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Facile Oxygen Exchanges of Phosphoenolpyruvate and Preparation of [^{18}O]Phosphoenolpyruvate†

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ABSTRACT: Phosphoenolpyruvate when heated in acidic solution exchanges its phosphoryl and carboxyl oxygens rapidly and its enolic oxygen much more slowly with oxygens from water. The incorporation of ^{18}O into phosphoenolpyruvate was measured by gas chromatography–mass spectrometry and phosphorus-31 nuclear magnetic resonance after heating in $H_2{}^{18}O$ at 98 °C. The rates of exchange of all six oxygens of phosphoenolpyruvate with water increase with increasing acidity, and the phosphoryl oxygens exchange more rapidly than the carboxyl oxygens. The rate of exchange of each oxygen of the phosphoryl group is 16-fold greater than the hydrolysis rate at 1 N HCl. This provides a simple and useful method for the synthesis of [^{18}O]phosphoenolpyruvate highly enriched in its phosphoryl-group oxygens. An enrichment of 89% was obtained with a 50% yield. The [^{18}O]-phosphoenolpyruvate showed a binomial distribution of ^{18}O in the phosphoryl-group oxygens. The exchange may be explained by the reversible formation of a transient cyclic phosphate and, for exchange of the enolic oxygen, a transient acyl phosphate. Preparation of [^{18}O]phosphoenolpyruvate from [^{18}O]P_i by a chemical synthesis from β-chlorolactate was not satisfactory because of drastic loss of ^{18}O during the procedures used. Some loss of ^{18}O also occurred during an enzymic synthesis with KCNO, [^{18}O]P_i, carbamate kinase, and pyruvate kinase.

For studies of oxygen exchanges accompanying cleavage of [^{18}O]ATP, it is desirable to have [^{18}O]phosphoenolpyruvate to serve for regeneration of the [^{18}O]ATP by the pyruvate kinase reaction. Phosphoenolpyruvate synthesized from [^{18}O]P_i by a chemical procedure was found to contain much less ^{18}O than the starting [^{18}O]P_i. This prompted us to examine the exchange properties of phosphoenolpyruvate in aqueous solution. The results reported herein show that the exchange of the phosphoryl-branch oxygens (those of the $-PO_3$ group) was more rapid than the hydrolysis and, further, that the carboxyl oxygens, and to a lesser extent the enolic bridge oxygen, also underwent exchange with water during heating at acid pH. These exchanges were in part predictable, on the basis of the exchange accompanying hydrolysis reported by Schray & Benkovic (1971) and the facile demethylation of (dimethylphospho)enolpyruvic acid in water. Promotion of demethylation by lower pH was noted by Clark & Kirby (1963) and documented more fully by Stubbe & Kenyon (1972). An even more rapid demethylation of (dimethylphospho)enol-3-bromopyruvic acid occurs (Stubbe & Kenyon, 1971). In addition to revealing interesting properties of phosphoenolpyruvate, the exchange serves as a method for preparation of highly ^{18}O-labeled phosphoenolpyruvate.

Experimental Procedures

Materials. The monopotassium salt of phosphoenolpyruvate, pyruvate kinase, glycerol kinase, ADP, and Sephadex were purchased from Sigma, and the ^{18}O-enriched water was from Norsk-Hydro and Monsanto. Dowex and Chelex resins were products of Bio-Rad. All other reagents were of the highest quality commercially available. For preparation of stock HCl in $H_2{}^{18}O$, for use in the synthesis of [^{18}O]-phosphoenolpyruvate, 0.4 mL of $H_2{}^{18}O$ of at least 96% enrichment was added to a small vial fitted with a Teflon-lined cap in a dry box. The vial was transferred to a hood and the cap replaced by a cork containing two small holes fitted with glass tubing drawn to a small diameter. One tube was connected by a piece of Teflon tubing to discharge near the rear of the hood; the other was connected, via tubing that had been flushed with nitrogen, to a tank of dry HCl gas. The surface of the enriched water was exposed to the dry HCl gas for 15 min and total acidity (about 12 N) determined by titration of a small aliquot.[1] A similar procedure was employed on

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a larger scale for preparation of a stock solution of HCl in $H_2{}^{18}O$ of 8% enrichment that was diluted with 8% $H_2{}^{18}O$ to give solutions of the desired acidities for use in the exchange experiments. A stock solution of 0.1 M potassium phosphoenolpyruvate, pH 8, was prepared in the enriched water by lyophilizing an unenriched sample at pH 8 and dissolving it in H_2O containing about 8% ^{18}O. This solution was used for all exchange experiments except for experiments at pH 0 where the phosphoenolpyruvate stock solution was prepared in unenriched H_2O. Phosphoenolpyruvate solutions were mixed with an HCl solution in $H_2{}^{18}O$ to give the desired final pH.

^{18}O Exchange Experiments. A 0.1-mL aliquot of a 0.1 M phosphoenolpyruvate solution at pH 8 and 98 °C was added to a known volume of about 2.0 mL of enriched water at the desired pH also at 98 °C in a 1.1 × 10 cm test tube covered with a conical glass stopper. Aliquots of about 0.1–0.2 mL were removed at various times and quenched within 3 s by injection into 5-mL conical glass vials that were partially immersed in dry ice. The frozen samples were lyophilized overnight and then ethylated with diazoethane in ether (Hackney et al., 1979). Appropriate precautions were taken with toxic, volatile, and explosive diazoethane. The excess reagent was removed by a stream of N_2, and sufficient dichloromethane was added to give a concentration of phosphoenolpyruvate of about 4 nmol/μL. A 1–3-μL sample was analyzed for ^{18}O content by gas chromatography–mass spectrometry (GC–MS). A Hewlett-Packard 5995A quadrupole GC–MS apparatus with electron-impact ionization and programmed for selected ion monitoring was used. Gas chromatography was on a 2.0 mm × 10 ft (3 ft for experiments at pH 0) glass column packed with 3% OV-275 on 40/60 Chromosorb T. The derivatized phosphate had a retention time of about 1.1 min, and the derivatized phosphoenolpyruvate had two distinct retention times of about 3.4 and 3.9 min, as analyzed with the 10-ft column at 30 mL/min helium carrier gas flow and for a temperature gradient of 200–210 °C at 4 °C/min.

The enrichments of the $H_2{}^{18}O$ and of the acid stock solutions were determined by GC–MS analysis of triethyl phosphate prepared by addition of 20 μL of the enriched water–acid to two to three crystals of PCl_5 in a 5-mL conical glass vial in a dry box. Controls showed that there was no change in the ^{18}O content of the solvent during the incubation at pH 0 and that phosphoenolpyruvate did not undergo oxygen exchange during lyophilization.

The average enrichment of ^{18}O in the oxygens of $[^{18}O]P_i$, E, was determined from the GC–MS results by using eq 1;

$$E = (4 \times P^{18}O_4 + 3 \times P^{18}O_3 + 2 \times P^{18}O_2 + P^{18}O_1)/4 \quad (1)$$

$P^{18}O_i$ (i = 0, 1, ..., 4) is the relative abundance of each of the different mass species present in the fragment monitored by GC–MS normalized to a total of 1. These were obtained by selected ion monitoring of masses 155, 157, 159, 161, and 163, which form a distribution corresponding to the diethyl phosphate ion fragment with 0, 1, 2, 3, or 4 atoms of ^{18}O per phosphate atom, respectively. These distributions have been corrected for contamination by unenriched P_i and for a small contribution, about 5%, due to spillover from a fragment at mass 153 that also contains four oxygens enriched with the same distribution pattern as the mass 155 series.

Hydrolysis Experiments. For measurement of the rates of hydrolysis, 10.2 mL of HCl of the desired acidity and 0.5 mL of 50 mM phosphoenolpyruvate, pH 8.0, were heated to 98 °C in 1.5 × 15 cm test tubes covered with conical glass stoppers. At zero time, the HCl was poured into the test tube containing the phosphoenolpyruvate. Approximately 1.0-mL aliquots were removed with a Pipetman and added to 1.0 × 7.5 cm plastic tubes immersed in ice and containing sufficient KOH or tris(hydroxymethyl)aminomethane (Tris) to neutralize the HCl. The exact size of the aliquot removed from the heated sample was determined by weight.

P_i formed was measured by the method of Sumner (1944). The first-order rate constants for hydrolysis was determined from measurements at 7–8 different time intervals, sufficient for up to 70% hydrolysis of the sample. The total amount of hydrolyzable phosphoenolpyruvate was measured by the P_i released after complete hydrolysis, which was accomplished by either prolonged heating at 98 °C or mercury cleavage of an aliquot of the sample (Benkovic & Schray, 1968b). For the latter reaction 0.25 volume of 0.5 M mercury(II) acetate, pH 4.0, was added to 1.0 volume of sample, and the mixture was incubated at room temperature for about 10 min. The rate constants were evaluated by a least-squares fit of the data to the expected first-order relationship.

pH Measurement. For oxygen exchange and hydrolysis experiments at pH values of 1 and greater, the pH of the sample remaining after the experiment was measured with a glass electrode. For experiments at pH values below 1, acidity was determined by KOH titration of an aliquot of the sample remaining after the experiment. For one experiment at low pH, the acidity was determined from that of the stock HCl used to prepare the sample. Where necessary, the amount of free acid was calculated from the total acidity with the pK values of phosphoenolpyruvate given by Benkovic & Schray (1968b) to correct for its protonation. At acidities of 1 and 5 N, no change in acidity is expected upon hydrolysis of phosphoenolpyruvate, and no change in acidity occurred during heating in any of the experiments.

Synthesis of [^{32}P]Phosphoenolpyruvate. The procedures of Lauppe et al. (1972) and Cheung & Marcus (1975) were used to synthesize [^{32}P]phosphoenolpyruvate from $^{32}P_i$ and β-chlorolactate. The sample was purified on a column of Dowex AG1-X4 with elution of the [^{32}P]phosphoenolpyruvate by 100 mM HCl.

Analysis of [^{18}O]Phosphoenolpyruvate. For analysis by a pyruvate kinase–glycerol kinase method (Webb, 1980), a reaction mixture contained 50 mM Tris, pH 8, 2 mM $MgCl_2$, 0.1 mM ADP, 2 mM D-glyceraldehyde, 50 mM KCl, 1.3 mM [^{18}O]phosphoenolpyruvate, a trace of [^{32}P]phosphoenolpyruvate, 50 μg of glycerol kinase, and 90 μg of pyruvate kinase in a final volume of 0.5 mL. The enzymes were prepared by dissolving the required amounts in less than 0.1 mL of 100 mM Tris-HCl, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol and centrifuging through a packed column of Sephadex G-50, 80 mesh, equilibrated with the buffer (Penefsky, 1977). The reaction was started by addition of ADP and carried out for 25 min

[1] An alternative method for acidification of the reaction mixture has been developed in this laboratory by Dr. W. E. Kohlbrenner. An all-glass vacuum system capable of maintaining a vacuum of less than 10 millitorr is set up to accommodate a 250-mL flask containing PCl_5, a 10-mL flask containing H_2O, and a 50-mL round-bottom flask containing the reaction mixture, all separated by stopcocks. All additions of reagents to flasks are done in a dry box. The H_2O is vacuum transferred to a large excess of PCl_5 with dry ice–2-propanol. The flask is warmed to room temperature and allowed to stand for 20 min (about 2 mmol of HCl gas is generated from 20 μL of H_2O and 0.5 g of PCl_5). The flask is refrozen in dry ice–2-propanol, and the HCl gas is transferred with liquid nitrogen in 20 min to the reaction mixture. The flask is then brought to room temperature, and the vacuum is released in a drybox.

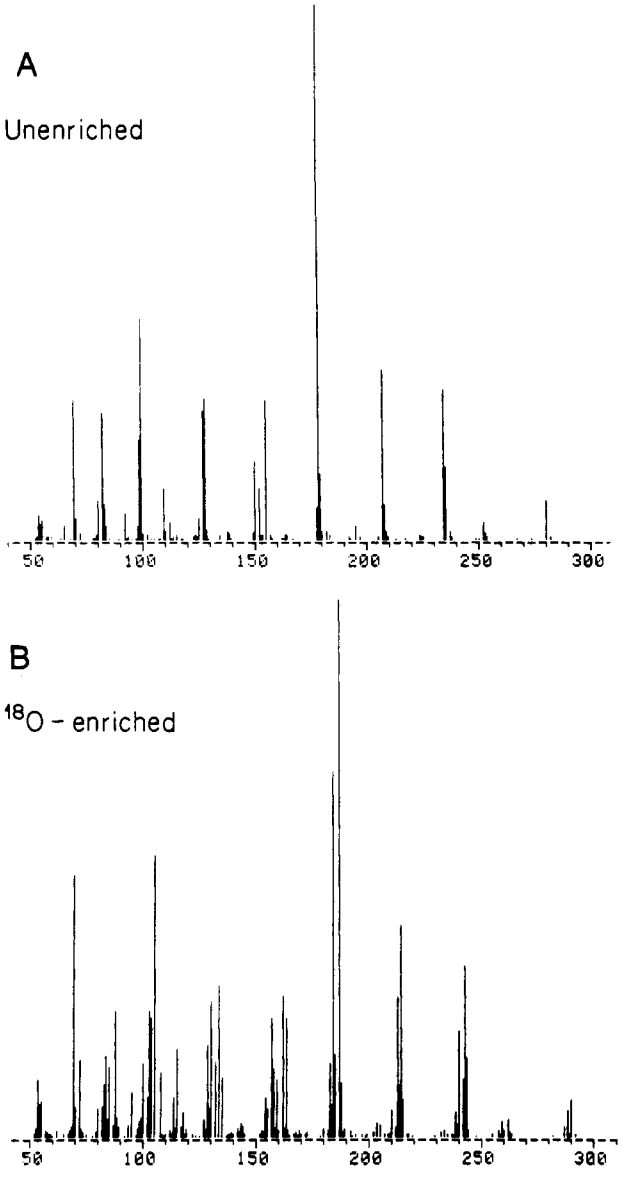


FIGURE 1: GC–MS spectra of major ethylated derivative of phosphoenolpyruvate. Scanned from 50–350 atomic mass units. (A) Natural abundance phosphoenolpyruvate. GC retention time of 1.86 min at 188 °C. Total abundance = 36 792. Abundance of base peak at mass 178.0 = 6889. (B) Phosphoenolpyruvate enriched with ^{18}O. GC retention time of 2.48 min at 180 °C. Total abundance = 56 164. Abundance of base peak at mass 186.1 = 5312.

at room temperature. The reaction was stopped by addition of 0.2 mL of chloroform with vortexing, 5 mL of water was added, and the protein was removed by centrifugation. The [^{18}O]P_i was purified by Dowex column chromatography (Hackney et al., 1979) prior to analysis by GC–MS. The ^{18}O content of all six oxygens of an acidified sample of [^{18}O]-phosphoenolpyruvate was analyzed by GC–MS as above by an equation analogous to eq 1 to calculate the average enrichment from the observed distribution of ^{18}O in the molecular ion (see Results).

Analysis of [^{18}O]phosphoenolpyruvate by ^{31}P NMR was carried out on a Bruker WM-500 NMR spectrometer operating at 202.5 MHz, in the Fourier-transform mode, at constant temperature, with deuterium lock, and single frequency decoupling of the methylene group protons. Volumes of about 0.6–1 mL in 5-mm sample tubes were used. Samples were treated with Chelex, the pH was adjusted to 8.5–9 with KOH, and K^+EDTA was added before analysis. Another sample of [^{18}O]phosphoenolpyruvate was analyzed on a Bruker HX-200 instrument operating at 81.0 MHz. Conditions were as above except that 10-mm tubes were used, and broad-band proton decoupling was employed.

Results

Other Methods of Synthesis of [^{18}O]Phosphoenolpyruvate. On approach explored was to synthesize phosphoenolpyruvate from [^{18}O]P_i and β-chlorolactate as described by Lauppe et al. (1972) and Cheung & Marcus (1975); however, the phosphoenolpyruvate was found to retain only about 4% of the ^{18}O content of the [^{18}O]P_i. The only oxygen source of sufficient size to account for the excessive exchange was the dimethyl sulfoxide, although we did not conclusively eliminate the possibility of water-oxygen contamination. Our observations may be related to those of Midelfort & Rose (1976), who noted some exchange of P_i oxygens during synthesis of [^{18}O]ATP with ADP–morpholidate in dimethyl sulfoxide.

[^{18}O]Phosphoenolpyruvate was also synthesized from carbamyl phosphate [prepared by reaction of [^{18}O]P_i and potassium isocyanate at pH 5.5 (Mokrasch et al., 1960)] by reaction with ADP and pyruvate, using pyruvate kinase and carbamate kinase to catalyze the coupled reactions at pH 9.2. The phosphoryl groups of the resulting [^{18}O]phosphoenolpyruvate and [^{18}O]ATP were equally enriched with ^{18}O but were 10–20% less enriched than expected. Further study might improve this method so that less exchange would be observed. The lack of success of these other methods of synthesis of [^{18}O]phosphoenolpyruvate makes the synthesis by exchange in hot acid the method of choice.

Mass Spectral Analysis of [^{18}O]Phosphoenolpyruvate. Figure 1 gives the mass spectrum of the volatile product formed by reaction of phosphoenolpyruvate and diazoethane.[2] The addition of four ethyl groups to phosphoenolpyruvate and subsequent ionization accounts for the molecular ion of mass 280, $C_{11}H_{22}O_6P^+$. Selected ion monitoring of the species at masses 280 (with no ^{18}O), 282 (with one ^{18}O), ..., and 292 (with six ^{18}O) was used to measure the total enrichment of the six oxygens of phosphoenolpyruvate with ^{18}O. Two fragments were also examined: phosphate ion, $H_4PO_4^+$, at mass 99 (with no ^{18}O) and diethyl phosphate ion, $(C_2H_5O)_2P(OH)_2^+$, at mass 155 (with no ^{18}O). In this mass region, the large number of fragments in the spectrum of ethylated [^{18}O]phosphoenolpyruvate compared to that of the unenriched compound makes further interpretation difficult. Analysis of highly enriched samples [^{18}O]phosphoenolpyruvate by both GC–MS and ^{31}P nuclear magnetic resonance (NMR) (Figure 3 and Table III) showed that the molecular ion (Table III), but not the two fragments (results not shown), gave an accurate determination of the ^{18}O content.[3] Also, analysis

[2] The spectra in Figure 1 were obtained in the scanning mode of the GC–MS apparatus. Intensity values are not as accurate in this method as in the selected ion-monitoring mode as used for other experiments.

[3] From the chemical structure of phosphoenolpyruvate, it is difficult to postulate fragments at masses 155 and 99 that could be labeled with four atoms of oxygen-18 other than diethyl phosphate ion and phosphate ion, respectively. This suggests that the lack of agreement between the GC–MS and ^{31}P NMR analyses was due to rearrangement of the oxygens of [^{18}O]phosphoenolpyruvate during the GC–MS analysis. Analysis of the ethylated derivatives of natural abundance phosphoenolpyruvate and highly labeled [^{18}O]phosphoenolpyruvate on an AEI MS-9 high-resolution mass spectrometer with a DS-50 data system revealed only single fragments at masses 99 and 107, respectively. Two fragments were apparent at mass 155 in the natural abundance sample; however, only one fragment was present in significant amounts at mass 163 in the enriched sample. This fragment had the correct exact mass for diethyl phosphate labeled with four atoms of ^{18}O. Thus, the high-resolution work substantiates the conclusion that rearrangement of the oxygens of phosphoenolpyruvate occurs during GC–MS analysis.

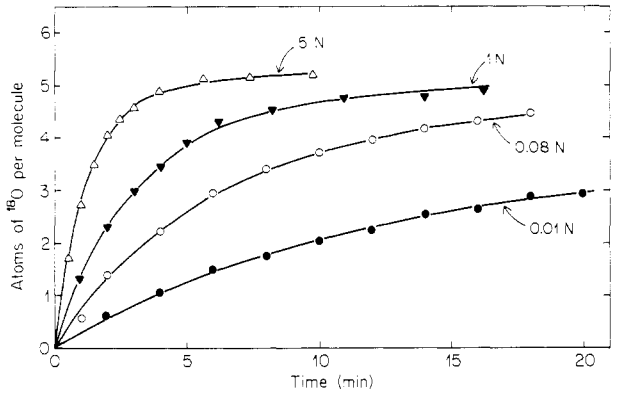


FIGURE 2: Enrichment of phosphoenolpyruvate with oxygen-18 at 98 °C and 0.01 N (●), 0.08 N (○), 1 N (▼), and 5 N (△) HCl. The symbols give the observed values of the number of atoms of oxygen-18 per molecule, and the lines are drawn through the calculated values.

of unenriched phosphoenolpyruvate by GC–MS established that about 1.2% of the molecules contained one atom of ^{18}O within experimental error, as expected from natural abundance. Hence, only the molecular ion data were used to analyze [^{18}O]phosphoenolpyruvate directly.

GC–MS analysis indicated that phosphoenolpyruvate forms at least two derivatives of the same mass upon reaction with diazoethane. This was shown by appearance of two peaks at mass 280 (molecular ion) that were largely resolved by GC with the 10-ft column, the second being somewhat smaller in size than the first. The ^{18}O enrichments of the two were the same within experimental error. The structures of the two molecular ions are not known, but a mass of 280 suggests that ethylated phosphoenolpyruvate contains four ethyl groups. One ethyl group could be attached to each of two phosphoryl oxygens, one of the carboxyl oxygens, and the methylene carbon of phosphoenolpyruvate. Cis–trans isomerization about the carbon–carbon double bond between C_2 and C_3 would account for the two observed derivatives. An alternative explanation is that ethylated phosphoenolpyruvate contains two ethyl groups and one butyl group. The butyl group could be attached to either a carboxyl- or phosphoryl-group oxygen giving rise to the two observed derivatives. In this regard, butyl phosphate has previously been observed in this laboratory during GC–MS analysis of phosphoric acid that had been derivatized with diazoethane. Each derivative gave rise to fragments at masses 99 and 155. Analysis of the complete mass spectrum of each derivative confirmed that the two gave rise to different fragmentation patterns (not shown).

Figure 2 describes the exchange of the six oxygens of phosphoenolpyruvate with 8% enriched water as a function of time and pH at 98 °C. The rate constants (Table I) governing this exchange reaction were calculated from a nonlinear least-squares fit (Ralston, 1979) of the data to eq 2, where

$$\text{atoms of } ^{18}O = 6 - 2e^{-k_1 t/2} - 3e^{-k_2 t/3} - e^{-k_3 t} \quad (2)$$

atoms of ^{18}O is the average number of atoms of oxygen per molecule that have been exchanged with water oxygens; k_1, k_2, and k_3 are the first-order rate constants for exchange of each oxygen of the carboxyl, phosphoryl, and enol groups, respectively; and t is the time in minutes. Only two rate constants were treated as variable parameters in a single fit, the value of the third being fixed (see Table I). The atoms of ^{18}O was calculated from eq 3, where E is the observed

$$\text{atoms of } ^{18}O = 6(E - E_{na})/(E_{eq} - E_{na}) \quad (3)$$

fractional enrichment of the parent ion, E_{na} the enrichment from the natural abundance of ^{18}O, and E_{eq} the enrichment at isotopic equilibrium (complete exchange). These rate constants (Table I) are for exchange of both labeled and unlabeled oxygen atoms, i.e., total exchange. The first-order rate constant for exchange of all of the oxygens of a particular group can be obtained by dividing the rate constant for exchange of each oxygen (Table I) by the number of oxygens in that group.

Table I: Rate Constants for Oxygen Exchange of Phosphoenolpyruvate with H_2O[a]

acidity	k_1 (-COOH oxygen) (min^{-1})	k_2 (-PO_3 oxygen) (min^{-1})	k_3 (-C-O-P oxygen) (min^{-1})
5.1 N[b]	1.57 ± 0.05	2.35 ± 0.08	0.024 ± 0.002
1.0 N[c]	0.40 ± 0.03	1.20 ± 0.07	0.00021 ± 0.00010
pH 1.1	0.16 ± 0.01	0.63 ± 0.03	nd[d]
pH 2.0	0.033 ± 0.004	0.28 ± 0.01	nd[d]

[a] For all acidities except 5.1 N HCl, fitting of the data to eq 2 gave rise to two solutions depending upon the relative values of the initial estimates of k_1 and k_2 used in the computer program; i.e., if k_1(initial) > k_2(initial), then k_1(final) > k_2(final), and vice versa. The solutions given above were chosen so that $k_1 < k_2$ in accord with analyses of highly enriched [^{18}O]phosphoenolpyruvate that showed that the phosphoryl-group oxygens were more enriched than those of the carboxyl group (Table III). At 1.0 N HCl and pH 1.1, these choices gave slightly better fits; at pH 2.0, the two solutions gave equally good fits. [b] At 5.1 N HCl, it was noted that $k_1/2 = k_2/3$; hence, these terms were equated in eq 2 so that the data were fit to only two rate constants. If the data were to fit to all three rate constants simultaneously, the resulting values of the rate constants were not significantly altered. [c] The value of k_3 at 1.0 N HCl was determined from analysis of a highly enriched sample by ^{31}P NMR (Figure 3 and Table III). [d] Not determined. The value of k_3 in eq 2 was set equal to 0. If $0 < k_3 < 0.002$, it has no significant effect on the evaluation of k_1 and k_2.

Table II: Rate Constants of Phosphoenolpyruvate Hydrolysis

acidity	k (min^{-1})[a]
4.9 N HCl	0.23
4.2 N HCl	0.16
1.0 N HCl	0.074
pH 0.9	0.068
pH 2.1	0.084

[a] The first-order rate constants were evaluated as described under Experimental Procedures. The uncertainty is estimated to be ±5%.

Rates of Phosphoenolpyruvate Hydrolysis. Table II gives the rates of hydrolysis of phosphoenolpyruvate at 98 °C and various pH levels. The effect of pH on the hydrolysis rate is qualitatively similar to that determined at 75 °C by Benkovic & Schray (1968a). The rates of hydrolysis are less than the rates of exchange of the three branch-phosphate oxygens of phosphoenolpyruvate at all pH levels examined. The pH dependences of hydrolysis and exchange differ, and the best condition for formation of [^{18}O]phosphoenolpyruvate by preferential exchange is near pH 0.

Preparation of Phosphoenolpyruvate Highly Enriched with ^{18}O. On the basis of the observed rates of exchange and hydrolysis, the following procedure is recommended for preparation of ^{18}O-labeled phosphoenolpyruvate. Approximately 40 mg of dry phosphoenolpyruvate monopotassium salt and a trace of [^{32}P]phosphoenolpyruvate solution (1 μL) are placed in a 1.5 × 9 cm Pyrex tube with a 14/20 greased ground-glass joint and stoppered. To this phosphoenolpyruvate in a drybox are added 0.5 mL of $H_2{}^{18}O$ and sufficient HCl prepared in $H_2{}^{18}O$ (see Experimental Procedures) to make the free acid concentration 1.0 N. The vial is capped with a glass stopper secured by wire and heated in a boiling water bath

Table III: GC-MS and ^{31}P NMR Analysis of [^{18}O]Phosphoenolpyruvate

atoms of ^{18}O per group analyzed[a]	GC-MS ($-PO_3$ group) obsd (%)	GC-MS ($-PO_3$ group) theory (%)[c]	^{31}P NMR[b] obsd (%)	GC-MS (parent ion) obsd (%)
sample 1				
0	0.00	0.00	0.00	0.00
1	3.41	3.70	4.15	0.00
2	25.33	25.65	25.2	1.59
3	71.25	71.15	70.6	13.82
4				39.96
5				44.33
6				0.290
sample 2				
0			0.00	0.00
1			2.61	0.00
2			24.61	1.24
3			72.64	9.04
4			0.646	38.77
5				50.55
6				0.385

[a] GC-MS ($-PO_3$ group) gives the ^{18}O content of the phosphoryl moiety of PEP; ^{31}P NMR, the ^{18}O content of the phosphate moiety of PEP; and GC-MS (parent ion), the ^{18}O content of all of the oxygens of PEP. [b] ^{31}P NMR analyzes the four oxygens bound to phosphorus for ^{18}O. Samples 1 and 2 were analyzed at 81.0 and 202.5 MHz, respectively. The ^{31}P NMR spectrum of sample 2 is given in Figure 3. [c] Binomial distribution corresponding to the observed average enrichment.

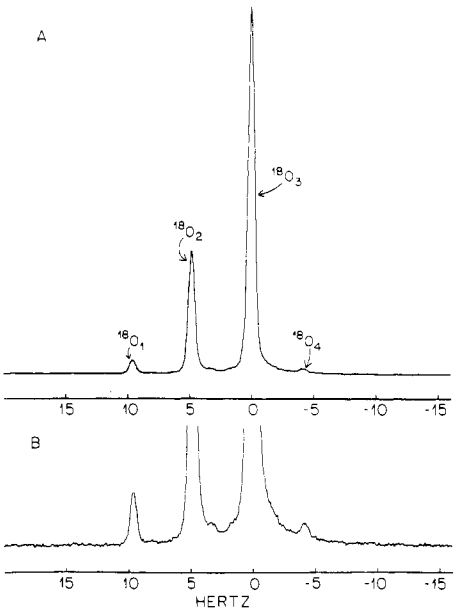


FIGURE 3: ^{31}P NMR spectra of [^{18}O]phosphoenolpyruvate at 202.5 MHz. The solution contained 12 μmol of K^+EDTA, about 0.17 mmol of [^{18}O]phosphoenolpyruvate, and about 0.4 mmol of [^{18}O]P_i and pyruvate, pH 8.5–9, in 99.8% D_2O with a final volume of 0.6 mL. The spectrum was acquired with 612 scans, a flip angle of 28.4°, a total data length of 16K zero-filled to 64K, and a spectral width of 1500 Hz. No exponential mutliplication was employed. (A) Complete spectrum; (B) spectrum expanded to show the most upfield peak due to the species with all four phosphate oxygens of phosphoenolpyruvate enriched.

for 5.5 min. The reaction is quenched by putting the vial in an ice–water mixture. The sample is frozen and evacuated and the $H_2{}^{18}O$ recovered by vacuum transfer to a vial immersed in liquid nitrogen and containing dry sodium glycinate, which neutralizes the HCl. From the values given in Tables I and II, it can be calculated that, after a 5-min incubation at 98 °C, 31% of the phosphoenolpyruvate would have undergone hydrolysis and the remaining phosphoenolpyruvate would have reached an enrichment of 86% of the equilibrium value. The actual value would be slightly less due to dilution of the ^{18}O content of $H_2{}^{18}O$ with the unenriched oxygens of phosphoenolpyruvate as a result of exchange. For a second cycle of enrichment, 0.5 mL of $H_2{}^{18}O$ and additional HCl are added to the phosphoenolpyruvate in the same vial in the drybox making the sample about 1 N in H^+. A second heating is carried out for 5.75 min and the $H_2{}^{18}O$ recovered as above.

The [^{18}O]phosphoenolpyruvate is separated from P_i and pyruvate by column chromatography at 4 °C. A cold mixture of 0.9 mL of 1 N Tris and 34 mL of H_2O is added to the sample to dissolve it and bring the pH to 8. The sample is applied to a 0.7 × 8 cm column of Dowex AG1-X4 resin in the chloride form. The sample is washed with 8 mL of H_2O and the P_i eluted with 40 mL of 30 mM HCl. About 24 mL of 50 mM HCl is applied; then, the [^{18}O]phosphoenolpyruvate is eluted with 28 mL of 100 mM HCl and the sample frozen. The excess HCl is removed by lyophilization transfer to sodium glycinate immersed in liquid nitrogen. The free acid of [^{18}O]phosphoenolpyruvate is then converted to the potassium salt by addition of an appropriate amount of cold KOH to bring the sample to pH 7.6. [^{18}O]Phosphoenolpyruvate prepared in this way and stored at −70 °C showed no loss of ^{18}O over a period of 1 year. The [^{18}O]phosphoenolpyruvate contained [^{18}O]P_i in an amount ≤0.4% of the total.

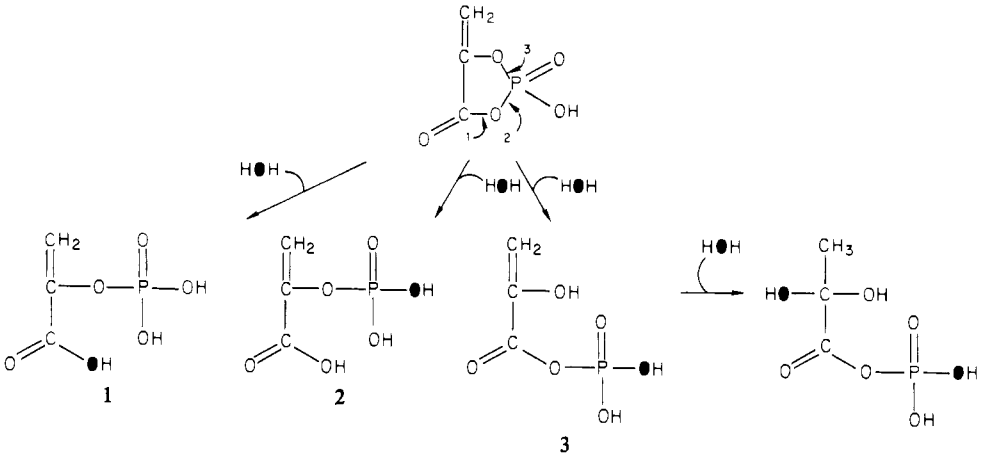
By use of this procedure and $H_2{}^{18}O$ of greater than 97% enrichment, [^{18}O]phosphoenolpyruvate was synthesized with an overall yield of 50% of the starting amount of phosphoenolpyruvate. Table III (sample 1) gives the distribution of ^{18}O in the phosphoryl oxygens of phosphoenolpyruvate determined by GC–MS analysis of the phosphate product of the pyruvate kinase–glycerol kinase catalyzed hydrolysis of purified [^{18}O]phosphoenolpyruvate. The enolic oxygen is not retained in the phosphate product of this reaction.[4] The three phosphoryl oxygens are enriched to 89.3%. A greater enrichment could be achieved by a longer time of incubation but with additional loss of product by hydrolysis. All three equivalent phosphoryl-group oxygens have the same probability of undergoing exchange; hence, the distribution should be the same as the binomial distribution for the average enrichment that is given for comparison. This agrees well with the observed distribution (Table III, sample 1).

Table III (sample 1) also gives the ^{18}O distribution of the parent ion. The distribution, as expected, is not binomial on a probability basis; the small amount of the species with six atoms of ^{18}O confirms the slow rate of exchange of the enolic oxygen relative to the other five (Table I). These results were confirmed by ^{31}P NMR.

^{31}P NMR Spectra. Table III (sample 1) gives the distribution of the phosphate-group oxygens determined by ^{31}P NMR at 81.0 MHz. This distribution agrees well with that

[4] Benkovic & Schray (1968b) have reported on the basis of the relative amounts of phosphate and methyl phosphate produced upon hydrolysis of phosphoenolpyruvate by mercury(II) ion (1.1-fold excess) in methanol–water at pH 4 that the enolic oxygen is retained in approximately 85% of the phosphate product. Hence, O–P bond cleavage occurred to an extent of about 15% under their conditions. Mercury(II)-catalyzed hydrolysis of phosphoenolpyruvate (0.9, 1.22, and 0.25 mM) by $HgAc_2$–NaAc, pH 4 (0.39 M, 0.24 M, and 0.092 M, respectively), in $H_2{}^{18}O$ (7.3%, 18%, and 6.1%, respectively) gave phosphate products in which the enolic oxygen was retained to extents of 26%, 66%, and 71%, respectively. Thus, O–P bond cleavage occurred 74%, 34%, or 29% of the time depending on conditions, compared to the value of 15% reported by Benkovic & Schray (1968b). Hence, analysis of [^{18}O]phosphoenolpyruvate by mercury cleavage was not used to determine the ^{18}O content of the enolic oxygen.

Scheme I: Possible Cleavage Patterns of Phosphoenolpyruvate[a]



[a] The ^{18}O is indicated by the filled oxygens. Cleavage at positions 1, 2, and 3 yields products 1, 2, and 3.

determined by GC–MS (Table III, sample 1). No peak indicative of the labeling of the enolic oxygen was apparent in the spectrum (not shown), suggesting that it had only been slightly labeled. For confirmation of the labeling of the enolic oxygen observed in the GC–MS results, a more concentrated sample was prepared and analyzed at 202.5 MHz. Figure 3 gives the ^{31}P NMR spectrum of the unpurified product of an exchange reaction of 100 mg of phosphoenolpyruvate and 1.0 mL of approximately 95% enriched $H_2{}^{18}O$ carried out for about 18.5 min at 1 N HCl and 98 °C. The distribution of the phosphate-group oxygens of this sample as determined by ^{31}P NMR is given in Table III (sample 2). The expanded ^{31}P NMR spectrum (Figure 3B) clearly shows the presence of a species containing four atoms of ^{18}O bonded to phosphorus in an amount, approximately 0.65%, that is greater than can be accounted for by the natural abundance of ^{18}O in P_i, 0.2035%. This is best explained by a slow exchange of the enolic oxygen with water oxygens at 1 N, confirming the slow exchange of the enolic oxygen detected at 1 and 5 N HCl by GC–MS. The small amount of the species with all six oxygens labeled with ^{18}O seen in the GC–MS distributions of the parent ion (Table III) is consistent with this result.

The differences in chemical shift between the species containing one and two, two and three, and three and four atoms of ^{18}O per molecule of phosphoenolpyruvate are 0.0235, 0.0238, and 0.210 ppm, respectively. These values can be compared to the corresponding isotopic shifts of phosphate, 0.0206 ppm, and of [β-^{18}O]ADP, 0.0166 ppm (α–β bridge oxygen) and 0.0215 ppm (branch oxygen), obtained by Cohn & Hu (1980).

Discussion

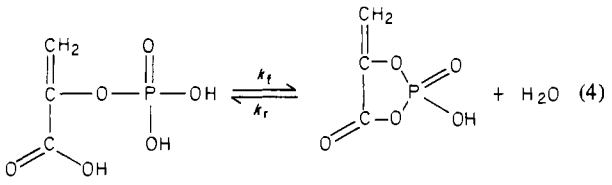
The results document a facile exchange of the phosphoryl and the carboxyl oxygens of phosphoenolpyruvic acid when heated in aqueous, acidic solution. The observed exchanges are most readily explained by the intermediate formation of a cyclic phosphate and its reversible cleavage to re-form the original phosphoenolpyruvate or a transient acyl phosphate. These exchanges occur orders of magnitude faster than exchanges of the oxygens of phosphoric acid (Bunton et al., 1961) or of carboxyl oxygens of aliphatic acids at similar acidities (Cohn & Urey, 1938).

From structural considerations and the observations of Clark & Kirby (1963) on the demethylation of (dimethylphospho)enolpyruvate, formation of a cyclic phosphate likely occurs by attack of a carboxyl oxygen on the phosphorus. The reason for water elimination with cyclization instead of phosphoryl transfer may be that the geometry prevents the C–O–P bond from assuming the appropriate apical position that favors O–P bond cleavage (Westheimer, 1968). This is also quite in accord with the observations of Stubbe and Kenyon on the rapid demethylation with (dimethylphospho)enol-3-bromopyruvic acid (Stubbe & Kenyon, 1971) or (dimethylphospho)enolpyruvic acid (Stubbe & Kenyon, 1972) is dissolved in water. They noted that incubation of (dimethylphospho)enol-3-bromopyruvic acid in water at room temperature for 0.5 h followed by removal of water gave the demethylated product.

From the data presented in Table I, it is apparent that the cyclization occurs most readily with the neutral fully protonated species. The small increase in exchange rate with acidity from 1 to 5 N HCl indicates that general acid catalysis does not have a prominent role. These results are consistent with those of Clark & Kirby (1963), who showed that (dimethylphospho)enolpyruvate loses one methyl group at pH 8 and both methyl groups at slightly acidic pH. Further, the difference in the time required for formation of phosphoenolpyruvic acid from (dimethylphospho)enolpyruvic acid at slightly acidic pH, 3 days (Clark & Kirby, 1963), and at pH near 0, 3 h (Stubbe & Kenyon, 1972), is analogous to the pH dependence of the oxygen exchange reactions of phosphoenolpyruvate (Table I).

The cyclic phosphate, once formed, may be regarded as undergoing cleavage at the three positions indicated by the arrows in Scheme I. Reversible cleavage at position 1 would incorporate water oxygen into the equivalent carboxyl oxygens formed. Reversible cleavage at position 2 would incorporate a water oxygen into the equivalent phosphoryl oxygens formed. Reversible cleavage at position 3 would similarly label the phosphoryl oxygens and would liberate the enolic product; the enol could reversibly ketonize and hydrate to incorporate a water oxygen into the enolic hydroxyl. These incorporations are indicated in Scheme I. With reversal of the cleavage at position 1 there would be one change out of two that the carboxyl group would retain an oxygen from water. With reversal at positions 2 or 3, there would be two chances out of three for retention of an oxygen in the phosphoryl group. Extent of labeling of the enolic oxygen accompanying each cleavage at position 3 would depend upon the relative rates of exchange of the ketonic oxygen and recyclization.

The rate constants of formation of the cyclic phosphate (Scheme I, eq 4) from the phosphoenolpyruvate species labeled at the three different positions are the rate constants for oxygen exchange given in Table I.[5] The sum of these constants then



gives k_f, the overall rate constant of formation of the cyclic phosphate. At 98 °C and 1 N HCl, the value of k_f is 1.6 min^{-1}. In eq 4, k_f(phosphoenolpyruvate) = k_r(cyclic phosphate) because the exchange occurs at chemical equilibrium. The competing hydrolysis reaction does not affect the evaluation of the rate constants for the exchange reaction; in the exchange experiment the average enrichment of the phosphoenolpyruvate remaining in the sample, not its concentration, was measured. The concentration of the small amount of cyclic phosphate likely present was not determined; thus, the rate constant for its cleavage, k_r, could not be evaluated. However, the relative values of the rate constants for cleavage of the cyclic phosphate at positions 1, 2, and 3 (Scheme I) are equal to the relative values of the first-order rate constants for oxygen exchange (Table I).[5]

With respect to the mass spectrometry cleavage patterns, it is fortunate that the molecular ion of the phosphoenolpyruvate derivative was present in sufficient amounts for satisfactory analysis. The uncertainty as to the apparent two types of ethylated derivatives giving the apparent molecular ion at mass 280 does not detract from the exchange analyses.

The preference for exchange over hydrolysis can serve for preparation of ^{18}O-labeled phosphoenolpyruvate but with the recognized loss of part of the product as higher ^{18}O enrichment is attained. Because of the dilution of the water oxygens by the oxygens of phosphoenolpyruvate, two or more cycles of exchange may be needed to obtain highly labeled phosphoenolpyruvate. Various convenient methods of separation could give the requisite product.

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Registry No. ^{18}O, 14794-71-8; $H_2{}^{18}O$, 14314-42-2; KNCO, 590-28-3; cyclic $CH_3C(OH)_2COOPO_3H_2$, 83928-97-6; $CH_3C(OH)_2COOPO_3H_2$, 83928-98-7; ADP, 58-64-0; phosphoenolpyruvate, 130-08-9; [^{18}O]phosphoenolpyruvate, 83928-96-5; [^{32}P]phosphoenolpyruvate, 56374-29-9; β-chlorolactate, 1713-85-5; carbonyl phosphate, 590-55-6; pyruvate, 127-17-3; monopotassium phosphoenolpyruvate, 4265-07-0.

[5] This assumes that the rates of keto–enol tautomerization and reversible hydration of the keto group are rapid compared to the rate of reformation of phosphoenolpyruvate. A rapid exchange of the carbonyl oxygen would be expected in hot, acidic solution (Cohn & Urey, 1938; Bell, 1966). If re-formation of phosphoenolpyruvate occurs more rapidly than the oxygen exchange, cleavage at position 3, Scheme I, would contribute to the rate of exchange of phosphoryl oxygens and not to that of enolic oxygens.

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