$ or ^{32}PP into phosphoenolpyruvate does not occur in the absence of CO_2. It also has been shown that phosphoenolpyruvate is required for the exchange of $^{32}P_i$ into PP_i. Thus there is a requirement for all components of the forward reaction; *i.e.*, P_i, CO_2, and phosphoenolpyruvate to obtain conversion or exchange of $^{32}P_i$ into phosphoenolpyruvate. For this reason the studies of Table V were set up with all components present and at approximately equilibrium concentrations (Wood *et al.*, 1966). However, the composition of the mixture changed significantly during the incubation because there was nonenzymic decarboxylation of the oxalacetate by Mg^{2+} during the extended incubation. Thus incorporation of tracer occurred in part by net synthesis as well as by exchange. Both $^{32}P_i$ and $^{14}CO_2$ were used to permit comparison of the two conversions: one series of the experiment contained $^{32}P_i$ and the other $^{14}CO_2$. The requirement for type I and type II metals was determined using EDTA-treated enzyme.

It is seen (Table V) when Mg^{2+} was present and Co^{2+} was omitted (the type II metal function being blocked by the EDTA) there was very little $^{32}P_i$ converted into PP_i (at 1 hr 5% in expt 20 compared with 34% in expt 19) and no ^{32}P was found in the phosphoenolpyruvate (0 compared with 5.4). Likewise when Mg^{2+} was omitted and Co^{2+} was present at 0.01 mM (expt 21), there was no conversion of $^{32}P_i$ into PP_i or phosphoenolpyruvate. In this case type I metal is lacking but the type II metal is free because the EDTA inhibition is removed by the Co^{2+}. Thus both type I and type II metals are required for the phosphate reactions. It is considered that the 5% conversion of $^{32}P_i$ into PP_i (expt 20) is due to incomplete inhibition of the type II function in the EDTA-treated enzyme.

The conversion of $^{14}CO_2$ into oxalacetate was greater in the complete mixture (expt 19, 20% at 1 hr) than when Co^{2+} was omitted (expt 20, 8% at 1 hr) which might be taken as an indication that type II metal is required for the CO_2 exchange into oxalacetate. However in these experiments, in contrast to those of Tables III and IV, there was some net synthesis of oxalacetate from the phosphoenolpyruvate, P_i, and CO_2. Note that the phosphoenolpyruvate fell from 1.20 μmoles at zero time to 1.03 μmoles in expt 19 at 1 hr. Therefore, CO_2 entered oxalacetate by both synthesis and exchange when the EDTA inhibition of type II metal was removed by addition of Co^{2+} (expt 19) whereas in the absence of Co^{2+}, $^{14}CO_2$ probably en-

TABLE V: Effect of Type I (Mg^{2+}) and Type II Metal on the Conversion of $^{32}P_i$ into PP_i and Phosphoenolpyruvate and of $KH^{14}CO_3$ into Oxalacetate under Approximately Equilibrium Conditions and with EDTA-Treated Enzyme.[a]

				Oxalacetate			Pyrophosphate			Phosphoenolpyruvate		
			Pyruvate				Sp Act.					
Expt	Omission	Time (hr)	(μmole/ml)	Amt (μmole/ml)	Sp Act. (cpm/μmole)	From CO_2 (%)[b]	Amt[c] (μmole/ml)	(cpm/μmole $\times 10^{-3}$)	From P_i (%)[c]	Amt μmole/ml	Sp Act. (cpm/μmole)	From PP_i (%)[d]
19	None	0.5	0.86	1.62	2000	**12**	0.35	274	**28**	1.10	4050	**3.7**
		1.0	1.41	1.06	3080	**20**	0.42	334	**34**	1.03	8050	**5.4**
20	Co^{2+}	0.5	0.81	1.65	1030	**6**	0.25	31	**3**	1.20	0	**0**
		1.0	1.26	1.14	1480	**8**	0.33	54	**5**	1.12	0	**0**
21	Mg^{2+}	0.5	0.24	2.36	208	**1**	0.23	0	**0**	1.22	0	**0**
		1.0	0.38	2.15	270	**1**	0.29	0	**0**	1.16	0	**0**

[a] The initial complete mixture contained 0.18 unit of EDTA-treated carboxytransphosphorylase/ml and the following in μmoles/ml: oxalacetate, 2.48; pyruvate, 0.24; Phosphoenolpyruvate, 1.20; PP_i, 0.25; $KH^{14}CO_2$ (22,000 cpm/μmole), 3.0; P_i, 1.2; $MgCl_2$, 4.0; $CoCl_2$, 0.01; and KCl, 26. A second series was set up to which $^{32}P_i$ was added containing 1.8×10^6 cpm/ml of reaction mixture and the $KHCO_3$ was unlabeled. Incubation was at 25°. Controls consisting of the complete medium without addition of enzyme were set up for determination of the initial values. The specific activity of the CO_2 was determined in a part of the final reaction by conversion into malate with excess carboxytransphosphorylase and malate dehydrogenase. [b] The conversion of CO_2 into oxalacetate equals specific activity of oxalacetate/specific activity of $CO_2 \times 100$. [c] The amount of PP_i was estimated to be equal to the micromoles added plus the decrease in phosphoenolpyruvate. The conversion of P_i into PP_i equals 0.5 specific activity of PP_i/specific activity of initial $P_i \times 100$. [d] The conversion of PP_i into phosphoenolpyruvate equals specific activity of phosphoenolpyruvate/0.5 specific activity of $PP_i \times 100$.

TABLE VI: Effect of Type I (Mg^{2+}) and Type II Metal on the Conversion of $^{32}PP_i$ into Phosphoenolpyruvate and P_i by Carboxytransphosphorylase.[a]

						PP_i		Phosphoenolpyruvate			P_i		
Expt	EDTA (mM)	Co^{2+} (mM)	Time (hr)	Pyruvate (μmole/ml)	Oxalacetate (μmole/ml)	μmole/ml	cpm/μmole $\times 10^{-3}$	μmole/ml	cpm/μmole $\times 10^{-3}$	% from PP_i[b]	μmole/ml	cpm/μmole $\times 10^{-3}$	% from PP_i[c]
22	0.1	0	0.5	1.21	0.66	0.25	1700	0.37	12	**1.4**	0.50	9.6	**1.1**
			1.0	1.56	0.54	0.25	1700	0.37	21	**2.4**	0.50		
23	0.1	1	0.5	1.51	0.34	0.33	780	0.29	270	**69**	0.42	236	**61**
			1.0	1.70	0.19	0.41	625	0.21	266	**85**	0.34	237	**76**
24	0	1	0.5	1.56	0.31	0.31	760	0.31	214	**56**	0.44	306	**80**
			1.0	1.88	0.17	0.43	678	0.19	251	**74**	0.32	303	**89**

[a] The initial mixtures except for Mg^{2+} or Co^{2+} were incubated 15 min at 25° to block the type II metal with EDTA and then the Mg^{2+} or Mg^{2+} and Co^{2+} were added to start the reaction. The mixtures contained 0.4 unit of untreated carboxytransphosphorylase/ml. After the 15-min preincubation the mixture contained in micromoles per milliliter, oxalacetate, 0.69; pyruvate, 1.15; phosphoenolpyruvate, 0.37; PP_i, 0.25; $KHCO_3$, 1.0; P_i, 0.5; $MgCl_2$, 4.0; KCl, 26.0; EDTA, 0.1; $^{32}PP_i$, 0.25 (1.74×10^6 cpm/μmole); and $CoCl_2$, 1.0 when indicated. Incubation was at 25°. [b] Phosphoenolpyruvate from PP_i equals specific activity of phosphoenolpyruvate/0.5 specific activity of $PP_i \times 100$. [c] P_i from PP_i equals specific activity of P_i/0.5 specific activity of $PP_i \times 100$.

tered mostly by exchange only and therefore less ^{14}C was incorporated.

It also was observed that a greater per cent of the PP_i was derived from $^{32}P_i$ in the complete mixture than was oxalacetate from $^{14}CO_2$. This fact does not necessarily indicate the rates of conversion were different. The pool size of oxalacetate was considerably greater than that of PP_i. Therefore, the same conversion of $^{32}P_i$ and $^{14}CO_2$ into PP_i and oxalacetate, respec-

tively, would lead to formation of a greater proportion of the PP_i from $^{32}P_i$ than of oxalacetate from $^{14}CO_2$.

The conversion of $^{32}P_i$ into phosphoenolpyruvate was quite low in Table V. Incorporation of PP_i into phosphoenolpyruvate was expected to be slow because the back-reaction, *i.e.*, the conversion of oxalacetate and PP_i into phosphoenolpyruvate, P_i, and CO_2, is only one-seventh as fast as the forward reaction (Lochmüller *et al.*, 1966). In order to study more directly the effect of type I and type II metals on the conversion of PP_i into phosphoenolpyruvate, it was desirable to use $^{32}PP_i$ as the primary source of label. Table VI presents results from such experiments. In these experiments the type II function was blocked by adding the EDTA (0.1 mM) directly to the reaction mixture. In a parallel experiment Co^{2+} (1 mM) was added to remove the EDTA from the type II metal. Experiment 24 was done without EDTA but with addition of the Co^{2+} as well as the Mg^{2+}. In addition to oxalacetate, pyruvate was included in the mixture as was done previously in the experiments of Tables I and II.

It is seen in expt 22 when EDTA was present and no Co^{2+} was added there was little conversion of $^{32}PP_i$ into phosphoenolpyruvate or P_i, the type II function being blocked. When the Co^{2+} was added to the EDTA-blocked enzyme to relieve the inhibition (expt 23), there was conversion of $^{32}PP_i$ into phosphoenolpyruvate and into P_i. At 1 hr there was 85% conversion of $^{32}PP_i$ into phosphoenolpyruvate in expt 23 compared with 2.4% in expt 22. The conversion was about the same with enzyme which was not treated with EDTA. These results confirm those of Table V showing that type II metal (as well as type I) is required for the phosphate transfer. The per cent of conversion and exchange was greater in the experiments of Table VI than of those of Table V; in part this is because more enzyme and a lower amount of $KHCO_3$ and oxalacetate was used in the experiments of Table VI.

Tests with Pyruvate-1-^{14}C to Determine If Pyruvate Is an Intermediate of the Reversible Oxalacetate Reaction. It is noted that pyruvate is not indicated as an intermediate in reactions 3 and 4. Although the catalysis of the pyruvate reaction might suggest such a role, there is considerable evidence against it. Carboxytransphosphorylase, unlike some of the other enzymes that fix CO_2, does not catalyze the decarboxylation of oxalacetate to pyruvate and CO_2. If pyruvate were an intermediate, it might be expected that the enzyme would catalyze this reaction. Likewise there is no catalysis of the conversion of pyruvate and PP_i into phosphoenolpyruvate, *i.e.*, the reverse of the pyruvate reaction. Furthermore reaction 8 which involves pyruvate as an intermediate has been excluded since PP_i is required for the $^{14}CO_2$ exchange into oxalacetate. Nevertheless it was possible that the oxalacetate reaction might occur as follows.

$$E + PEP + P_i \rightleftharpoons E\begin{matrix}\diagup PEP\\ \diagdown P\end{matrix} \rightleftharpoons E\begin{matrix}\diagup Pyr\\ \diagdown PP_i\end{matrix} \longrightarrow$$

$$E\begin{matrix}\diagup Pyr\\ \diagdown \end{matrix} + PP_i \rightleftharpoons E + Pyr + PP_i \quad (9)$$

$$E\begin{matrix}\diagup Pyr\\ \diagdown PP_i\end{matrix} + CO_2 \rightleftharpoons E\begin{matrix}\diagup OA\\ \diagdown \end{matrix} + PP_i \rightleftharpoons E + PP_i + OA \quad (10)$$

The decarboxylation of the oxalacetate in reaction 10 might provide sufficient energy to cause linkage of the PP_i to the enzyme complex whereas this linkage might not be formed starting with pyruvate and PP_i (reaction 9). Thus the pyruvate reaction would be irreversible. If the enzyme–pyruvate–PP_i intermediate occurred as shown in reaction 10, pyruvate might dissociate reversibly from the complex and in this case pyruvate-1-^{14}C would exchange into oxalacetate. The mixture to test this exchange contained the following in micromoles per milliliter: phosphoenolpyruvate, 2.5; pyruvate-1-^{14}C (47,000 cpm/μmole), 1.0; $MgCl_2$, 4.0; $KHCO_3$, 3.0; oxalacetate, 3.1; PP_i, 0.25; P_i, 0.4; KCl, 26.0; and $CoCl_2$, 0.1. Three experiments were done; (1) with β-mercaptoethanol, 1.0 μmole/ml; (2) without mercaptoethanol; and (3) as a control with no enzyme. Carboxytransphosphorylase (0.69 unit) was used per milliliter and it was not treated with EDTA. After 2.75 hr at 25° the mixtures were acidified to inactivate the carboxytransphosphorylase. The oxalacetate was converted into aspartate and purified as described under Methods and its ^{14}C specific activity was determined. The phosphoenolpyruvate was also purified and its ^{14}C was determined. There was no significant incorporation of ^{14}C in the products beyond that of the control value which was 1.3 cpm above background.

Similar results were obtained when tests were made with pyruvate-1-^{14}C under conditions similar to the tests of Table II for the pyruvate reaction. The mixtures contained in micromoles per milliliter: phosphoenolpyruvate, 1.0; pyruvate (50,000 cpm/μmole), 1.0; $MgCl_2$, 4.0; and P_i, 0.5. CO_2 was removed from the system by gassing with N_2. Thus there was no evidence of the formation of pyruvate as an intermediate under conditions favoring the pyruvate reaction.

Discussion

Cohen (1963) has classified enzymes which utilize Mn^{2+} or Mg^{2+} into two groups based on their response to Ca^{2+}. The first group consists of enzymes in which the metal complexes with the substrate but the metal is not linked directly to the enzyme. This group is activated by Ca^{2+}. The second group includes enzymes in which the metal is linked directly to the enzyme; it may or may not serve as a bridge between the enzyme and substrate. This group is inhibited by Ca^{2+}. Since carboxytransphosphorylase is inhibited by Ca^{2+} (Lochmüller *et al.*, 1966), we have tentatively assumed that the type I metal binds directly to carboxytransphosphorylase and forms a bridge to P_i or PP_i as shown in reactions 3 and 4. The type II metal is tightly bound to carboxytransphosphorylase.

It has been found that exchange of $^{14}CO_2$ into the β-carboxyl group of oxalacetate occurs when the type II metal is blocked with EDTA. The type I metal (Mg^{2+}) and PP_i are required for this exchange (Table IV). The requirement of PP_i excludes the sequence shown in reactions 7 and 8 but is in accord with the sequence shown in reaction 4. An enzyme–Mg–pyrophosphate complex is proposed which combines with oxalacetate to form Mg–pyrophosphoenol oxalacetate which is reversibly decarboxylated. No linkage with type II metal is shown in reaction 4 since there is no inhibition by the chelator and thus there is no indication of a type II function.

The conversion or exchange of $^{32}P_i$ into PP_i requires both type I and type II metals and phosphoenolpyruvate. This excludes the sequence shown in reactions 5 and 6. Furthermore, the conversion of $^{32}PP_i$ into P_i and phosphoenolpyruvate re-

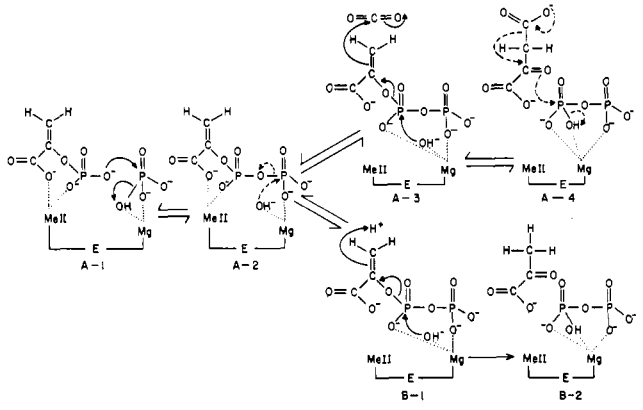


FIGURE 2: Possible role of the dissociable type I metal (Mg^{2+}) and the unidentified tightly bound type II metal (Me II) of carboxytransphosphorylase (E) in the oxalacetate reaction (A-1 to A-4) and the pyruvate reaction (A-1 to B-2). Conversions A-1 into A-4 are reversible. The dotted arrows indicate electron shifts when the conversion is from right to left and the solid arrows when it is from left to right. Conversion of A-4 into A-3 requires only the type I metal (Mg^{2+}). Thus CO_2 exchanges with the carboxyl group of oxalacetate when the type II metal is blocked by chelators. On the other hand, the phosphate cleavage requires both the type I and type II metals. Thus $^{32}P_i$ exchange occurs only when both metals are free. The pyruvate reaction A-1 to B-2 is experimentally irreversible and $^{32}PP_i$ is not converted into phosphoenolpyruvate by this sequence.

quires both type I and type II metals and CO_2. The CO_2 apparently is required to form oxalacetate *via* reaction 1 and then the $^{32}PP_i$ is converted into phosphoenolpyruvate by reverse of this reaction. The pyruvate reaction (reactions 2 or 3) is irreversible, so $^{32}PP_i$ cannot enter phosphoenolpyruvate *via* this reaction in the absence of CO_2.

A more detailed mechanism for the reactions is shown in Figure 2 which involves enzyme complexes A-1, A-2, A-3, and A-4 for the oxalacetate reaction and complexes A-1, A-2, B-1, and B-2 for the pyruvate reaction. A-1, A-2, and A-3 or B-1 are common to both reactions, A-3 being used to illustrate CO_2 fixation and B-1 the proton addition of the pyruvate reaction. The scheme serves only as an illustration of the requirements and as a working model. Type II tightly bound metal, Me II, is shown linked to phosphoenolpyruvate and the type I metal, Mg^{2+}, linked to orthophosphate giving A-1 which is converted into A-2 and then dissociates to A-3. A-3 can combine with CO_2 without the involvement of Me II to yield oxalacetate and PP_i as shown in A-4. This sequence accounts for requirements of both type I and type II metals in the over-all reactions and also accounts for the partial reactions. The solid arrows indicate electron shifts when the conversion is from left to right, and the dashed arrows when it is from right to left.

Free CO_2 is shown as the reactant in A-3 of Figure 2. This is in accord with the observations of Cooper *et al.* (1968) who have demonstrated that CO_2 is the active species in the carboxytransphosphorylase reaction. The mode of binding of the CO_2 or β-carboxyl of oxalacetate by the enzyme is not considered in the scheme.

The occurrence of the same intermediate complexes A-1 and A-2 in both the oxalacetate and pyruvate reactions may account for the reciprocal relationship of these two reactions with increase in CO_2 concentration (Figure 1). However, there are factors other than CO_2 concentration which control these reactions. Evidence has been presented (Davis *et al.*, 1969; Willard *et al.*, 1969) that the pyruvate reaction may involve a heavy metal which is complexed by thiol and is thus inhibited. Therefore, in the presence of thiols the pyruvate reaction is practically eliminated when the concentration of CO_2 is high (Figure 1B).

Pyruvate has not been indicated as an enzyme-bound intermediate since there is no incorporation of ^{14}C from pyruvate-1-^{14}C into oxalacetate or phosphoenolpyruvate during the reversible oxalacetate reaction. The exchange technique for detection of the enzyme-bound pyruvate requires that there is dissociation of the enzyme–pyruvate intermediate and thereby exchange with the pyruvate-1-^{14}C of the solvent. It is possible that the conformation of the protein is such that the pyruvate is not available for exchange and thus escapes detection or that the reaction occurs by a concerted mechanism not involving a pyruvate intermediate. Somewhat similar tests were done by Salles *et al.* (1950) with malate enzyme in an attempt to prove that oxalacetate is an intermediate of the reduction of pyruvate and $^{14}CO_2$ to malate by NADPH. No evidence was found that oxalacetate is an intermediate even though the malate enzyme does decarboxylate oxalacetate to pyruvate and CO_2.

The scheme shown in Figure 2 indicates that conversion of B-1 into B-2 is irreversible, thus $^{32}P_i$ which enters $^{32}PP_i$ *via* the pyruvate reaction cannot enter the phosphoenolpyruvate whereas the conversion of A-3 into A-4 is reversible so $^{32}P_i$ exchange can occur in this case. As noted previously, the decarboxylation of the oxalacetate may make it thermodynamically feasible to form the pyrophosphoenol pyruvate bond (A-4 to A-3) whereas it cannot occur with pyruvate *per se* (B-2 to B-1).

The pyruvate reaction by carboxytransphosphorylase is in some respects similar to that by pyruvate kinase. Mn^{2+} or Mg^{2+} and a monovalent ion are required by pyruvate kinase; in contrast monovalent ions are not required by carboxytransphosphorylase (Lochmüller *et al.*, 1966; Willard *et al.*, 1969). The mechanism of the pyruvate kinase reaction has been studied carefully by Mildvan and Cohen (1966) by measurement of the enhancement of the relaxation rate of the nuclear spins of water protons in the ternary enzyme–manganese–substrate complexes. The mechanism of the pyruvate reaction shown in Figure 2 is similar to that of Mildvan and Cohen (1966) except that formation of OH^- is not postulated in the pyruvate kinase reaction. The kinase reaction has been shown to be reversible (McQuate and Utter, 1959; Lardy and Ziegler, 1945). Rose (1960) has shown that pyruvate kinase catalyzes an exchange of hydrogen with the methyl group of pyruvate. Such an exchange may not occur with carboxytransphosphorylase in the absence of CO_2 since B-1 to B-2 is irreversible.

The fixation of CO_2 as catalyzed by carboxytransphosphorylase and that catalyzed by phosphoenolpyruvate carboxykinase are quite similar as seen by comparison of reactions 1 and 11.

$$\text{phosphoenolpyruvate} + \text{IDP (or GDP)} + CO_2 \rightleftarrows \text{oxalacetate} + \text{ITP (or GTP)} \quad (11)$$

Chang *et al.* (1966) have investigated the mechanism of this reaction by exchange studies with labeled substrates and by kinetic measurements. They have proposed a mechanism quite similar to that of Figure 2 which involves ITP–enolpyruvate as

an intermediate, instead of pyrophosphoenolpyruvate. However, Miller and Lane (1968) recently have used $H_2{}^{18}O$ to study this reaction. No incorporation of ^{18}O was observed in the products and they have proposed that the over-all reaction occurs by a single concerted mechanism which does not involve hydrolysis. In contrast the carboxytransphosphorylase reaction appears to occur by consecutive steps since only the type I metal is required for $^{14}CO_2$ exchange into oxalacetate but both the type I and type II metals are required for the pyrophosphate cleavage as illustrated in the conversion of A-2 into A-1. Thus the two steps are distinctly separated.

It is to be noted that there is an inherent assumption in the experiments of Miller and Lane (1968). The assumption is that the enzyme-bound water (*e.g.*, that bound to the Mn) is in equilibrium with the ^{18}O of the solvent water. It is possible that an OH might be removed from a substrate at one step of the reaction and remain bound to the Mn and be returned in a second step without significant interchange with the ^{18}O of the solvent. It is noteworthy that Chang *et al.* (1966) found that the exchange of $^{14}CO_2$ was much more rapid with oxalacetate than that of IDP-^{14}C with ITP or phosphoenolpyruvate-^{14}C with oxalacetate. The differences in rates of exchange presumably are due to differences in the rate of dissociation of the CO_2, IDP, and phosphoenolpyruvate from the enzyme, since these products would be formed simultaneously on the enzyme from oxalacetate and GTP by the proposed concerted reaction.

The role of the tightly bound Mn of pyruvate carboxylase in the fixation of CO_2 in oxalacetate has been studied by Mildvan *et al.* (1966). This reaction involves biotin with bicarbonate as the active species (Cooper *et al.*, 1968), thus the sequence is quite different from that discussed above for carboxytransphosphorylase. HCO_3^- is likewise involved in the reaction catalyzed by propionyl-CoA carboxylase (Kaziro *et al.*, 1962) and phosphoenolpyruvate carboxylase (Maruyama *et al.*, 1966).

It is noteworthy that Webster (1965) has found that acetyl-CoA synthetase has a dual requirement for divalent metals. This reaction as well as that of carboxytransphosphorylase involves PP_i as a reactant. Webster could separate the synthetase reaction into two partial reactions and show that both types of metals are required for one partial reaction and only one type for the other partial reaction. A similar situation applies to carboxytransphosphorylase since only type I metal is required for the exchange of $^{14}CO_2$ but both type I and type II for the exchange of $^{32}P_i$ into phosphoenolpyruvate.

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