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Phosphoenolpyruvate Carboxytransphosphorylase VII.
An Investigation of the Reaction Mechanism with ^{18}O *

Subtitle: Enolpyruvate and CO_2 Fixation

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ABSTRACT

Phosphoenolpyruvate carboxytransphosphorylase catalyzes the formation of oxalacetate and PP_i from P-enolpyruvate, P_i and CO_2 and in the absence of CO_2 , the products are pyruvate and PP_i . Wood et al. (1969a) had 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 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 (1973) 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 enolpyruvate and that the enolpyruvate is carboxylated while attached to the enzyme yielding oxalacetate. Alternatively, in the absence of CO_2 , enolpyruvate dissociates from the enzyme and then is protonated to yield keto pyruvate. The P-enolpyruvate carboxykinase and the P-enolpyruvate carboxylase reactions may likewise occur by mechanisms involving an enzyme-bound enolpyruvyl moiety.

Phosphoenolpyruvate carboxytransphosphorylase (pyrophosphate:oxalacetate carboxylase phosphorylating, EC 4.1.1.38) catalyzes reactions 1 and 2, referred to as the oxalacetate and pyruvate reactions respectively.

The enzyme has been obtained 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 Fig. 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 with PP_i or 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 Fig. 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 Fig. 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 Fig. 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 C-O 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 Fig. 1 involves the formation of enolpyruvate as an intermediate and is suggested by analogy with pyruvate kinase (Rose, 1960 and Reynard *et al.*, 1961) and oxalacetate decarboxylase (Kosicki, 1968). Since water is not involved, ^{18}O from solvent water should not be incorporated into any of the products. Also, since the phosphoryl group is cleaved from P-enolpyruvate, the mechanism involves P-O bond breakage.

EXPERIMENTAL PROCEDURE

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

Chemicals. Isotopically enriched water was from BioRad 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 0.12M, 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 x 20 cm were routinely used and were equilibrated with water. A 1 liter linear gradient of 0 to 0.4M 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 0.2N formic acid. The formic acid was 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 crystallized 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 crystallized 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 deter-

mined with P-enolpyruvate carboxytransphosphorylase by making the P-enolpyruvate the limiting reaction component. Acetate was determined enzymically by the method of Schulman and Wood (1971). Malonic acid was determined by celite chromatography (Swim and Krampitz, 1954).

RESULTS

Reactions with P-enolpyruvate Carboxytransphosphorylase in $H_2^{18}O$. The results of the experiments in $H_2^{18}O$ enriched media are summarized in Table I. Mechanisms A and B of Fig. 1 were tested in experiments 1 and 2. No significant incorporation of ^{18}O from solvent water was observed. The double displacement mechanism C of Fig. 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 moles ^{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 2) probably 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 Fig. 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 % 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 non-enzymic hydrolysis of the P-enolpyruvate during storage following its synthesis. The chemically cleaved material yielded phosphate with 2.86 ^{excess} atom % 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 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, oxygen-phosphorus bond cleavage occurs primarily and as the pH rises, carbon-oxygen 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

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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 carboxy-transphosphorylase reaction.

In experiment 9 of Table III, the 1.1 % atom percent 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 μmoles of malonic acid were subjected to purification and analysis, only 78% of the label was recovered but when

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336 μ moles was used, the percent 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 $[\text{}^{18}\text{O}]\text{-P-enolpyruvate}$.

DISCUSSION

The experiments in H_2^{18}O and with $[\text{}^{18}\text{O}]$ P-enolpyruvate demonstrate that the carboxytransphosphorylase reaction proceeds without the mechanistic involvement of water and occurs by phosphoryl transfer from P-enolpyruvate. Of the mechanisms considered in Fig. 1, only D is consistent with these observations, since mechanisms A, B and C all involve hydrolytic steps. Mechanism C is further eliminated by the observation of O-P bond cleavage. In making this conclusion, we have assumed that the hydrolytic agent is in equilibrium with H_2^{18}O . If the reaction occurred in a hydrophobic crevice of the enzyme, a possibility that seems unlikely in view of the polyanionic nature of the reactants, the exchange with H_2^{18}O might occur very

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slowly and the ^{18}O from H_2^{18}O not enter the products. In studies with aconitase, in which a dehydration and rehydration occur in the conversion of citrate to isocitrate, the transferred hydrogen was fully retained whereas the hydroxyl showed extensive exchange with the solvent (Rose and O'Connell, 1967).

In mechanism D, enolpyruvate is displaced by an initial nucleophilic attack of P_i on the phosphoryl group of P-enolpyruvate. This mechanism has the advantage over mechanisms A, B and C because the enolate is an energetically more favorable leaving group from P-enolpyruvate than hydroxyl (in the case of A and B) or P_i (in the case of C). The enolpyruvate is carboxylated to oxalacetate while bound to the enzyme, or in the absence of CO_2 , dissociates into the medium where protonation to ketopyruvate occurs. This aspect of the mechanism is based on the results of Willard and Rose (1973) who have determined the stereochemistry of the pyruvate reaction and have proposed that enolpyruvate is an intermediate which is protonated non-stereospecifically in the solvent. In view of their results, since pyruvate exists largely as the keto form in solution, the previous failure by Wood et al. (1969a) to find exchange of ^{14}C -pyruvate into oxalacetate may have been because of the concentration of enolpyruvate was far below the K_m of the enolate species.

The requirement of PP_i for CO_2 exchange into oxalacetate

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(Wood et al. 1969a) may arise from a PP_i induced conformational change in the enzyme. The effect of mercaptoethanol in increasing the oxalacetate reaction and decreasing the pyruvate reaction may be due to an increased affinity of the enzyme for the enolate intermediate; this may occur either via a conformational change or a metal effect, as suggested by Davis et al. (1969). The inhibition of pyruvate formation by CO_2 (Wood et al. 1969a) is explained by trapping of the enolate moiety at the active site rather than allowing it to dissociate and become protonated.

The pyruvate kinase reaction and the carboxytransphosphorylase pyruvate reaction are similar in that pyruvate is a product of both and the energy of the P-enolpyruvate is conserved in ATP in the one case and PP_i in the other. Rose (1960) and Reynard et al. (1961) have proposed a mechanism for pyruvate kinase with many features in common with that of D in Fig. 1. Lardy and Zeigler (1945) were the first to show that the pyruvate kinase reaction is reversible and McQuate and Utter (1959) were able to demonstrate the formation of P-enolpyruvate using pyruvate and ATP. Thus far, the reversibility of the pyruvate reaction of carboxytransphosphorylase has not been demonstrated (Wood et al. 1969a). There are three factors which explain why that carboxytransphosphorylase, unlike pyruvate kinase, has not been shown to be reversible; (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) and may only bind the enol form; (2) with pyruvate kinase, McQuate and Utter (1959) could use substantial concentrations of ATP to assist in the reversal whereas carboxytransphosphorylase is inhibited by very low concentrations of PP_i (Davis et al., 1969); (3) the free energy of hydrolysis of PP_i is lower than that of ATP, thus the equilibrium is less favorable for P-enolpyruvate formation from PP_i and pyruvate than from ATP and pyruvate.

The reaction catalyzed by P-enolpyruvate carboxykinase, i.e., the conversion of P-enolpyruvate plus ADP or GDP and CO_2 to oxalacetate and ATP or GTP, also 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, Kurahashi, and Rose, 1954); (4) both reactions to oxalacetate are readily reversible with ΔG° values of -0.1 and -0.3 k cal 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. However, based on our observations and those of Willard and Rose (1973) with carboxytransphosphorylase, it seems quite possible that the reaction catalyzed by carboxykinase may also involve two steps and formation of an enolpyruvate intermediate with ADP or GDP as the phosphoryl acceptor in place of P_i .

The formation of oxalacetate from P-enolpyruvate by P-enolpyruvate carboxylase differs from those above but may occur by a somewhat analogous mechanism. Muruyama et al. (1966) using $\text{HC}^{18}\text{O}_3^-$ have found that the reaction involves HCO_3^- as the reactive species, and that all three oxygens of $\text{HC}^{18}\text{O}_3^-$ were 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. It does seem possible, however, that the reaction might proceed as follows: an initial attack of the bicarbonate hydroxyl on the P-enolpyruvate phosphoryl displacing enolpyruvate which is subsequently carboxylated by the CO_2 generated in the initial attack leading to oxalacetate and P_i containing the three oxygens of $\text{HC}^{18}\text{O}_3^-$. The second step would have to occur without exchange of the CO_2 with solvent H_2O .

¶ 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 hydroxyl of bicarbonate. Secondly, this initial attack generates enolpyruvate (the stability of which depends on local conditions at the active site of the enzyme) which 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

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of such a speculation obviously requires much further characterization
of the enzymes involved.

Fig. 1. Mechanisms which have been considered for the carboxy-transphosphorylase reactions: (A) via a pyrophosphorylenolpyruvate intermediate as proposed by Wood et al. (1969); (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.

TABLE I

PHOSPHOENOLPYRUVATE CARBOXYTRANSPHOSPHORYLASE REACTION IN H_2^{18}O

Reaction	Atom % excess ^{18}O Found in					Theoretical atom % excess ^{18}O based on mechanisms of Fig. 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	0.73	0	0
2. Pyruvate	4.00	-	0.24	-	-	0	0	2.00	0
3. Oxalacetate	4.57	2.16	-	-	-	2.28*	2.28*	3.42*	2.28*
4. Pyruvate	4.00	-	-	-	0.0	0	2.00	0	0

*Calculated on the basis of total exchange of HCO_3 with H_2^{18}O .

TABLE I

Experiment 1. The mixture contained in μ moles; KHCO_3 , 60; P_i , 40; MgCl_2 , 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. The mixture was saturated with CO_2 as above. The reaction was for three hours and 45 minutes and 10.5 μ moles of pyrophosphate were formed. Catalase was added and the H_2^{18}O was recovered by lyophilization.

Experiment 2. The mixture contained in μ moles: P-enolpyruvate, 100; P_i , 100; H_2O_2 , 100; MgCl_2 , 40; H_2^{18}O , ~10 atom percent, 8.0 ml in a final total volume of 20 ml. Nitrogen was bubbled through the mixture for 20 minutes. Inorganic pyrophosphatase (4 units), and ^{P-enolpyruvate}carboxytransphosphorylase (5 units) were added followed by incubation was at 25° under nitrogen. The acetate yield was 46 μ moles.

Experiment 3. The mixture contained in μ moles; KHCO_3 , 275; P-enolpyruvate 116; MgCl_2 , 108; P_i , 60; H_2O_2 , 980 and P-enolpyruvate carboxytransphosphorylase, 17.5 units, SA = 15; pyrophosphatase, 135 units; H_2^{18}O , 12 atom %, 3 ml; in a volume of 7.0 ml. Before addition of enzymes, CO_2 was bubbled through the mixture for 15 minutes. The reaction proceeded for four hours at which time 0.02 mg catalase was added and the mixture was lyophilized to recover the H_2^{18}O . 103 μ moles of phosphoenolpyruvate were consumed.

Experiment 4. The mixture contained in μ moles: P-enolpyruvate 40; P_i , 200; $MgCl_2$, 80; $H_2^{18}O$, 10 atom percent, 8.0 ml in a final total volume of 20 ml. Nitrogen was passed through the mixture for 20 minutes 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 five hours, pyruvate production ceased at 36 μ moles and the reaction was terminated by adding 20 μ moles of EDTA and chilling. The pyruvate was isolated by chromatography. The pyruvate was lyophilized and crystallized as the silver salt and the dry salt decarboxylated by gentle heating. The resulting CO_2 was analyzed for its isotope content.

TABLE II

Degradation of Phosphoenolpyruvate Labeled
with ^{18}O in the Enolester Oxygen

Treatment	^{18}O found in phosphate	Theoretical
	atom % excess	
5. Alkaline Phosphatase	0.25	0.0
6. $\text{Hg}(\text{ClO}_4)_2$	2.86	3.3

TABLE II

Experiment 5. 109 μ moles 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 three hours and 45 minutes, the reaction was terminated. 102.5 μ moles of P-enolpyruvate were utilized.

Experiment 6. To 30 μ moles of [^{18}O] P-enolpyruvate dissolved in 0.5 ml H_2O was added 0.5 ml of 0.08 M $\text{Hg}(\text{ClO}_4)_2$ and one drop of 5 N HCl (final pH was 1 or less). After 12 minutes, 0.1 ml conc 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 N NH_4OH and the supernatant liquids combined. The inorganic phosphate was purified and crystallized in both experiments as described in "Experimental Procedure".

TABLE III

Incorporation of ^{18}O into the Products from P-enolpyruvate Labeled in the Enolester Oxygen

Reaction	Atom % excess ^{18}O Found in				Theoretical atom % excess ^{18}O based on mechanisms of Fig. 1			
	P-enol pyruvate	Malonate from oxalacetate	Acetate from pyruvate	Phosphate from pyrophosphate	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

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TABLE III

Experiment 7. The mixture contained in μ moles; ^{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 "Experimental Procedure".

Experiment 8. The mixture contained in μ moles; 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 μ moles of PP_i .

Experiment 9. The mixture contained in μ moles; P-enolpyruvate, 284.8; KHCO_3 , 600; MgCl_2 , 240; P_i , 140; H_2O_2 , 2000; and P-enolpyruvate carboxytransphosphorylase, 32 units; pyrophosphorylase, 216 units; in a volume of 20.1 ml. CO_2 was passed through the mixture for 10 minutes prior to addition of enzymes. The reaction proceeded for nine hours at 25° and then 0.03 mg of catalase was added. Malonate was purified as described in the "Experimental Procedure". 280 μ moles of P-enolpyruvate were consumed.

Experiment 10. The mixture was identical to Experiment 8 except that four units of pyrophosphorylase was added. Acetate was isolated as described in "Experimental Procedure". The yield was 13 μ moles.

REFERENCES

- Benkovic, S.J., and Schray, K.J. (1968), Biochemistry 11, 4097.
- Benkovic, S.J., and Schray, K.J. (1969), J. Amer. Chem. Soc. 91, 5653.
- Bondinell, W.E., Vnek, J., Knowles, P.F., Sprecher, M., and Sprinson, D.B. (1971), J. Biol. Chem. 246, 6191.
- Boyer, P.D., Graves, D.J., Suelter, C.H., and Dempsey, M.E. (1961), Anal. Chem. 33, 1906.
- Clark, V.M., and Kirby, A.J. (1963), J. Amer. Chem. Soc. 85, 3705.
- Cohn, M. (1949), J. Biol. Chem. 180, 771.
- Cooper, T.G., Tchen, T.T., Wood, H.G., and Benedict, C.R. (1968), J. Biol. Chem. 246, 5486.
- Davis, J.J., Willard, J.M., and Wood, H.G. (1969), Biochemistry 8, 3127.
- DeLeo, A.B., Dayan, J. and Sprinson, D.B. (1973), J. Biol. Chem. 248, 2344.
- Dempsey, M.E., Boyer, P.D., and Benson, E.S. (1963), J. Biol. Chem. 238, 2708.
- Gomori, J. (1941), J. Lab. Clin. Med. 27, 955.
- Haberland, M.E., Willard, J.M., and Wood, H.G. (1972), Biochemistry 11, 712.
- Kosicki, G.W. (1968), Biochemistry 7, 4310.
- Lochmüller, H., Wood, H.G., and Davis, J.J. (1966), J. Biol. Chem. 241, 5678.
- Lardy, H.A., and Ziegler, J. (1945), J. Biol. Chem. 234, 2151.
- McQuate, J.T., and Utter, M.F. (1959), J. Biol. Chem. 243, 6041.
- Muruyama, H., Easterday, R.L., Chang, H-C., and Lane, M.D. (1966), J. Biol. Chem. 241, 2405.

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- Reynard, A.M., Hass, L.F., Jacobsen, D.D., and Boyer, P.D. (1961),
J. Biol. Chem. 236, 2277.
- Robinson, J.L., and Rose, I.A. (1972), J. Biol. Chem. 247, 1096.
- Rosé, I.A. (1960), J. Biol. Chem. 235, 1170.
- Rose, I.A., and O'Connell, E.L. (1967), J. Biol. Chem. 242, 1870.
- Rose, I.A., O'Connell, E.L., Noce, P., Utter, M.F., Wood, H.G.,
Willard, J.M., Cooper, T.G., and Benziman, M. (1969),
J. Biol. Chem. 244, 6130.
- Schulman, M., and Wood, H.G. (1971), Anal. Biochem. 39, 505.
- Swim, H.F., and Krampitz, L.O. (1954), J. Bacteriol. 67, 426.
- Utter, M.F., and Kurahashi, K. and Rose, I.A. (1954), J. Biol. Chem.
207, 803.
- Utter, M.F., and Kolenbrander, K. (1972), in The Enzymes, VI, 3rd
Edition, Academic Press, New York, 117.
- Willard, J.M., and Rose, I.A. (1973), ~~Previous paper.~~
- Wood, H.G., Davis, J.J., and Lochmüller, H. (1966), J. Biol. Chem.
241, 5696.
- Wood, H.G., Davis, J.J., and Willard, J.M. (1969a), Biochemistry
8, 3145.
- Wood, H.G., Davis, J.J., and Willard, J.M. (1969b), Methods of Enzymol.
13, 297.
- Zalkin, H., and Sprinson, D.B. (1966), J. Biol. Chem. 241, 1067.