Phosphoenolpyruvate Carboxytransphosphorylase. An Investigation of the Mechanism with ^{18}O†

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ABSTRACT: Phosphoenolpyruvate carboxytransphosphorylase catalyzes the formation of oxalacetate and PP_i from phosphoenolpyruvate, P_i, and CO_2; in the absence of CO_2, the products are pyruvate and PP_i. Wood *et al.* have proposed that the carboxytransphosphorylase reaction involves pyrophosphorylenolpyruvate as an intermediate. This mechanism involves a hydrolytic step and thus predicts the incorporation of ^{18}O from $H_2{}^{18}O$ into the products. It has now been shown that this does not occur and with phosphoenolpyruvate (P-enolpyruvate) containing ^{18}O in the enolester linkage the ^{18}O is converted to the carbonyl group of oxalacetate or pyruvate. Thus the conversion involves an O–P cleavage of the enolester. In conjunction with the studies of Willard and Rose the results indicate that this cleavage occurs by attack of P_i on the phosphoryl group of P-enolpyruvate with formation of PP_i and oxalacetate. Two mechanisms are considered. In the first, enolpyruvate is generated and carboxylated while attached to the enzyme yielding oxalacetate; in the absence of CO_2 the enolpyruvate dissociates from the enzyme and is protonated nonenzymatically to yield keto pyruvate. The second mechanism involves the formation of pentavalent pyrophosphoenolpyruvate which then is carboxylated to yield oxalacetate and PP_i. In the absence of CO_2, the intermediate breaks down irreversibly to enolpyruvate and PP_i. This latter mechanism is in accord with all available data.

Phosphoenolpyruvate carboxytransphosphorylase (phosphoenolpyruvate carboxykinase (pyrophosphate), EC 4.1.1.38) catalyzes reactions 1 and 2, referred to as the oxalacetate and pyruvate reactions, respectively. The enzyme has been ob-

$$\text{P-enolpyruvate} + CO_2 + P_i \rightleftharpoons \text{oxalacetate} + PP_i \quad (1)$$

$$\text{P-enolpyruvate} + P_i \longrightarrow \text{pyruvate} + PP_i \quad (2)$$

tained in crystalline form from *Propionibacterium shermanii* (Lochmüller *et al.*, 1966). It is a rhomboid tetramer of molecular weight 4.0×10^5 and dissociates to dimers and monomers, all of which are active (Haberland *et al.*, 1972).

The purpose of the present investigation was to study the mechanism of the reaction. Wood *et al.* (1969a) proposed that the reaction occurs with the formation of pyrophosphorylenolpyruvate as an intermediate, as illustrated in mechanism A of Figure 1. This mechanism was proposed because of a requirement for PP_i in the exchange of CO_2 with the β-carboxyl of oxalacetate under conditions where there was no exchange of [^{14}C]pyruvate with oxalacetate. Furthermore, the CO_2–oxalacetate exchange occurred in the presence of EDTA but there was no exchange of $^{32}P_i$ with PP_i or phosphoenolpyruvate[1] (P-enolpyruvate). Finally, under the same conditions (*i.e.*, the presence of CO_2, EDTA, and mercaptoethanol) no significant decarboxylation of oxalacetate to pyruvate and CO_2 was observed. However, Willard and Rose (1973) have now demonstrated that oxalacetate is decarboxylated to pyruvate and CO_2 under different conditions. Since mechanism A of Figure 1 involves a hydrolysis of the pyrophosphoenolpyruvate intermediate, there would be incorporation of ^{18}O from solvent water into pyrophosphate product.

S. J. Benkovic (private communication), on the basis of model studies of the hydrolysis of P-enolpyruvate (Benkovic and Schray, 1969), suggested that the carboxytransphosphorylase reaction might occur with the formation of a cyclic anhydride between the enolphosphoryl and the carboxyl of P-enolpyruvate (mechanism B of Figure 1). Cyclic anhydride intermediates of this type were initially proposed by Clark and Kirby (1963) in the hydrolytic reactions of dimethyl or diphenyl P-enolpyruvate. In mechanism B the intermediate is hydrolyzed by solvent water with incorporation of ^{18}O from $H_2{}^{18}O$ into the pyruvate carboxyl.

Mechanism C of Figure 1 is based on the work of Bondinell *et al.* (1971) and DeLeo *et al.* (1973). Bondinell *et al.* (1971) found the catalysis by 5-enolpyruvylshikimate 3-phosphate synthetase involves the displacement of phosphate, rather than phosphoryl, from P-enolpyruvate. Likewise, DeLeo *et al.* (1973) found that the 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthetase catalyzes carbon–oxygen bond cleavage of the P-enolpyruvate and displacement of phosphate. An analogous mechanism for carboxytransphosphorylase was considered in which a double displacement reaction occurs. In this mechanism the initial attack on the P-enolpyruvate is by hydroxide ion, with incorporation of ^{18}O from solvent water into the enol oxygen of pyruvate (or oxalacetate). Furthermore, since phosphate is the leaving group from P-enolpyruvate this mechanism (to the exclusion of all others) involves carbon–oxygen (C–O) bond cleavage, as opposed to phosphorus–oxygen (P–O) bond cleavage in which phosphoryl is the leaving group.

Mechanism D of Figure 1 involves the formation of enolpyruvate as an intermediate and is suggested by analogy with pyruvate kinase (Rose, 1960; Reynard *et al.*, 1961) and oxalacetate decarboxylase (Kosicki, 1968). Since water is not involved, ^{18}O from solvent water should not be incorporated

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[1] Abbreviation used is: P-enolpyruvate, phosphoenolpyruvate.

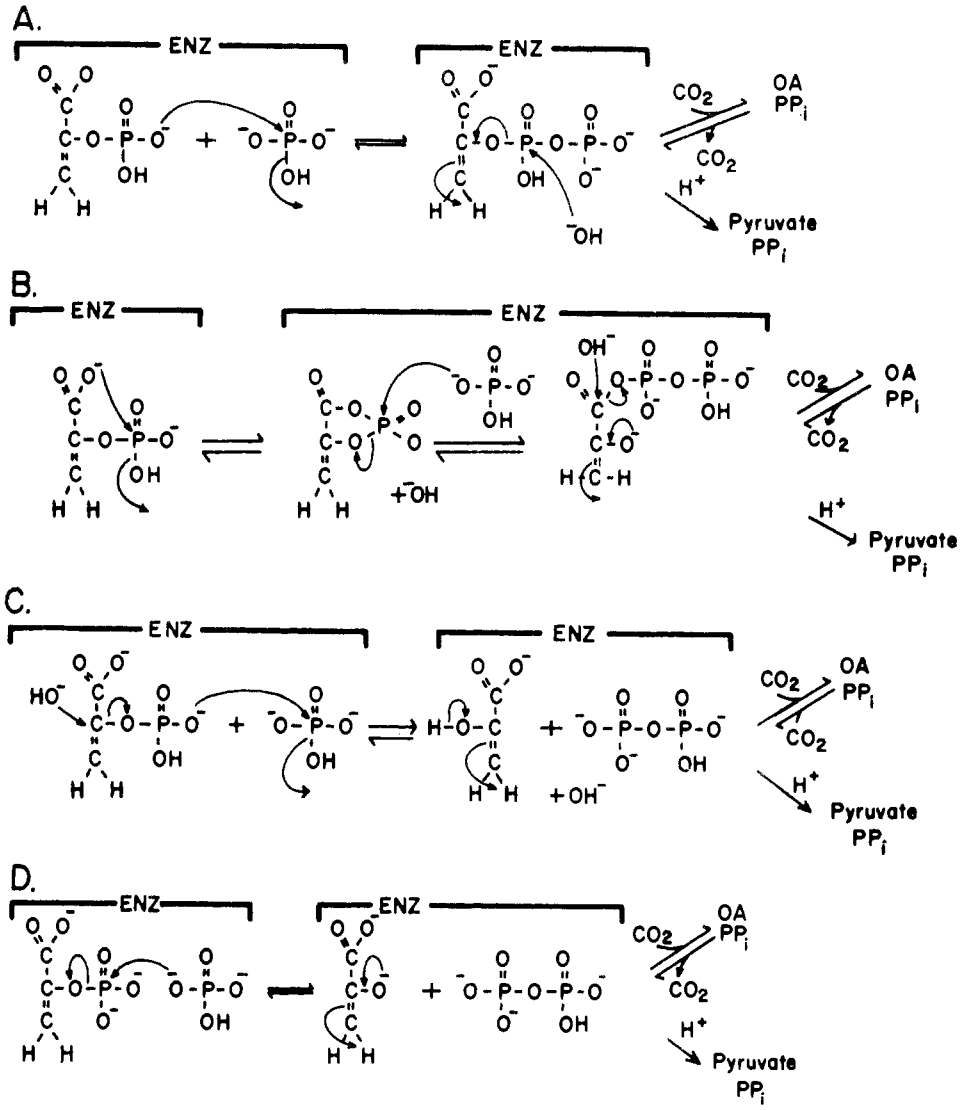

FIGURE 1: Mechanisms which have been considered for the carboxytransphosphorylase reactions: (A) *via* a pyrophosphorylenolpyruvate intermediate; (B) *via* a cyclic anhydride as suggested by Benkovic (private communication); (C) enolate generation *via* P_i displacement; and (D) enolate generation *via* phosphoryl transfer.

into any of the products. Also, since the phosphoryl group is cleaved from P-enolpyruvate, the mechanism involves P–O bond breakage.

Experimental Section

Enzymes. Phosphoenolpyruvate carboxytransphosphorylase was isolated and assayed as described by Wood *et al.* (1969b). Malate dehydrogenase was from Boehringer and catalase, inorganic pyrophosphatase, and alkaline phosphatase were from Worthington.

Chemicals. Isotopically enriched water was from Bio-Rad Laboratories, 5 atom % (Low-D), or from Miles Laboratories, 10 atom % (normalized). P-enolpyruvate labeled with ^{18}O in the carboxyl and enolester oxygens was kindly supplied by Dr. D. B. Sprinson and was prepared as described by Bondinell *et al.* (1971). The carboxyl oxygens contained 8.84 atom % excess ^{18}O and the enolester oxygen, 13.2 atom % excess. Other chemicals were reagent grade obtained from commercial sources.

Use of H_2O_2 to Obtain the Carbonyl Oxygens of Oxalacetate and Pyruvate in Stable Forms. Carbonyl oxygens exchange very rapidly with the oxygens of water and to determine their ^{18}O content conversion to a stable form by oxidation or reduction is necessary. Zalkin and Sprinson (1966) have employed H_2O_2 to investigate isocitrate dehydrogenase, the α-ketoglutarate being converted to succinate in which the carbonyl oxygen is stabilized in the carboxyl of the resulting succinate. These workers established that the reaction of H_2O_2 with α-keto acids occurs without exchange of oxygen with the water during the decarboxylation and ^{18}O is not lost from the carbonyl oxygen during the reaction. We have found that carboxytransphosphorylase is active when H_2O_2 is present at 0.12 M; hence we employed this reagent in our studies. In the presence of H_2O_2, the products were malonate from oxalacetate and acetate from pyruvate.

Purification of Reaction Components. QAE-Sephadex was prepared in either the chloride or bicarbonate form. Columns 0.9 × 20 cm were routinely used and were equilibrated with water. A 1-l. linear gradient of 0–0.4 M triethylammonium bicarbonate (pH 7) was employed to elute, in the following order, acetate, phosphate, P-enolpyruvate, and pyrophosphate. The triethylammonium bicarbonate was removed by lyophilization and the residue stored *in vacuo* over P_2O_5. When malonic acid was to be isolated, it was eluted from QAE-Sephadex with 0.2 N formic acid. The formic acid was

TABLE I: Phosphoenolpyruvate Carboxytransphosphorylase Reaction in $H_2{}^{18}O$.[a]

Reaction[a]	Atom % Excess ^{18}O Found in					Theoretical Atom % Excess ^{18}O Based on Mechanisms of Figure 1			
	H_2O	Malonate from Oxalacetate	Acetate from Pyruvate	Phosphate from Pyrophosphate	Pyruvate Carboxyl	A	B	C	D
1. Oxalacetate	5.87			0.0		0.73	0	0	0
2. Pyruvate	2.00				0.0	0	1.00	0	0
3. Oxalacetate	4.57	2.16				2.28[b]	2.28[b]	3.42[b]	2.28[b]
4. Pyruvate	3.39		0.24			0	0	1.70	0

[a] Experiment 1: The mixture contained in μmoles: $KHCO_3$, 60; P_i, 40; MgCl, 40; P-enolpyruvate, 18.4; H_2O_2, 195; $H_2{}^{18}O$, 3.6 ml; and P-enolpyruvate carboxytransphosphorylase, 5.8 units in a total volume of 4.0 ml. Before addition of enzymes, CO_2 was bubbled through the mixture for 15 min. The reaction was for 3.75 hr and 10.5 μmol of pyrophosphate was formed. Catalase was added and the $H_2{}^{18}O$ was recovered by lyophilization. Experiment 2: The mixture contained in μmoles: P-enolpyruvate, 40; P_i, 200; $MgCl_2$, 80; $H_2{}^{18}O$, 5 atom %, 8.0 ml in a final total volume of 20 ml. Nitrogen was passed through the mixture for 20 min to remove any dissolved CO_2 and then inorganic pyrophosphatase, 4 units, and P-enolpyruvate carboxytransphosphorylase, 1.5 units, were added and the mixture was incubated at 25° under N_2. After 5 hr, pyruvate production ceased at 36 μmol and the reaction was terminated by adding 20 μmol of EDTA and chilling. The pyruvate was isolated by chromatography. The pyruvate was lyophilyzed and crystallized as the silver salt and the dry salt decarboxylated by gentle heating. The resulting CO_2 was analyzed for its isotope content. Experiment 3: The mixture contained in μmoles: $KHCO_3$, 275, P-enolpyruvate, 116; $MgCl_2$, 108; P_i, 60; H_2O_2, 980; and P-enolpyruvate carboxytransphosphorylase, 17.5 units (specific activity 15); pyrophosphatase, 135 units; $H_2{}^{18}O$, 12 atom %, 3 ml; in a volume of 7.0 ml. The mixture was saturated with CO_2 as above. The reaction proceeded for 4 hr at which time 0.02 mg of catalase was added and the mixture was lyophilized to recover the $H_2{}^{18}O$; 103 μmol of phosphoenolpyruvate was consumed. Experiment 4: The mixture contained in μmoles: P-enolpyruvate, 100; P_i, 100; H_2O_2, 100; $MgCl_2$, 40; $H_2{}^{18}O$, 10 atom %, 4.0 ml in a final total volume of 10 ml. Nitrogen was bubbled through the mixture for 20 min. Inorganic pyrophosphatase (4 units) and P-enolpyruvate carboxytransphosphorylase (5 units) were added followed by incubation which was at 25° under nitrogen. The acetate yield was 46 μmol. [b] Calculated on the basis of total exchange of HCO_3^- with $H_2{}^{18}O$.

removed by lyophilization and the residue extracted with diethyl ether. The ether was removed under a stream of nitrogen and the malonic acid stored *in vacuo* over P_2O_5. Phosphate was isolated and then precipitated as the monopotassium salt from ethanol as described by Dempsey *et al.* (1963). Isolated pyrophosphate was converted to phosphate by treatment with inorganic pyrophosphatase and then precipitated as the monopotassium salt.

Determination of ^{18}O. For determination of the ^{18}O in phosphate, water, acetate, or malonate, the samples were pyrolyzed in guanidine-HCl as described by Boyer *et al.* (1961). The mass ratio of the resulting CO_2 was determined on a CEC cycloidal mass spectrometer Model No. 21-130. Atom % excess ^{18}O was calculated from the relationship

$$\text{atom \% excess} = \frac{R - 0.0042}{2 + R - 0.0042} \times 100$$

where R is the measured mass 46:44 ratio (Boyer *et al.*, 1961).

Analytical Methods. Inorganic phosphate was determined by the method of Gomori (1941) and inorganic pyrophosphate by the same technique after treatment with pyrophosphatase. P-enolpyruvate was determined with phosphoenolpyruvate carboxytransphosphorylase by making the P-enolpyruvate the limiting reaction component. Acetate was determined enzymatically by the method of Schulman and Wood (1971). Malonic acid was determined by Celite chromatography (Swim and Krampitz, 1954).

Results

Reactions with Phosphoenolpyruvate Carboxytransphosphorylase in $H_2{}^{18}O$. The results of the experiments in $H_2{}^{18}O$ enriched media are summarized in Table I. Mechanism A of Figure 1 was tested in experiment 1 and mechanism B in experiment 2. No incorporation of ^{18}O from solvent water was observed in either experiment. The double displacement mechanism C of Figure 1 was tested in experiments 3 and 4. In experiment 3, significant incorporation of ^{18}O into malonate (arising from oxalacetate) is seen and the 2.16 atom % excess corresponds to an incorporation of 1.9 mol of ^{18}O from $H_2{}^{18}O$. This label most likely was in the β-carboxyl of oxalacetate and was incorporated by rapid exchange of HCO_3^- with $H_2{}^{18}O$ prior to fixation of the CO_2. The small incorporation of ^{18}O into acetate *via* the pyruvate reaction (experiment 4) provably is not mechanistically significant and adds support to the conclusions regarding experiment 3.

The above experiments with $H_2{}^{18}O$ indicate that a hydrolytic step is not involved in the enzymic reaction and suggest that the carboxytransphosphorylase reaction does not occur by the mechanisms A, B, or C of Figure 1.

Determination of the ^{18}O *in P-enolpyruvate Labeled in the Enolester.* The [^{18}O]P-enolpyruvate used in these studies was degraded enzymatically with alkaline phosphatase which cleaves the enolester bond of P-enolpyruvate at the O–P position (Cohn, 1949) and chemically with Hg^+ at low pH which cleaves the C–O bond (Benkovic and Schray, 1968). The results of these experiments are given in Table II. Some ^{18}O (0.25 atom % excess) was observed in the phosphate from the enzymatically cleaved [^{18}O]P-enolpyruvate. This small amount of ^{18}O in the phosphate may have resulted from nonenzymic hydrolysis of the P-enolpyruvate during storage following its synthesis. The chemically cleaved material yielded phosphate with 2.86 atom % excess which is equivalent to 11.4 atom % excess ^{18}O in the enolester oxygen of the P-enolpyruvate. This may be considered as a minimal

TABLE II: Degradation of Phosphoenolpyruvate Labeled with ^{18}O in the Enolester Oxygen.[a]

Treatment	Atom % Excess	
	^{18}O Found in Phosphate	Theoretical
5. Alkaline phosphatase	0.25	0.0
6. $Hg(ClO_4)_2$	2.86	3.3

[a] Experiment 5: 109 μmol of [^{18}O]P-enolpyruvate and 4.5 units of alkaline phosphatase were placed in 6 ml of 0.1 M Tris-Cl (pH 8.0). After 3.75 hr, the reaction was terminated; 102.5 μmol of P-enolpyruvate was utilized. Experiment 6: To 30 μmol of [^{18}O]P-enolpyruvate dissolved in 0.5 ml of H_2O was added 0.5 ml of 0.08 M $Hg(ClO_4)_2$ and one drop of 5 N HCl (final pH was 1 or less). After 12 min, 0.1 ml of concentrated NH_4OH was added and the solution cooled to precipitate the perchlorate and the precipitate was removed by centrifugation. The precipitate was washed once with 5 M NH_4OH and the supernatant liquids were combined. The inorganic phosphate was purified and precipitated in both experiments as described in the Experimental Section.

value since some O–P bond cleavage may have occurred during the chemical dephosphorylation and would dilute the label in the final product. At pH values below 2.0, C–O bond cleavage occurs primarily and as the pH rises, O–P bond cleavage increases and may account for 30% of the reaction (Benkovic and Schray, 1968). The value of 13.2 is used throughout this report as the true value of the atom % excess in the enolester oxygen of the P-enolpyruvate.

^{18}O in the Products from P-enolpyruvate with ^{18}O in the Enolester. The results are shown in Table III. In experiments 7 and 8, the reaction was conducted in the absence of pyrophosphatase and the resulting pyrophosphate was analyzed for ^{18}O. The very small incorporation observed probably is of no significance since the atom % excess in the phosphate in experiment 5 of Table II was 0.25 whereas the atom % excess in the pyrophosphate in experiment 7 was $0.12 \times 2 = 0.24$. The factor of 2 is required since one phosphate of the pyrophosphate arises from the inorganic phosphate in the carboxytransphosphorylase reaction.

In experiment 9 of Table III, the 1.1 atom % excess found in the malonate represents a 59% conversion of the label of the enolester oxygen of P-enolpyruvate to the carbonyl of the oxalacetate. This calculation is based on an ^{18}O content of 13.2 atom % excess in the enolester. The P-enolpyruvate used in experiment 9 was diluted prior to use with unlabeled P-enolpyruvate and the atom % excess was calculated using the dilution factor.

The recovery of only 59% of the label in experiment 9 and only 23% in experiment 10 is disappointingly low but probably is due to the small amount of material available from the enzymic reactions. We were not able to recover 100% of the ^{18}O from malonate or acetate samples unless large quantities were used in the procedure. For example, when 100 μmol of malonic acid was subjected to purification and analysis, only 78% of the label was recovered but when 336 μmol was used, the per cent recovery was 94. Similar problems were encountered with acetate. It also is possible that the reaction between the oxalacetate or pyruvate and H_2O_2 was not sufficiently rapid to prevent some exchange between the carbonyl oxygens and the solvent water. We conclude from the data in Table III that the cleavage of the enolester bond of P-enolpyruvate occurs between the oxygen and phosphorus. This conclusion is supported by the fact that essentially no ^{18}O was found in the phosphate moieties which were isolated from the reactions involving [^{18}O]P-enolpyruvate.

Discussion

The following observations must be accounted for in relation to the carboxytransphosphorylase reaction. (1) The oxalacetate reaction apparently occurs in two steps. The first can be demonstrated by use of the $^{14}CO_2$–oxalacetate exchange reaction and it occurs in the presence of metal chelators such as EDTA. The second step, which involves cleavage of the pyrophosphate, does not occur in the presence of the chelators, there being no transfer of ^{32}P from PP_i into P_i or P-enolpyruvate (Wood *et al.*, 1969a). (2) PP_i is required for the exchange of $^{14}CO_2$ into oxalacetate (Wood *et al.*, 1969a) and the analogs methylene diphosphonate and imidodiphosphate do not substitute for the PP_i but are competitive inhibitors with respect to PP_i (Willard and Rose, 1973). (3) The exchange of CO_2 into oxalacetate is not accompanied by exchange of [^{14}C]pyruvate into oxalacetate (Wood *et al.*, 1969a). (4) P-enolpyruvate and P_i are irreversibly converted to pyruvate and PP_i in the absence of CO_2 (Davis *et al.*, 1969). With an increasing concentration of CO_2 the pyruvate reaction decreases and the oxalacetate reaction increases (Wood *et al.*, 1969a). (5) The oxalacetate reaction is favored by the presence of thiols whereas the pyruvate reaction is inhibited (Davis *et al.*, 1969), and in the presence of thiols with saturating concentrations of CO_2, there is little or no pyruvate reaction (Wood *et al.*, 1969a). In addition, under these conditions, the decarboxylation of oxalacetate to pyruvate and CO_2 is negligible. (6) The carboxylation of P-enolpyruvate to yield oxalacetate involves CO_2 rather than HCO_3^- (Cooper *et al.*, 1968). (7) The carboxylation of P-enolpyruvate is stereospecific occurring on the ψ side of the molecule (Rose *et al.*, 1969) whereas the protonation of the P-enolpyruvate in the pyruvate reaction is not stereospecific (Willard and Rose, 1973). (8) Oxygen from $H_2{}^{18}O$ is not incorporated in the products of the carboxytransphosphorylase reaction and there is O–P cleavage of the ester bond of the P-enolpyruvate (present communication). Thus, of the mechanisms considered in Figure 1, only D is consistent with the ^{18}O results.

The assumption has been made in the studies with $H_2{}^{18}O$ that the hydrolytic agent at the active site of the enzyme is in equilibrium with the solvent water. If the reaction occurred in a hydrophobic crevice of the enzyme, the exchange with $H_2{}^{18}O$ might occur very slowly and the ^{18}O from $H_2{}^{18}O$ not enter the products. Because of the polyanionic nature of the reactants, it seems unlikely, however, that the active site would be hydrophobic. With aconitase, in which there is a dehydration, Rose and O'Connell (1967) have shown that the hydroxyl exchanges extensively with the solvent but the transferred hydrogen is fully retained.

Although mechanism D is attractive, Willard and Rose (1973) have noted that it is surprising that neither methylene diphosphonate nor imidodiphosphate substituted for the requirement of PP_i in the oxalacetate exchange reaction even though the analogs are bound to the PP_i site. These results suggest that the PP_i may be required to form a direct chemical derivative with the oxalacetate rather than have an indirect effect on the enzyme by a conformation change. In addition, we

TABLE III: Incorporation of ^{18}O into the Products from P-enolpyruvate Labeled in the Enolester Oxygen.[a]

Reaction	Atom % Excess ^{18}O Found in: P-enol pyruvate	Malonate from Oxalacetate	Acetate from Pyruvate	Phosphate from Pyrophosphate	Theoretical Atom % Excess ^{18}O Based on Mechanisms of Figure 1: A	B	C	D
7. Oxalacetate	13.2			0.12	0	0	1.65	0
8. Pyruvate	13.2			0.11	0	0	1.65	0
9. Oxalacetate	7.46	1.1			1.86	1.86	0	1.86
10. Pyruvate	13.2		1.56		6.6	6.6	0	6.6

[a] Experiment 7: The mixture contained in μmoles: [^{18}O]P-enolpyruvate, 16.5; $KHCO_3$, 100; $MgCl_2$, 40; P_i, 23.3; H_2O_2, 326; and P-enolpyruvate carboxytransphosphorylase, 2.9 in a volume of 6 ml. Pyrophosphate was purified and treated as described in the Experimental Section. Experiment 8: The mixture contained in μmoles: P-enolpyruvate, 100; P_i, 100; $MgCl_2$, 40; H_2O_2, 100; and P-enolpyruvate carboxytransphosphorylase, 5 units, in a volume of 10 ml. When P-enolpyruvate was no longer consumed, the reaction was terminated by addition of EDTA and catalase and the PP_i isolated on QAE-Sephadex. The yield was 13 μmol of PP_i. Experiment 9: The mixture contained in μmoles: P-enolpyruvate, 284.8; $KHCO_3$, 600; $MgCl_2$, 240; P_i, 140; H_2O_2, 2000; P-enolpyruvate carboxytransphosphorylase, 32 units; and pyrophosphorylase, 216 units; in a volume of 20.1 ml. CO_2 was passed through the mixture for 10 min prior to addition of enzymes. The reaction proceeded for 9 hr at 25° and then 0.03 mg of catalase was added. Malonate was purified as described in the Experimental Section; 280 μmol of P-enolpyruvate was consumed. Experiment 10: The mixture was identical with experiment 8 except that 4 units of pyrophosphorylase was added. Acetate was isolated as described in the Experimental Section. The yield was 13 μmol.

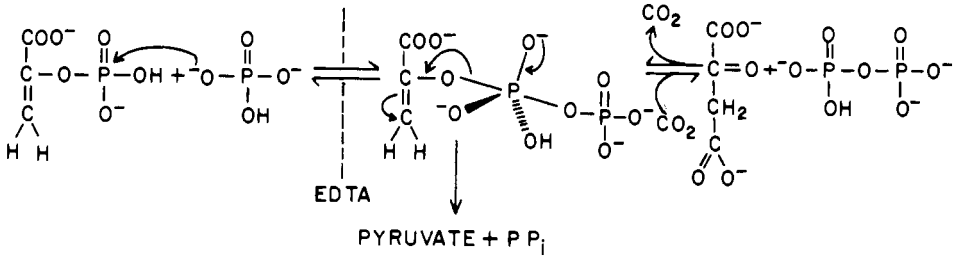

FIGURE 2: Proposed mechanism for the carboxytransphosphorylase reaction involving an intermediate with a pentacoordinated phosphorus.

have reinvestigated the exchange of [^{14}C]pyruvate with oxalacetate during the exchange of CO_2 with oxalacetate and have found that even when pyruvate is present at a high level (0.12 M) there is little or no exchange. Although the enzyme does not bind the keto form of pyruvate or catalyze its tautomerization it would be expected that at this high concentration there would be a significant concentration of enolate present. If there were an enolate site on the enzyme, some exchange would have been expected.

For the above reasons, other possible mechanisms need to be considered. One mechanism which is in accord with most observations is that shown in Figure 2 which has been considered by Willard and Rose (1973).

In this mechanism, we envision an initial attack of P_i on the P-enolpyruvate phosphoryl leading to a pentavalent pyrophosphoenolpyruvate intermediate with the bridge atoms of enolpyruvate and PP_i in apical positions. This species collapses to oxalacetate and PP_i in the presence of CO_2, or in its absence, enolpyruvate and PP_i are formed. The enolpyruvate is released from the enzyme and protonated in solution. In this respect, the mechanism is reminiscent of the 6-P-gluconate hydratase reaction studied by Meloche and Wood (1964).

Although this mechanism appears analogous to mechanism A of Figure 1, it differs in two significant respects. It does not require the energetically unfavorable displacement of hydroxide ion and does not involve a subsequent hydrolytic step. Thus, there is no prediction of the incorportion of ^{18}O from solvent water. An attractive feature of this mechanism is that it accounts for the requirement of PP_i for the exchange of $^{14}CO_2$ into oxalacetate in the presence of EDTA and the failure of ^{32}P from PP_i to exchange into P-enolpyruvate under these conditions. Furthermore, this mechanism also accounts for the observation that the competitive inhibitors, methylene diphosphonate and imidodiphosphate, fail to replace PP_i in the $^{14}CO_2$–oxalacetate exchange reaction. If a pentavalent species involving these compounds were possible, it would be expected to be somewhat different than that formed by PP_i.

In the mechanism of Figure 2, enolpyruvate would not be a direct intermediate of the reaction, but would rather be formed by a side reaction through breakdown of the pentavalent intermediate which would accumulate in the absence of CO_2.

The pyruvate reaction is similar to the pyruvate kinase reaction, P_i being the phosphate acceptor rather than ADP. The pyruvate kinase reaction has been shown to be reversible (Lardy and Ziegler, 1945; McQuate and Utter, 1959) but thus far the reversibility of pyruvate reaction has not been demonstrated with carboxytransphosphorylase (Wood *et al.*, 1969a). There are three factors in addition to possible fundamental differences in the mechanisms which explain why reversibility has not been possible with carboxytransphosphorylase: (1) pyruvate kinase has tautomerase activity (Robinson and Rose, 1972) and apparently binds the keto form of pyruvate whereas carboxytransphosphorylase does

not have such activity (Willard and Rose, 1973); (2) with pyruvate kinase, McQuate and Utter (1959) could use substantial concentrations of ATP to assist in the reversal whereas carboxytransphosphorylase is inhibited by even low concentrations of PP_i (Davis *et al.*, 1969); (3) the free energy of hydrolysis of PP_i is lower than that of ATP (Wood *et al.*, 1966); thus the equilibrium is less favorable for P-enolpyruvate formation from PP_i and pyruvate than from ATP and pyruvate.

The reaction catalyzed by phosphoenolpyruvate carboxykinase, *i.e.*, the conversion of P-enolpyruvate plus ADP or GDP and CO_2 to oxalacetate and ATP or GTP, is similar to the oxalacetate reaction of carboxytransphosphorylase: (1) both enzymes utilize CO_2 rather than HCO_3^- as the substrate for carboxylation (Cooper *et al.*, 1968); (2) both show the same stereochemistry (Rose *et al.*, 1969); (3) carboxykinase catalyzes a partial reaction in which pyruvate is formed by the dinucleotide-dependent decarboxylation of oxalacetate (Utter *et al.*, 1954); (4) both reactions to oxalacetate are readily reversible with ΔG° values of -0.1 and -0.3 kcal for carboxykinase and carboxytransphosphorylase, respectively (Wood *et al.*, 1966). The properties of both enzymes have been recently reviewed (Utter and Kolenbrander, 1972) and it seems likely that the two enzymes may have a similar reaction mechanism. Miller and Lane (1968) demonstrated that ^{18}O of $H_2{}^{18}O$ is not incorporated into the products of the carboxykinase reaction and they postulated that the mechanism might occur by a concerted or one-step mechanism. By analogy with carboxytransphosphorylase, based on our observations and those of Willard and Rose (1973), it seems quite possible that the reaction catalyzed by carboxykinase may also involve two steps with ADP or GDP as the phosphoryl acceptor in place of P_i.

The formation of oxalacetate from P-enolpyruvate by phosphoenolpyruvate carboxylase differs from those above but may occur by a somewhat analogous mechanism. Muruyama *et al.* (1966) using $HC^{18}O_3^-$ have found that the reaction involves HCO_3^- as the reactive species, and that all three oxygens of $HC^{18}O_3^-$ are incorporated into products, one into phosphate and two into the β-carboxyl of oxalacetate. Cooper and Wood (1971) by spectrophotometric techniques have confirmed that HCO_3^- is the active species. Muruyama *et al.* (1966) proposed that the mechanism involves an irreversible concerted reaction of P-enolpyruvate and HCO_3^- *via* formation of a transient six-membered ring. From stereochemical reasoning, such a mechanism does not seem feasible. In the case of phosphorus, the attacking and leaving groups must be in line (180°), a condition not possible in a six-membered transition state.

We suggest that all three carboxylation reactions involving P-enolpyruvate share two features in common. First, all involve an initial attack of a nucleophile on the phosphoryl of P-enolpyruvate; in the carboxytransphosphorylase reaction, this is P_i, in the carboxykinase reaction, it is ADP or GDP, and in the carboxylase reaction, it is the oxyanion of bicarbonate. Second, all may involve a two-step mechanism. In the first step, P-enolpyruvate and the attacking nucleophile form a covalent intermediate and in the second step this intermediate is carboxylated to yield oxalacetate.

It is interesting to speculate on the evolutionary significance of these three reactions. There is an increase in complexity of the attacking nucleophile from HCO_3^- and P_i to ADP. If such an evolutionary relationship could be demonstrated at the structural level, it might provide further support for the hypothesis of the involvement of PP_i as a primitive energy reservoir. Confirmation of such a speculation obviously requires much further characterization of the enzymes involved.

Acknowledgment

The authors thank Professor S. J. Benkovic for his assistance in determining the ^{18}O in the carboxyl group of pyruvate and for helpful discussion.

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Porphobilinogen Oxygenase from Wheat Germ: Isolation, Properties, and Products Formed†

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ABSTRACT: The enzymatic oxidation of porphobilinogen by a new enzyme isolated from wheat germ is described. The name of porphobilinogen oxygenase was proposed for the enzyme. An exchange on DEAE-cellulose followed by filtration through DEAE-Sephadex A25 allowed to separate the enzyme from a proteic inhibitor present in the crude extracts. Porphobilinogen oxygenase belonged to the family of the pyrrolooxygenases, and required the presence of oxygen and of a reducing agent. The enzyme existed in several molecular aggregation forms. Three multiple weight active forms were obtained which interconverted among them. The oxygenase had an allosteric kinetics and was desensitized by the addition of Co^{2+} or Ni^{2+} or by freezing. It is a metalloenzyme containing iron as ferrous ion, and was inhibited when the metal was removed by exhaustive dialysis against 1 mM EDTA. Activity was restored by addition of various metal ions. Metal-chelating substances inhibited the enzymatic activity. Porphobilinogen oxygenase had a relatively broad specificity for alkylpyrroles containing a free α position. The oxidation of porphobilinogen produced 2-hydroxy-5-oxoporphobilinogen as the major product and 5-oxoporphobilinogen as the minor product.

Porphobilinogen, 2-aminomethyl-3-carboxymethyl-4-carboxyethylpyrrole, is the universal precursor of heme, chlorophylls, porphyrins, and the corrin nucleus (Lascelles, 1964). As such, it is the only known monopyrrole in primary metabolism and has no other biological analogs. It is originated in the self-condensation of two units of δ-aminolevulinic acid. The δ-aminolevulinic acid synthetase, which is the enzyme responsible for the metabolic formation of the latter, is apparently also the one which regulates the amount of porphobilinogen formed (De Matteis, 1967). Porphobilinogen itself is consumed by a complex of two enzymes, porphobilinogen deaminase and uroporphyrinogen III cosynthetase, which transforms it into uroporphyrinogen III, the first cyclic tetrapyrrole intermediate in the biosynthetic sequence leading to the natural porphyrins (Lascelles, 1964).

Of the two enzymes mentioned above, only porphobilinogen deaminase consumes porphobilinogen. The purified enzyme from wheat germ had a K_m of the order of 10^{-5} (Frydman and Frydman, 1970), suggesting that under physiological conditions the concentration of porphobilinogen may be very small. During the metabolic disorders known as hepatic porphyrias a considerable increase in the physiological concentrations of both δ-aminolevulinic acid and porphobilinogen takes place, a phenomenon that also occurs during the induction with drugs of the so-called experimental porphyrias (De Matteis, 1967). An analogous increase in the concentration of porphobilinogen in plant material is not known.

We already described in a preliminary note (Frydman *et al.*, 1972a) the existence in plants and animals of a new enzyme which oxidized porphobilinogen, for which we proposed the name of porphobilinogen oxygenase. The enzyme belonged to the general type of the pyrrolooxygenases, a new group of enzymes recently described (Frydman *et al.*, 1972b). The oxidation of porphobilinogen appears as an alternative pathway for porphobilinogen, diverting it from its well-known function as porphyrin precursor. The properties of the enzyme isolated from wheat germ will be described, as well as the nature of the new products formed by it.

Materials and Methods

Materials

Porphobilinogen (**1**)[1] and porphobilinogen lactam **2** were obtained by synthesis (Frydman *et al.*, 1969). 2-Aminomethyl-3-carboxymethylpyrrole (**3**) and its lactam **4**, 2-aminomethyl-4-methyl-3-carboxymethylpyrrole (**5**) and its lactam **6**, 2-aminomethyl-4-ethyl-3-carboxymethylpyrrole (**7**) and 2-methyl-3-carboxymethyl-4-carboxyethylpyrrole (**8**) were of synthetic origin (Frydman *et al.*, 1973).

Sodium dithionite, *p*-dimethylaminobenzaldehyde, and all other chemical reagents were commercial products of analytical grade. Wheat germ was a gift of Molinos Rio de la

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[1] Abbreviations used are: PBG, porphobilinogen, Paa, 2-aminomethyl-3-carboxymethylpyrrole; MPaa, 2-aminomethyl-3-carboxymethyl-4-methylpyrrole; PBGL, porphobilinogen lactam; MPaaL, 2-aminomethyl-3-carboxymethyl-4-methylpyrrole lactam.