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Formation of Enolpyruvate in the Phosphoenolpyruvate Carboxytransphosphorylase Reaction†

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ABSTRACT: Carboxytransphosphorylase converts phosphoenolpyruvate and P_i to pyruvate and PP_i. In the presence of CO_2, oxalacetate and PP_i are formed. The addition of a proton to yield pyruvate is nonstereospecific whereas the addition of CO_2 is only to the *si* face of phosphoenolpyruvate to yield oxalacetate. These results are explained by a mechanism in which phosphoryl transfer to P_i gives rise to enzyme-bound enolpyruvate which is specifically carboxylated. Pyruvate is formed from enolpyruvate after it has dissociated from the enzyme and the keto form of pyruvate never occurs on the enzyme. That the formation of the keto form of pyruvate is nonenzymatic is shown by the nonstereospecificity and the failure of carboxytransphosphorylase to detritiate [3-^{3}H]-pyruvate. According to this mechanism the requirement for pyrophosphate for $^{14}CO_2$–oxalacetate exchange should not involve phosphoryl transfer but may be indirect or synergistic. Contrary to expectation, however, neither methylene diphosphonate nor imidodiphosphate which were competitive inhibitors was effective in replacing pyrophosphate for this reaction, and are presumed not to contribute the degree of fit required for the synergistic role. A mechanism in which one phosphorus of pyrophosphate forms a pentacovalent adduct with the C-2 oxygen of enolpyruvate in the decarboxylation step might be much less favorable with the pyrophosphate analogs due to the polarity rules that govern the stereochemistry of such compounds. The nonstereoselectivity of pyruvate formation could then result from the decomposition of this metastable intermediate by a partially nonenzymatic route.

Phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38) is known to catalyze reactions 1 and 2 in which CO_2 and proton appear to act as alternate electrophiles (Davis *et al.*, 1969; Wood *et al.*, 1969a,b) for the activated carbon 3 of phosphoenolpyruvate[1] (P-enolpyruvate). The stereochemistry of CO_2 addition has been examined (Rose *et al.*, 1969)

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[1] The abbreviations used are: PCP, methylene diphosphonate; PNP, imidodiphosphate; P-enolpyruvate, phosphoenolpyruvate.

TABLE I: Stereochemistry of Pyruvate and Oxalacetate Formation from P-enolpyruvate.

Expt[a]	P-enolpyruvate Substrate Configuration	P-enolpyruvate Substrate cpm/μmol	Product of Enzyme Reaction	Product of Enzyme Reaction cpm/μmol	Malate Formed from Pyruvate cpm/μmol	Distribution of ^3H in Malates: ^3H in C_3 *pro-R* (%)	Distribution of ^3H in Malates: ^3H in C_3 *pro-S* (%)
1	*E*	6,450	Pyruvate		4,400	51.7	48.3
2	*Z*	37,500	Pyruvate		21,300	49.5	50.5
3a	*Z*	98,400	Pyruvate	100,000	75,000	50.0	50.0
3b	*Z*		Malate	110,000		98.0	2.0

[a] Conditions of experiments: experiments 1 and 2, (*E*)-[3-^{3}H]P-enolpyruvate (1 mM, 6450 cpm/μmol or (*Z*) [3-^{3}H]P-enolpyruvate (1 mM, 37,500 cpm/μmol) + KP_i (10 mM, pH 6.9) + $MgCl_2$ (12 mM) + DPNH (0.4 mM) + malate dehydrogenase (10 units) + lactate dehydrogenase (30 units) + carboxytransphosphorylase (0.3 unit as measured in the pyruvate reaction, *i.e.*, no CO_2). All reagents were prepared in D_2O and vigorously gassed with N_2. All enzymes were equilibriated in D_2O containing 0.5 mM KP_i by passage through a 0.25 × 8 cm Sephadex G-50 column. The reactions were conducted under N_2 and followed at 340 nm in 3-ml cuvettes of 1-cm light path. Malate dehydrogenase was included to convert any oxalacetate to malate formed due to trace amounts of CO_2. Experiment 3, (*Z*)-[3-^{2}H,^{3}H]P-enolpyruvate (1 mM, 98,400 cpm/μmol) + KP_i (10 mM, pH 7.4) + $MgCl_2$ (12 mM) + DPNH (0.5 mM) + malate dehydrogenase (0.3 unit) + $CoCl_2$ (0.1 mM) + carboxytransphosphorylase (0.02 unit) in H_2O. The reaction was followed at 340 nm in a 2-mm light path cuvette. About 0.25 μmol of malate was formed in the 1-ml incubation.

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

$$\text{P-enolpyruvate} + P_i \xrightarrow[H^+]{} \text{pyruvate} + PP_i \quad (2)$$

and the initial purpose of the present study was to determine that of the proton addition, in the formation of the methyl group of pyruvate. This problem has been approached by the use of the kinetic isotope effect in the malate synthase reaction (Cornforth *et al.*, 1969; Luthy *et al.*, 1969) and the pyruvate carboxylase reaction (Rose, 1970) to determine the stereochemistry of isotopically enantiomorphic methyl groups. The results of this study demonstrate that protonation is completely nonstereospecific, suggesting that enolpyruvate is ketonized after leaving the enzyme.

Materials and Methods

Crystalline phosphoenolpyruvate carboxytransphosphorylase (21 units/mg of protein) was purified from *Propionibacterium shermanii* and assayed as described by Wood *et al.* (1969a). Malate dehydrogenase and lactate dehydrogenase were obtained from Boehringer Mannheim. Imidodiphosphate was a kind gift of Dr. Ralph Yount and methylene diphosphonate was a product of Miles Laboratories. All other compounds were reagent grade obtained from commercial sources. Oxalacetic acid, assayed with malate dehydrogenase, was used within 3 hr of preparation. PP_i concentrations were determined with inorganic pyrophosphatase (Nutritional Biochemicals) and subsequent analysis of P_i by the method of Taussky and Schorr (1953).

Preparation of Labeled P-enolpyruvate. (*Z*)- and (*E*)-[3-^{3}H]PEP were prepared as described by Rose *et al.* (1969). (*Z*)- and (*E*)-[3-^{2}H,^{3}H]P-enolpyruvate were prepared by the following sequence. A mixture of 2- and 3-phosphoglycerate containing deuterium and tritium at C-3 in the (3*S*) and (3*R*) relation were prepared as given in Rose (1970). These were converted to the appropriate P-enolpyruvate[1] by equilibration with phosphoglycerate mutase and enolase at 55° as described by Cohn *et al.* (1970).

Purification of Products. Acetate, lactate, malate, pyruvate, and P-enolpyruvate were isolated on Dowex 1 (Cl^-) (1 × 3 cm) by elution with dilute HCl (0.001 N for acetate and lactate; 0.005 N for malate; 0.01 N for pyruvate; and 0.04 N for P-enolpyruvate.

Conversion of Lactate and Pyruvate to Malate. Following chromatography, lactate was converted to pyruvate by reaction of lactate dehydrogenase in the presence of phenazine methosulfate: in 1 ml, 50 mM triethanolamine (pH 7.5), 1 mM EDTA, 5 mM DPN, about 1 mg of lactate dehydrogenase, and 0.2 mM phenazine methosulfate. After the mixture was stirred for 25 min in the dark at 25°, H_2O_2 (100 μmol) was added and the acetate recovered by ion exchange chromatography. The acetate was converted to malate by the reaction sequence: acetate kinase, transacetylase, and malate synthase. Pyruvate was converted to malate by reaction of pyruvate carboxylase coupled to malate dehydrogenase. Both procedures followed the description given by Rose (1970).

The distribution of tritium in malate was determined with fumarase incubated in 0.05 M Tris-Cl (pH 7.5). The samples (0.4 ml), to which 60 μmol of sodium acetate was added, were lyophilized in a closed system to separate the water (C_3 *pro-R* position) from the nonvolatile residue (representing tritium derived from the C_3 *pro-S* position of malate as well as nonvolatile contaminating radioactivity). Both fractions were counted. The presence of sodium acetate prevented the loss of radioactivity by adsorption to glass. The malates were better than 97% free of radioactive contaminants in all cases, as judged by the ability of malate dehydrogenase alone to labilize this amount of radioactivity under alkaline conditions as given by Rose (1970).

Results

Table I summarizes studies on the stereochemistry of the formation of pyruvate from P-enolpyruvate labeled with isotopes of hydrogen at C-3. Two methods of analysis were used. In the first (experiments 1 and 2), the pyruvate formed in D_2O by reaction with [3-^{3}H]P-enolpyruvate was converted to lactate by lactate dehydrogenase in order to prevent its further reaction, if any, with the carboxytransphosphorylase. The lactate

was isolated and converted to malate for analysis with the fumarase reaction by the series of reactions in which it was first oxidized to pyruvate which was converted to acetate and then to malate by way of malate synthase. In the second method of analysis (experiment 3a), the pyruvate formed from [3-^{2}H,^{3}H]P-enolpyruvate was not trapped but was isolated as such and converted to malate with pyruvate carboxylase plus malate dehydrogenase. In both cases the malates were analyzed with fumarase and malate dehydrogenase to determine the fraction of the C-3 tritium counts of malate that were present in the *pro-R* position. Both approaches clearly indicate the unexpected fact that the transcarboxylase-catalyzed conversion of P-enolpyruvate to pyruvate includes a nonstereospecific step. From previous experiments (Rose, 1970) with these two analytical methods, the ratio of *pro-R* to *pro-S* C-3 tritiated malate formed from pyruvate generated in the pyruvate kinase reaction from (*E*)-[3-^{2}H,^{3}H]P-enolpyruvate was 32:68 by the malate synthase route and 36:64 by the pyruvate carboxylase route. For (*Z*)-[3-^{2}H,^{3}H]P-enolpyruvate the values were 84:16 and 77.5:22.5, respectively.

Previous studies on the stereochemistry of the carboxylation to oxalacetate indicated a degree of nonspecificity for the CO_2 addition varying from 20 to 68% (Rose *et al.*, 1969). In view of the complete nonspecificity of the proton addition, the small amount of malate formed in experiment 3 due to the presence of CO_2 was isolated and analyzed as before to re-examine this previous result. Table I, 3b, shows that the carboxylation is much more stereoselective than previously determined and probably completely stereospecific.[2] This new result indicates that the two faces of the P-enolpyruvate molecule are clearly distinguished in the binding and that intermediates of the pathway to pyruvate that are common to the carboxylation pathway must have no randomization of the two hydrogen positions of P-enolpyruvate. It seems very unlikely, given the expected highly unsymmetrical nature of the active sites, that protonation, either by an acidic group on the enzyme or from general acids in the surrounding medium, could occur in a completely nonstereospecific manner while the intermediate was bound to the enzyme. It is more likely that the result is strong evidence for the dissociation of the intermediate as enolpyruvate, followed by rapid and essentially irreversible ketonization in the medium.

If ketonization is a nonenzymatic process, it follows that the enzyme must not recognize the keto form of pyruvate as a substrate for enolization. Previous studies with a complete reaction mixture have failed to detect incorporation of [^{14}C]-pyruvate into either P-enolpyruvate or oxalacetate (Wood *et al.*, 1969b). In further support of this, it has been shown that [3-^{3}H]pyruvate is not detritiated by carboxytransphosphorylase. In a typical experiment, PP_i (0.5 mM), $MgCl_2$ (12 mM), orthophosphate (1 mM), $KHCO_3$ (30 mM), carboxytransphosphorylase (10 units), and 1.2×10^6 cpm of pyruvate (specific activity of 3.35×10^6 cpm/μmol) were incubated at pH 6.8 and 25° for 2 and 5 hr, respectively. Following removal of the pyruvate by adsorption on Dowex 1-acetate, no tritium was detected in the eluted void volume. Subsequently, pyruvate at 10 mM was found not to inhibit the oxalacetate to P-enolpyruvate reaction rate with oxalacetate at its K_m concentration, 0.47 mM.

TABLE II: Replacement of PP_i by Analogs in the $^{14}CO_2$–Oxalacetate Exchange in the Presence of EDTA.[a]

Expt	Additions	Units of Enzyme in Reaction Mixture	Total Fixed Cpm Greater Than 0 min Control: 30 min	60 min
1	None	1.15	2,200	4,570
2	PP_i (0.25 mM)	0.23	50,000[b]	70,400
3	PNP (0.25 mM)	0.23	2,650	4,800
4	PNP (0.25 mM)	1.15	4,070	8,100
5	PCP (0.25 mM)	1.15	1,480	2,240

[a] All incubations in a final volume of 1.0 ml contained in μmoles: oxalacetate, 1.8; KCl, 25; P_i, 5; $MgCl_2$, 4; EDTA, 0.1; $KH^{14}CO_3$, 3 (specific activity 253,300 cpm/μmol); the indicated additions and units of carboxytransphosphorylase. The pH was 6.7 by adjustment prior to the addition of carboxytransphosphorylase. After incubation at 25° the reactions were terminated by addition of 0.3 ml of 1 *N* HCl to 0.4-ml samples. CO_2 was removed with a stream of N_2 and the residual radioactivity determined. Zero time, with acid added before enzyme, corresponded to 250 cpm that were nonvolatile in acid. At 60 min oxalacetate was present at 1.3 mM in the incubation lacking PP_i and 1.09 mM in experiment 2. [b] 50,000 cpm/ml corresponds to an exchange rate of about 0.01 μmol min^{-1} ml^{-1} of incubation.

If enzyme-bound enolpyruvate is an intermediate common to the carboxylation and protonation paths, the transfer of the phosphoryl group from P-enolpyruvate to P_i must occur prior to, not concerted with, carboxylation. However, as previously reported, CO_2 is necessary to obtain $^{32}PP_i$–P-enolpyruvate exchange (Wood *et al.*, 1969b) and PP_i is necessary for $^{14}CO_2$–oxalacetate exchange (Wood *et al.*, 1969b). These requirements suggest that phosphoryl transfer might be concerted with carboxylation. Contrary to this, the conversion of P-enolpyruvate and P_i to pyruvate and PP_i occurs well in the absence of CO_2. Thus the role of CO_2 in the P-enolpyruvate–PP_i exchange may simply be a kinetic one whereby CO_2 reacts with the enolpyruvate and hence prevents its irreversible conversion to keto pyruvate by the formation of the enzyme–enolpyruvate–CO_2 complex. Likewise, the PP_i requirement for $^{14}CO_2$–oxalacetate exchange may be due to an indirect or synergistic effect.

A test of whether the PP_i structural analogs methylene diphosphonate and imidodiphosphate (PCP and PNP, respectively)[1] could replace PP_i in the $^{14}CO_2$–oxalacetate exchange reaction is shown in Table II. EDTA was present to prevent the net production of P-enolpyruvate in the presence of PP_i (Davis *et al.*, 1969). PCP was clearly without effect. A slight stimulation of exchange by PNP compared with a control was suggestive, however, of a synergistic role. How-

[2] Although in the previous experiments (Rose *et al.*, 1969) mercaptoethanol was present in the phosphoenolpyruvate carboxytransphosphorylase stock used for the incubations, and not present in experiment 3, subsequent studies without mercaptoethanol have given precisely the result obtained here. A possible explanation for the high and variable amount of apparently random carboxylation in the published experiments is that prior to shipping the samples from Cleveland to Philadelphia, the malate dehydrogenase had not been fully inactivated. Prolonged incubation of specific [3-^{3}H]malate with malate dehydrogenase and DPN^+ would lead to racemization at C-3 due to enolization of free oxalacetate that would continuously mix by action of the enzyme with the malate. This is consistent with the fact that the malates isolated had a significantly lower specific activity than the starting P-enolpyruvate, which is not found to be the case in usual studies of this kind or in subsequent experiments with P-enolpyruvate carboxytransphosphorylase.

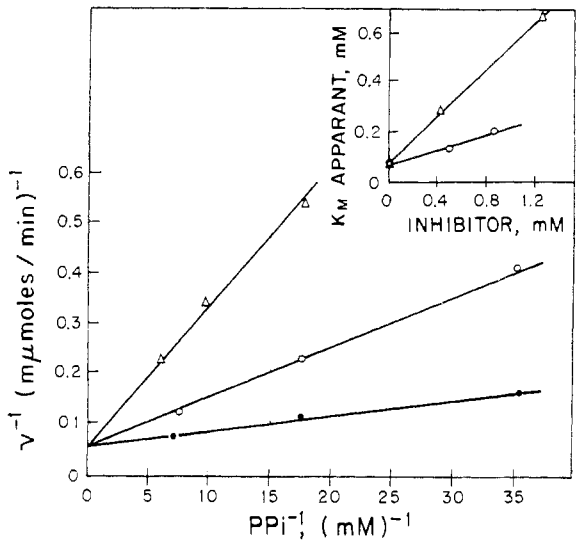


FIGURE 1: Inhibition by PNP and PCP of the conversion of oxalacetate to P-enolpyruvate. Reaction mixtures of 0.7 ml contained in mM concentrations: oxalacetate, 3.0; $MgCl_2$, 4.0; PP_i was varied as indicated; Tris-HCl (pH 7.7), 15; ADP, 0.5; glucose, 1.0; TPN^-, 0.3; KCl, 20; and the following units of enzymes: pyruvate kinase, 2.0; hexokinase, 3.0; glucose-6-P dehydrogenase, 1.5; and 5.5 μg of crystalline carboxytransphosphorylase (specific activity 21). The commercial preparations of pyruvate kinase, hexokinase, and glucose-6-P dehydrogenase were dialyzed 2 hr against 30 mM Tris-HCl (pH 7.7) before use. Carboxytransphosphorylase was added last to initiate reaction. Initial velocities, determined at 340 nm, were proportional to carboxytransphosphorylase concentration. PCP (△) at 1.26 mM and PNP (○) at 0.86 mM were tested. Additional concentrations were tested and a plot of the calculated apparent K_m's *vs.* inhibitor concentration is in the inset.

ever, it remained possible that the PNP might be contaminated by PP_i; therefore studies were repeated in the absence of EDTA and with pyruvate kinase and ADP present. In this case the carboxytransphosphorylase and pyruvate kinase would convert any PP_i impurity to P_i plus ATP. Indeed, when EDTA was absent and ADP and pyruvate kinase added to trap P-enolpyruvate and deplete PP_i, the PNP was found not to support the exchange reaction (Table III). Similar negative results were obtained at pH 6.8 and 8.7 with PNP.

Evidence for Binding by PCP and PNP. The failure of the PP_i analogs to substitute for PP_i in the $^{14}CO_2$–oxalacetate exchange raises the question of whether they are indeed suitable for interaction with the active site. That these compounds have affinity was shown by demonstrating that they both act as competitive inhibitors with respect to PP_i (Figure 1). The

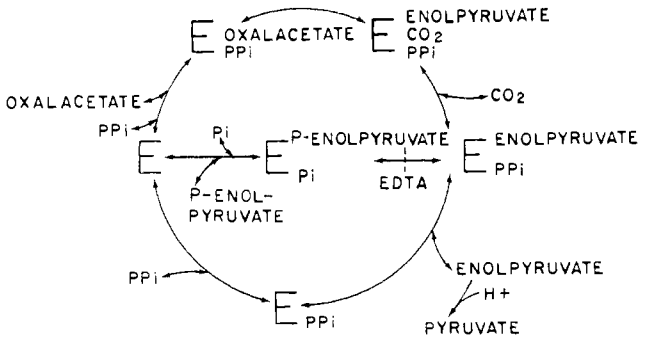


FIGURE 2: Scheme for P-enolpyruvate carboxytransphosphorylase reactions.

TABLE III: $^{14}CO_2$–Oxalacetate Exchange after Removal of PP_i from PNP by Carboxytransphosphorylase Plus Pyruvate Kinase.[a]

Expt	pH	Activators added: 0–20 min	Activators added: After 20 min	Total Fixed Cpm Greater Than Control
6	6.8	PP_i		90
7	6.8	PNP		9
8	6.8	PP_i + PNP		360
9	6.8	None	PP_i + EDTA	133,000[b]
10	8.7	PNP	EDTA	83
11	8.7	PNP	PP_i + EDTA	31,600

[a] A preliminary 20 min incubation of 1 ml contained, in addition to the noted activators at 0.25 mM, the following in μmoles: oxalacetate, 1.8; KCl, 25; P_i, 5; $MgCl_2$, 4; ADP, 2.0; 0.75 unit of pyruvate kinase, and 1.15 unit of carboxytransphosphorylase when assayed in the forward direction which is about 14 times faster than the reaction in the reverse direction at the concentration of oxalacetate used. The pH was 6.8 or 8.7, as noted, by adjustment with dilute triethanolamine. After 20 min at 25° a further 30 min incubation included $KH^{14}CO_3$, 3 μmol at 253,000 cpm/μmol, plus the noted 20 min additions, column 4. When present, EDTA was 0.1 mM. The radioactivity fixed was determined as in Table II. Experiments 9 and 11 were included to demonstrate that preincubation did not destroy capacity for exchange under conditions of a stable PP_i concentration, that is, in the presence of EDTA which prevents conversion of PP_i to P-enolpyruvate by the carboxytransphosphorylase. [b] 133,000 cpm/ml corresponds to an exchange rate of about 0.035 μmol min^{-1} ml^{-1} of incubation, or about 3% of the forward rate at saturation.

kinetic constants determined at pH 7.7 were K_m (PP_i) = 70 μM, K_i (PCP) = 150 μM, and K_i (PNP) = 460 μM.

Formation of Pyruvate from Oxalacetate. The only process of the scheme shown in Figure 2 that has not been demonstrated in earlier studies of carboxytransphosphorylase is the direct decarboxylation of oxalacetate to pyruvate. Table IV shows that in the presence of EDTA, which prevents P-enolpyruvate formation presumably by inhibiting phosphoryl transfer (Davis *et al.*, 1969), the rate of pyruvate formation from oxalacetate is quite comparable to P-enolpyruvate production in the absence of EDTA. In these experiments oxalacetate was used at about its K_m concentration of 0.47 mM in order to limit excessive nonenzymatic decarboxylation and mercaptoethanol, which has been shown to inhibit the formation of pyruvate but increase oxalacetate formation from P-enolpyruvate (Davis *et al.*, 1969), was omitted from the incubations.

Discussion

In the formation of pyruvate from P-enolpyruvate or oxalacetate by carboxytransphosphorylase, pyruvate itself does not appear to be the immediate enzymatic product. The nonstereoselectivity of the protonation reaction suggests that the enzymatic product is protonated subsequent to leaving the enzyme. Free enolpyruvate or some derivative thereof such as pyrophosphorylenolpyruvate would be suitable enzymatic

TABLE IV: Formation of P-enolpyruvate and Pyruvate from Oxalacetate.

Expt	Method of Assay[a]	Conditions for Reaction	Product Determined μmol min^{-1} mg^{-1} of Protein	
			P-enol-pyruvate	Pyruvate
12	A	No EDTA	1120	
13	B	0.67 mM EDTA	0	800
14	B	0.67 mM EDTA (no enzyme)	0	78

[a] Method A is a continuous spectrophotometric assay in which a final volume of 0.6 ml of reaction mixture contained: Tris-HCl (pH 7.4), 15 mM; oxalacetate (9.6 mM); PP_i (0.5 mM); $MgCl_2$ (4 mM); ADP (0.5 mM); KCl (20 mM); glucose (1 mM); TPN^+ (0.3 mM); 1.6 units of glucose-6-P dehydrogenase, and 6.5 μg of carboxytransphosphorylase (specific activity 21). Method B is a discontinuous assay in which aliquots are removed, the reactions terminated with acid, and the products determined spectrophotometrically. In a final volume of 0.89 ml reaction mixture contained: Tris-HCl (pH 7.4) (20 mM); oxalacetate (0.6 mM); PP_i (0.5 mM); $MgCl_2$ (4 mM); EDTA as indicated; and 26 μg of carboxytransphosphorylase; 0.2-ml aliquots were removed at 0, 1.5, 3, and 5 min and added to cold 0.1 N HCl, then oxalacetate, pyruvate, and P-enolpyruvate were determined by the successive use of malate dehydrogenase, lactate dehydrogenase, and pyruvate kinase as previously described.

products with the qualification in the latter case that its hydrolysis must be rapid enough to account for the linear formation of pyruvate in the absence of CO_2. Experiments reported in the following paper (O'Brien *et al.*, 1973) using $H_2{}^{18}O$ rule out any such product that requires hydrolysis for the generation of pyruvate.

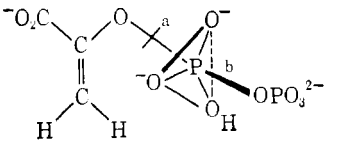
Enolpyruvate itself seems the most likely enzymatic product. As generated on the enzyme it would preserve the arrangement of isotopic hydrogens introduced in the P-enolpyruvate substrate as required by the observed stereospecificity of carboxylation. Enolpyruvate–enzyme intermediates have been proposed for the pyruvate kinase (Rose, 1960) and oxalacetate decarboxylase (Kosicki, 1968) catalyzed reactions based on the observation of catalyzed proton exchanges between water and pyruvate under conditions that preclude product formation. Both of these enzymes catalyze reactions which have their close analogies in steps proposed in Figure 1 for the carboxytransphosphorylase. Pyruvate kinase is known to protonate its bound enolpyruvate stereospecifically (Rose, 1970) and is presumed to have a catalytic group for this function as expected from the promotion of proton exchange. The absence of a similar group on the carboxytransphosphorylase seems indicated.

The failure of pyruvate at 10 mM to show significant inhibition of the oxalacetate–P-enolpyruvate conversion is unexpected confirmation of the nonenzymatic nature of pyruvate formation. Such weak binding through the available carboxyl and carbonyl groups common to oxalacetate indicates that the low K_m of 0.45 mM for oxalacetate may be attributable in large part to its β-carboxyl. Weak interaction must also be attributed to the enol oxygen of enolpyruvate to explain its loss from the enzyme at rates similar to the rate of decarboxylation. This suggests that this oxygen may be protonated in the intermediate.

The reaction: P-enolpyruvate + P_i + enzyme → enolpyruvate–enzyme–PP_i, presumed to be a first step in the reversible carboxylation reaction, must be readily reversible. However, the failure to observe $^{32}PP_i$–P-enolpyruvate exchange in the absence of CO_2 (Wood *et al.*, 1969b) suggests that in the absence of CO_2 either the steady-state concentration of enolpyruvate–enzyme is too low after PP_i dissociation for exchange incorporation of $^{32}PP_i$ or else that PP_i dissociates after enolpyruvate, and ketonization of the enolpyruvate makes that step irreversible. The presence of CO_2 is apparently necessary for the reversible dissociation of formed PP_i. This may simply reflect a stabilization by CO_2 of the enzyme–enolpyruvate complex, thus increasing its concentration for reaction with $^{32}PP_i$ from the medium, or else it might be supposed that oxalacetate formation is required in order to achieve the dissociation of PP_i and reincorporation into P-enolpyruvate. Either suggestion for the role of CO_2 in the $^{32}PP_i$–P-enolpyruvate exchange implies a structural rather than a chemical role for PP_i in the reversible $^{14}CO_2$–oxalacetate exchange.

On the other hand, two "chemical" mechanisms to explain the PP_i requirement for CO_2–oxalacetate exchange independent of P–O cleavage may also be considered. The first of these involves enolpyruvate as an obligatory intermediate, and the second as a side product of the main path. In earlier studies with pyruvate kinase it was found that in the enolization of pyruvate for which K^+, Mg^{2+}, and ATP were required, the ATP could be replaced by any of a large number of dianions such as orthophosphate, phosphoramidate, monoalkyl phosphates, phosphonates, and carbonate (Rose, 1960). Using $H_2{}^{18}O$ it could be shown that the P–O bond was not cleaved when P_i acted as the activator. It was suggested that the central atom of the activators might function as the electrophile for polarization of the carbonyl in an enolization step that preceded the ATP specific phosphoryl transfer in the normal reaction. The PP_i dependent decarboxylation of oxalacetate by carboxytransphosphorylase to give bound enolpyruvate may be viewed as a formal analogy to the ATP dependent enolization of pyruvate by pyruvate kinase. The electrophilic character of phosphorus in methylene diphosphonate and imidodiphosphate should be less than that of PP_i as judged from the electronegativity of O > N > C and the reported pK_a PP_i < PNP < PCP (Irani and Callis, 1961; Yount *et al.*, 1971; Grabenstetter *et al.*, 1967). Nevertheless, by analogy with the pyruvate kinase study they should be able to participate perhaps at one-tenth the rate of PP_i. It was therefore surprising that neither PCP nor PNP satisfied the role of PP_i in the $^{14}CO_2$ exchange although both analogs are bound well to the PP_i site.

A possible alternative to the enolpyruvate mechanism that might explain failure of the analogs is the following. The enzymatic phosphoryl transfer in the carboxytransphosphorylase reaction may involve a pentacoordinate phosphorus intermediate. Since bond cleavage occurs only at one of the two apical ligands, the optimal arrangement for such an intermediate would be the in-line configuration as shown. Cleavage



at bond a would be concerted with carboxylation and at bond b would give P_i and P-enolpyruvate. If the initial attack by the C_2–O of oxalacetate of PP_i were such that the bridge atom of PP_i were apical, one would avoid the necessity for a pseudorotation in the subsequent step. Such a complex might be much less likely to form with PCP or PNP in view of the polarity rule (Muetterties and Schunn, 1966; Westheimer, 1968) which states that the apical positions are occupied preferentially by the most electronegative ligands. In this mechanism, enolpyruvate is not an intermediate in the overall carboxylation of P-enolpyruvate. The nonstereoselective formation of pyruvate would require that enolpyruvate be formed in a side reaction resulting from cleavage at a without carboxylation. This might occur on the enzyme with subsequent dissociation of enolpyruvate or subsequent to the occasional dissociation from the enzyme of the metastable intermediate itself.[3]

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[3] With these considerations in mind, PNP and PCP were tested as activators of pyruvate enolization by muscle pyruvate kinase. These compounds were found to be at least as effective as PP_i in the exchange detritiation of [^{3}H]pyruvate. However, they were less active than the same concentration of P_i when all were tested well below saturation. The apparent absence of a "ligand effect" suggests that PP_i has no advantage over P_i in replacing ATP as the activator and hence one may not expect a distinction to be made between PP_i and its analogs.

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