Synthesis and Study of Phosphoenolthiopyruvate†

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Received July 10, 1987; Revised Manuscript Received October 16, 1987

ABSTRACT: Phosphoenolthiopyruvate, the analogue of phosphoenolpyruvate in which the bridging oxygen of the phosphate ester is replaced by sulfur, has been synthesized from methyl acrylate and dimethyl (chlorothio)phosphonate. The compound is a substrate for alkaline phosphatase, pyruvate kinase, enolase, and phosphoenolpyruvate carboxylase. Both pyruvate kinase and phosphoenolpyruvate carboxylase convert the compound to thiopyruvate, which is a substrate for lactate dehydrogenase. Phosphoenolpyruvate carboxylase is slowly inactivated by phosphoenolthiopyruvate.

Comparative studies of phosphate and thiophosphate esters have provided interesting information about mechanisms of enzymatic phosphate transfer. Nonbridging thiophosphates,[1] which contain a C—O—P=S linkage, have found wide use in studies of the stereochemistry of phosphate transfer (Eckstein, 1985), and these compounds react anywhere from several fold to several orders of magnitude more slowly than the corresponding phosphates. Nonbridging thiophosphates are hydrolyzed roughly 100 times more slowly than the corresponding phosphates by alkaline phosphatase from *Escherichia coli* (Breslow & Katz, 1968; Mushak & Coleman, 1972), and similar results are obtained with other enzymes (Knowles, 1980; Domanico et al., 1986). The acid phosphatases are exceptional: in this case, thiophosphates and phosphates undergo hydrolysis at roughly equal rates (Neumann, 1968).

Bridging thiophosphate monoesters, which contain a C—S—P=O linkage, have been little studied, perhaps in part because of their acid lability. Intestinal alkaline phosphatase is the only enzyme that has been shown to hydrolyze *S*-alkyl thiophosphates readily (Neumann, 1968). Alkaline phosphatases from other sources are inert toward bridging thiophosphates (Rossomando et al., 1983; Neumann et al., 1976; Bhanot & Weeks, 1985). Hexokinase-catalyzed phosphate transfer from 6-thioglucose 6-phosphate to ADP is 100 000 times slower than with the natural substrate (Knight & Cleland, 1985), and glycerokinase reacts with (*R*,*S*)-1-thioglycerophosphate 1/1000 as fast as with its oxygen-containing counterpart (Knight & Cleland, 1985). On the other hand, phosphoglucomutase transfers the phosphate between the 6 and 1 positions of 6-thioglucose at rates similar to those of glucose (Knight & Cleland, 1985). Interestingly, bridging thio analogues of nucleoside di- and triphosphates do not appear to be known, but the bridging thio analogue of pyrophosphate has recently been reported (Loewus & Eckstein, 1983).

Thiophosphoenolpyruvate, the analogue of phosphoenolpyruvate (PEP)[2] in which a nonbridging phosphate oxygen is replaced by sulfur, has been used for investigating phosphorus stereochemistry (Orr et al., 1978; Hansen & Knowles, 1982).

phosphoenolthiopyruvate (S-PEP) thiophosphoenolpyruvate

This compound is a substrate for PEP carboxylase, enolase, PEP carboxykinase, and pyruvate kinase (Orr et al., 1978; Hansen & Knowles, 1982; Sheu et al., 1984; Lee et al., 1985). In the case of PEP carboxylase, the thio compound is about 10% as reactive as PEP (J. R. Knowles, private communication). Rates have not been measured for other enzymes. We were interested in the synthesis and study of phosphoenolthiopyruvate (S-PEP), the PEP analogue in which the bridging oxygen of the phosphate ester has been substituted by sulfur, because reaction of this compound with a variety of enzymes should generate a highly reactive thio ketone, or the enethiolate thereof, and these compounds might serve as enzyme inactivators or undergo other interesting reactions. We report here the synthesis of S-PEP and study of its reactions with alkaline phosphatase, pyruvate kinase, and phosphoenolpyruvate carboxylase.

Experimental Procedures

Materials. Sulfur (Mallinckrodt), L-serine (Sigma), sodium thiophosphate decahydrate (Alfa), sodium iodoacetate (Aldrich), trisodium phosphoenolpyruvate (Sigma), Na_2NADH (Sigma), K-ADP (Sigma), NAD (Sigma), 4,4′-dipyridyl disulfide (Aldrich), 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma), thiolactate (Aldrich), and sodium sulfide (Mallinckrodt) were used as supplied. Buffers (HEPES and MES) were purchased from Sigma or U.S. Biochemicals. Trimethyl phosphite (Aldrich) was allowed to stand over sodium metal for 2 days and distilled. Sulfuryl chloride (Aldrich) was distilled immediately before use. Methyl acrylate (Aldrich) was washed with 1 M NaOH and water, dried over $CaCl_2$, and distilled under reduced pressure. Diisopropylethylamine (Aldrich) was refluxed over CaH_2 for 1 h and distilled. Triethylamine (Aldrich) was distilled from CaH_2. Trimethylsilyl bromide (Aldrich) was distilled from 1-decene. Potassium deuterioxide was prepared by addition of potassium metal (Mallinckrodt) to D_2O (Aldrich) and filtered through a Swinny filter with

† This work was supported by Grant 83-CRCR-1-1297 from the Competitive Research Grants Office, U.S. Department of Agriculture.

[1] The nomenclature in this field is unfortunate and tends to confuse all except the specialists: Compounds containing a nonbridging sulfur in a C=S or P=S double bond should be referred to by the prefix *thion*; compounds containing a bridging C—S or P—S single bond should be referred to as *thiol* compounds; the prefix *thio* is noncommital as to bonding. We choose simply to call our compounds thio and use other means to describe the position of sulfur substitution.

[2] Abbreviations: PEP, phosphoenolpyruvate; S-PEP, phosphoenolthiopyruvate; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; P_i, inorganic phosphate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; PEI, poly(ethylene imine).

0006-2960/88/0427-1342$01.50/0

a Metricel membrane, and its concentration was determined by measuring the pH of an aliquot diluted with water. Pyridine (Aldrich) was refluxed over KOH or BaO and distilled. Benzene (Aldrich) was distilled from CaH_2. Methylene chloride (Mallinckrodt) was shaken with concentrated H_2SO_4, washed with H_2O, neutralized with 5% NaOH, washed with H_2O, dried with $CaCl_2$, and distilled. All distillations were performed under anhydrous conditions. Solvents were stored over molecular sieves. For flash chromatography, CH_2Cl_2 was distilled and diethyl ether (Fisher) was used as supplied. Water was purified with a Millipore Super Q water purification system. Triethylammonium bicarbonate was prepared by bubbling CO_2 from subliming dry ice through an aqueous solution of triethylamine.

Alkaline phosphatase (bovine intestinal mucosa, type VII-N), lactate dehydrogenase (bovine heart, type III), pyruvate kinase (rabbit muscle, type II), hexokinase (yeast, type IV), glucose-6-phosphate dehydrogenase (*L. mesenteroides*, type XXIV), malate dehydrogenase (porcine heart mitochondrial), and enolase (yeast, type III) were purchased from Sigma Chemical Co. PEP carboxylase was isolated from maize as described by Díaz et al. (1988).

Methods. All new compounds were characterized by IR, NMR, and (where possible) mass spectra. Fourier transformed ^1H NMR spectra were taken on a Bruker WP200SY spectrometer. Chemical shifts are given in parts per million downfield from internal tetramethylsilane. Proton-decoupled 80-MHz ^{31}P and 50-MHz ^{13}C NMR spectra were taken on a JEOL FX 200 spectrometer and are reported in parts per million downfield from 85% H_3PO_4 and tetramethylsilane, respectively. Phosphorus chemical shifts and integrations were measured relative to a PP_i internal standard. IR spectra were taken on a Beckman Acculab 7 spectrophotometer. Mass spectra were taken on the DS-55 mass spectrometry data system at The University of Wisconsin—Madison. UV spectra were taken on a Cary 118 spectrophotometer thermostated at 25.0 °C.

Flash chromatography was performed on Baker flash chromatography silica gel. Thin-layer chromatography was performed on glass plates or plastic sheets precoated with 0.2-mm silica gel 60 (E. Merck) or PEI–cellulose (Baker) containing a fluorescent indicator. Ion-exchange chromatography was performed on DEAE-Sephadex A-25 (Pharmacia) or Dowex 1-X8 (200–400 mesh). Dowex resins were cleaned with an alkaline solution of bromine before use.

Syntheses. (*a*) *Trimethyl Thionophosphate.* Elemental sulfur (0.25 mol, 8 g) was heated at 75 °C for 0.5 h under nitrogen and then allowed to cool to room temperature, 100 mL of dry pyridine was added via syringe, and the solution was cooled to 0 °C. Trimethyl phosphite (0.25 mol, 31 g) in 100 mL of dry pyridine was added dropwise via cannula over 0.5 h at 0 °C. After the addition was complete, the pyridine was removed under vacuum to give an 85% yield of trimethyl thionophosphate, which was vacuum-distilled (bp 38 °C at 2 mmHg) (Ailman & Magee, 1976; Loewus & Eckstein, 1983) and then stored as a solution in dry benzene at −15 °C.

(*b*) *Dimethyl (Chlorothio)phosphonate.* Freshly distilled sulfuryl chloride (101 mmol, 13.6 g) in 30 mL of dry CH_2Cl_2 was added dropwise via cannula over 1 h to 101 mmol (10.93 g) of trimethyl thionophosphate in 30 mL of dry CH_2Cl_2 at −20 °C with vigorous stirring under nitrogen. The reaction solution became yellow as dimethyl (chlorothio)phosphonate was formed.

(*c*) *Trimethyl S-Phospho-2-mercapto-3-chloropropanoate.* The dimethyl (chlorothio)phosphate was immediately added dropwise via cannula over 1 h to 150 mmol of methyl acrylate (12.9 g) in 30 mL of CH_2Cl_2 at −20 °C with stirring under nitrogen. The yellow reaction solution was allowed to warm to room temperature and was stirred for 15 h under nitrogen.

The slightly yellow reaction solution was washed with 5% sodium bicarbonate until the washes were neutral. The aqueous washes were combined and extracted with ether. The ether extracts of the aqueous washes and the CH_2Cl_2 reaction solution were combined, dried over sodium sulfate, and concentrated on the rotary evaporator to give a slightly cloudy colorless liquid. The liquid was placed on a 9.8 × 8.5 cm column of flash chromatography silica gel and eluted with 1 L of 15% ether in CH_2Cl_2 and then with 1 L of 40% ether in CH_2Cl_2. Sixty-milliliter fractions were collected and analyzed by thin-layer chromatography (ethyl acetate solvent). The product-containing fractions were combined and concentrated on the rotary evaporator to give 6.14 g (48%) of a slightly yellow liquid. The 200-MHz ^1H NMR ($CDCl_3$) indicated the presence of trimethyl S-PEP, trimethyl *S*-phospho-2-mercapto-3-chloropropanoate, and trimethyl *S*-phospho-3-mercapto-2-chloropropanoate in a ratio of 2:1:4.

(*d*) *Trimethyl S-PEP.* The mixture of phosphate esters from the above reaction (6.1 g total) was dissolved in 90 mL of dry CH_2Cl_2, and 16.8 mmol (2.2 g) of diisopropylethylamine in 60 mL of dry CH_2Cl_2 was added dropwise via cannula over 1.5 h with vigorous stirring under N_2 at 0 °C. The yellow solution was reduced in volume on the rotary evaporator and placed on a 9 × 9 cm column of flash chromatography silica gel. The column was eluted with 2 L of 40% ether in CH_2Cl_2 and then with 1 L of 60% ether in CH_2Cl_2. Sixty-milliliter fractions were collected and analyzed by thin-layer chromatography (ethyl acetate solvent). The ^1H NMR indicated that trimethyl S-PEP and trimethyl *S*-phospho-3-mercapto-2-chloropropanoate were present in a ratio of 2.1:1. The two compounds were separated (with difficulty) by flash chromatography on a 4.5 × 10 cm column of silica gel eluted with 20% ether in CH_2Cl_2. A total of 1.5 g of trimethyl S-PEP was obtained. The compound was distilled in a Kugelrohr apparatus at 100 °C at 0.1 mmHg and then stored as a solution in dry benzene or CH_2Cl_2 at −15 °C.

^1H NMR ($CDCl_3$): δ 6.82 (1 H, d, J = 3.5 Hz), 6.40 (1 H, d, J = 3.7 Hz), 3.86 (6 H, d, J = 12.8 Hz), 3.84 (3 H, s). ^{13}C NMR ($CDCl_3$): δ 164.2 (d, J = 4.4 Hz), 136.2 (d, J = 7.2 Hz), 128.6 (d, J = 7.4 Hz), 54.2 (d, J = 5.9 Hz), 52.9 (s). ^{31}P NMR ($CDCl_3$): δ 25.3 (s). IR (neat): 1725 (s), 1600 (w), 1440 (m), 1265 (s), 1125 (s), 1025 (s), 875 (w) cm^{-1}. UV (CH_3OH): 243.5 nm. MS (% of base peak) 226 (12), 142 (22), 127 (12), 109 (100), 93 (10), 79 (22), 58 (34).

(*e*) *Tripotassium S-PEP.* Trimethyl S-PEP (0.16 mmol, 37 mg) was dissolved in 1.6 mL of dry CH_2Cl_2, and 0.99 mmol (6 equiv, 0.13 mL) of trimethylsilyl bromide was added via syringe under nitrogen with stirring at room temperature; stirring was continued for 12 h. The solvent was removed under a nitrogen stream, and the unreacted trimethylsilyl bromide was removed under oil pump vacuum to give a slightly yellow viscous liquid. The bis(trimethylsilyl) monomethyl ester thus produced was hydrolyzed with 0.82 mmol (5 equiv) of 1 M KOD added via a Pasteur pipet under nitrogen with rapid stirring at room temperature. The resulting cloudy slightly yellow solution was filtered through a Swinny filter with a Metricel membrane. The filter was rinsed with 0.5 mL of 0.1 mM EDTA. The pH was lowered to 11 with Dowex 50 (H^+), and this solution was stored at −78 °C for later use.

^1H NMR (1 M KOD): δ 6.09 (1 H, s), 6.02 (1 H, d, J = 0.9 Hz). ^{31}P NMR (1.3 M KOD): 13.0 ppm. ^{13}C NMR (1.3

M KOD): 140.1 (d, J = 5 Hz), 120.0 ppm (d, J = 3 Hz). No peak was observed for the carboxyl carbon, presumably because of its long relaxation time. UV (H_2O, pH 8): 261 nm (1000 M^{-1} cm^{-1}).

(*f*) *Trisodium Phosphothioglycolate.* Sodium iodoacetate (0.25 mmol, 52 mg) in 0.5 mL of D_2O was added to 0.25 mmol (100 mg) of trisodium thiophosphate (decahydrate) in 0.5 mL of D_2O at room temperature. The product was purified by precipitation with alcohol followed by ion-exchange chromatography on Dowex 1-X8 (200–400 mesh) with a linear gradient of 375 mL of KCl (0.1–1.0 M) at pH 9.0 at 5 °C. Fractions were assayed with alkaline phosphatase and 4,4′-dipyridyl disulfide.

(*g*) *Concentrations of Thiophosphates.* Concentrations of phosphothioglycolate and of S-PEP were determined by integration of the ^{31}P NMR spectrum following addition of a known concentration of inorganic pyrophosphate. Alternatively, concentrations were determined by treating the compounds with alkaline phosphatase and reacting the resulting thiol compounds with 4,4′-dipyridyl disulfide. For S-PEP, the concentration was also determined by observing the change in absorption at 340 nm following treatment of the compound with alkaline phosphatase, lactate dehydrogenase, and NADH at pH 8. All methods gave consistent results.

(*h*) *Hydrolysis of S-PEP.* Reactions were studied in 0.1 M buffers at 25 °C by measuring the decrease in absorbance at 260 nm vs time.

Enzyme Studies. (*a*) *Alkaline Phosphatase.* Reactions with alkaline phosphatase (1 unit per assay) were carried out at pH 8, 25 °C, in the presence of 0.1 M HEPES buffer, 6 mM $MgCl_2$, and 0.1 mM EDTA. Reactions were followed in the presence of 4,4′-dipyridyl disulfide, with spectrophotometric detection at 324 nm, or else (for S-PEP) with lactate dehydrogenase (5 units) and 0.2 mM NADH.

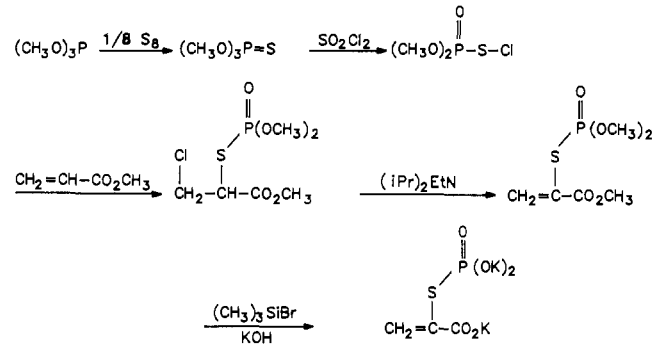
In the case of the lactate dehydrogenase assay, the product of reduction was chromatographed on PEI–cellulose TLC plates with acetate or formate buffers, pH 3, 4, or 5. The plates were sprayed with 5,5′-dithiobis(2-nitrobenzoic acid) (Glaser et al., 1970) or sodium nitroferricyanide (Toennies & Kolb, 1951) to give yellow and purple spots, respectively. The product solution was chromatographed alongside lactate and thiolactate standards. Lactate was not detected with these two reagents but could be visualized by reaction with I_2 or by fluorescence quenching on plates treated with a fluorescent dye. In every case, the reaction product cochromatographed with authentic thiolactate. Production of thiolactate was determined to be quantitative by reaction of the product with dithiobis(2-nitrobenzoic acid) and comparison of the yield of that reaction with the loss of NADH during the original reduction.

(*b*) *Pyruvate Kinase.* Pyruvate kinase was assayed spectrophotometrically at 340 nm in the presence of 0.05 M HEPES, pH 7.5, with 4 mM $MgCl_2$, 0.3 mM ADP, 0.1 mM EDTA, 100 mM KCl, 0.2 mM NADH, and various concentrations of PEP and inhibitor and coupled to lactate dehydrogenase (5 units). The computer programs of Cleland (1979) were used to obtain kinetic parameters.

For studies of ATP formation, the assay solution contained 50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM ADP, 5 mM glucose, 1 mM NAD^+, 7 units of hexokinase, 5.5 units of glucose-6-phosphate dehydrogenase, 4.0 mM $MgCl_2$ or 3.0 mM $MnCl_2$, various concentrations of S-PEP, and pyruvate kinase.

To determine whether S-PEP was an irreversible inhibitor of pyruvate kinase, 1.4 units of enzyme was incubated at pH 7.5 with 0.66 mM inhibitor, and aliquots were taken for assay at intervals.

Scheme I



(*c*) *Phosphoenolpyruvate Carboxylase.* Unless otherwise noted, this enzyme was assayed spectrophotometrically at 340 nm in the presence of 0.05 M HEPES, pH 7.5 or pH 8, 5.4 mM $MgCl_2$, 5.4 mM $NaHCO_3$, 1 mM PEP, 0.2 mM NADH, and 22 units of malate dehydrogenase. Reactions were initiated by addition of PEP carboxylase.

For studies of S-PEP as a substrate for PEP carboxylase, the assay solution contained 200 mM HEPES, pH 8.0, 0.1 mM EDTA, 4.3 mM $NaHCO_3$, 0.2 mM NADH, 5 units of lactate dehydrogenase, 0.16 unit of PEP carboxylase, and 4.3 mM $MgCl_2$ or 0.8 mM $MnCl_2$. Reactions were initiated by addition of substrate. The reaction was linear with enzyme concentration over the range from 0.025 to 0.100 units of enzyme per assay. No rate was observed when malate dehydrogenase was substituted for lactate dehydrogenase.

For studies of irreversible inhibition of PEP carboxylase, the incubation solution contained 235 mM HEPES, pH 8.0, 0.1 mM EDTA, 4.1 mM $MgCl_2$, 4.1 mM $NaHCO_3$, 0.78 mM S-PEP, and 0.16 units of PEP caboxylase. Aliquots were withdrawn at intervals and assayed for PEP carboxylase activity in the standard assay. A similar experiment was carried out with 3.7 mM Na_2S as the inhibitor.

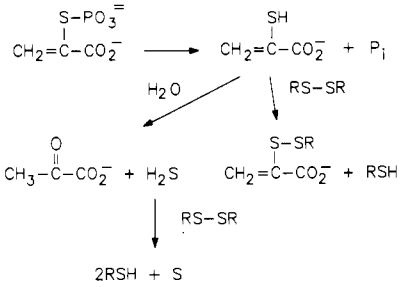
RESULTS

Synthesis of Phosphothioglycolate. This compound was synthesized in 100% yield by the reaction of sodium iodoacetate with trisodium thiophosphate in aqueous solution at room temperature (eq 1) (Akerfeldt, 1962).

$$ICH_2CO_2Na + Na_3SPO_3 \rightarrow O_3^{2-}PSCH_2CO_2^- + 3Na^+ \quad (1)$$

Synthesis of S-PEP. The synthetic sequence is given in Scheme I. Trimethyl thionophosphate was synthesized in 85% yield by the reaction of trimethyl phosphite with elemental sulfur in pyridine (Ailman & Magee, 1976; Loewus & Eckstein, 1983). This material was converted to dimethyl (chlorothio)phosphate by reaction with sulfuryl chloride (Loewus & Eckstein, 1983). Because of its instability, this compound was not isolated but was reacted immediately with methyl acrylate, giving a 2:1 ratio of *S*-phospho-2-mercapto-3-chloropropanoate and *S*-phospho-3-mercapto-2-chloropropanoate. These two compounds could not be readily separated. Instead, diisopropylethylamine was added to this mixture. Hydrogen chloride was selectively eliminated from the *S*-phospho-2-mercapto-3-chloropropanoate isomer to give a mixture of trimethyl S-PEP and trimethyl *S*-phospho-3-mercapto-2-chloropropanoate (Thaler et al., 1968; Kuhle, 1971). The mixture was separated by flash chromatography. Demethylation was achieved via the bis(trimethylsilyl) thio-

Scheme II



phosphate ester, which was synthesized by reaction with trimethylsilyl bromide (Borecka et al., 1979) and then hydrolyzed by treatment of this material with KOH (Bartlett & Chouinard, 1983). The final product proved difficult to purify. Ion-exchange chromatography gave solutions from which the eluting buffer could not be removed without significant decomposition. Cyclohexylammonium salts were not pure and could not be readily recrystallized.

Stability of S-PEP. The hydrolysis of S-PEP under acidic conditions at 25 °C was studied by observing the changes in the UV spectrum between 240 and 320 nm with time. At pH 3 the half-life for hydrolysis is about 0.5 h, increasing to over 2 h at pH 6. At pH 7.5, the half-life is approximately 30 h. At pH 13, the half-life is approximately 10 days at 5 °C. The stability was not affected by the presence of 4 mM Mg^{2+}, but rapid hydrolysis (and precipitation) occurred in the presence of 3 mM Mn^{2+}.

Reaction of S-PEP with Alkaline Phosphatase. When phosphothioglycolate was treated with alkaline phosphatase at pH 8 in the presence of 4,4′-dipyridyl disulfide, absorbance at 324 nm gradually developed due to formation of 4-thiopyridone. Thus, thiophosphoglycolate is a substrate for alkaline phosphatase.

Similarly, treatment of S-PEP with alkaline phosphatase in the presence of 4,4′-dipyridyl disulfide resulted in the gradual development of an absorbance at 324 nm. Similar results were obtained with dithiobis(2-nitrobenzoic acid) as the SH detection reagent. With the latter compound, the observed rate was proportional to the concentration of alkaline phosphatase. Thus, S-PEP is also a substrate for alkaline phosphatase. When S-PEP was treated with alkaline phosphatase in the presence of lactate dehydrogenase and NADH, the NADH absorbance gradually disappeared.

The stoichiometry of the alkaline phosphatase catalyzed hydrolysis of S-PEP was compared by using three measurement systems. First, the initial concentration of S-PEP was obtained from integration of its ^{31}P NMR spectrum prior to hydrolysis. Second, the concentration of thiopyruvate produced after hydrolysis was obtained from the change in NADH concentration in the presence of lactate dehydrogenase and NADH. Third, the concentration of SH groups produced following treatment of the substrate with alkaline phosphatase was determined by reaction of the product with 4,4′-dipyridyl disulfide. The stoichiometry of this reaction is of particular interest. The initial product of the hydrolysis is the enethiol (Scheme II), which might react directly with the sulfhydryl reagent. Alternatively, the enethiol might tautomerize to thiopyruvate, which could then undergo reaction with water to form H_2S. The former reaction would give 1 equiv of 4-thiopyridone/equiv of S-PEP, whereas the latter reaction would give 2 equiv/equiv of S-PEP (Svensen, 1980; Nashef et al., 1977). A comparison of the results of the thiol assay with those of the phosphorus NMR revealed that the concentration of the chromophoric mercaptan was the same as

Table I: Kinetic Parameters for Interaction of Pyruvate Kinase with Phosphothioglycolate and Phosphoenolthiopyruvate at pH 7.5 in the Presence of Mg^{2+} and Mn^{2+} [a]

	phosphothioglycolate		S-PEP	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
K_i vs PEP	2 mM	0.6 mM	110 μM	70 μM
K_m as substrate			230 μM	80 μM
V_{max} relative to PEP			0.0002	0.004

[a] Velocities are given relative to PEP = 1.0.

Table II: Kinetic Parameters for Interaction of PEP Carboxylase with Phosphothioglycolate and Phosphoenolthiopyruvate at pH 8.0 in the Presence of HCO_3^- and Mg^{2+} or Mn^{2+} [a]

	phosphothioglycolate		S-PEP	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
K_i vs PEP	16 mM	1.4 mM	2 mM	0.25 mM
K_m as substrate			5.3 mM	32 μM
V_{max} relative to PEP			0.09	0.02

[a] Velocities are given relative to carboxylation of PEP = 1.0. The product of PEP carboxylase acting on S-PEP is thiopyruvate (see text).

(rather than twice) the concentration of substrate. Thus, it appears that the thiol reagents react with thiopyruvate or its enethiol rather than with H_2S (Scheme II).

It is also clear that reaction of S-PEP with alkaline phosphatase produces a material which can be reduced by lactate dehydrogenase and NADH. The concentration of NADH consumed is the same as the concentration of the starting compound and the same as the thiol concentration [by reaction with dithiobis(2-nitrobenzoic acid)] after the reaction was complete. This equivalency indicates that thiopyruvate is being formed and that this material is being reduced to thiolactate.

In order to confirm that thiopyruvate was being reduced to thiolactate by lactate dehydrogenase, the reduction product was chromatographed on PEI–cellulose plates with acetate and formate buffers at various concentrations and pH's. The product was detected with dithiobis(2-nitrobenzoic acid) (Glaser et al., 1970) or sodium nitroferricyanide (Toennies & Kolb, 1951), and the product coeluted with authentic thiolactate.

In separate experiments, we attempted to oxidize thiolactate using NAD^+ and lactate dehydrogenase. At pH 9.3 with 5 mM NAD^+ and 5 mM thiolactate, conditions under which lactate dehydrogenase oxidized lactate, no NADH was formed. However, this presumably reflects the unfavorable equilibrium for thiolactate oxidation rather than the inherent reactivity.

Reaction of S-PEP with Pyruvate Kinase. Phosphothioglycolate and S-PEP are inhibitors of pyruvate kinase. Inhibition constants are shown in Table I.

S-PEP is itself a substrate for pyruvate kinase. Activity could be measured as appearance of material reactive with 4,4′-dipyridyl disulfide, as material reducible by lactate dehydrogenase and NADH, or as material that could be coupled to hexokinase in the presence of glucose-6-phosphate dehydrogenase and NAD^+. The observed rate was linear with pyruvate kinase concentration (according to the ATP-coupled assay), and the same rate was obtained with both enzymatic assays. Kinetic parameters, together with corresponding parameters for phosphoenolpyruvate, are given in Table I.

Pyruvate kinase was not significantly inactivated by a 1.5-h incubation with 0.7 mM S-PEP in the presence of 4 mM Mg^{2+} and 1 mM ADP at pH 7.5.

Reaction of S-PEP with PEP Carboxylase. Both phosphothioglycolate and S-PEP are inhibitors of phosphoenol-

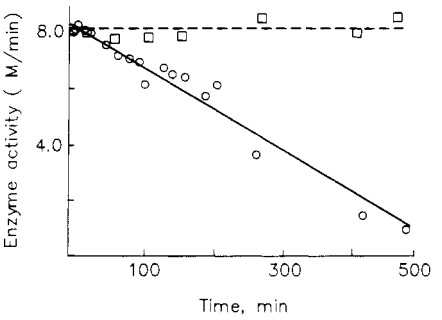


FIGURE 1: Irreversible inactivation of PEP carboxylase by S-PEP. Activity of PEP carboxylase in the presence of S-PEP (solid line) and absence of S-PEP (broken line) with time. The incubation solution contained 235 mM HEPES, pH 8.0, 0.1 mM EDTA, 4.1 mM $MgCl_2$, 4.1 mM $NaHCO_3$, 0.78 mM S-PEP, and 0.16 unit of PEP carboxylase. The control was the same as the incubation solution, except S-PEP was replaced by water.

pyruvate carboxylase. Kinetic data are summarized in Table II.

In the presence of either Mg^{2+} or Mn^{2+} and HCO_3^-, phosphoenolpyruvate carboxylase catalyzes the conversion of S-PEP into thiopyruvate, which is in turn reduced by lactate dehydrogenase and NADH. In these experiments, NADH disappearance was linear with time, without any significant induction period. When malate dehydrogenase was used instead of lactate dehydrogenase, no change in NADH absorbance was observed. Kinetic parameters are summarized in Table II.

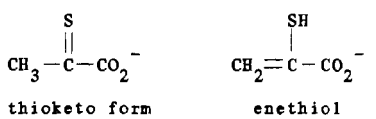
In the presence of 4 mM Mg^{2+} and 4 mM HCO_3^-, phosphoenolpyruvate carboxylase was inhibited irreversibly to the extent of 90% by 0.78 mM S-PEP in 8 h at pH 8 (Figure 1). Hydrogen sulfide, which might be generated by the hydrolysis of thiopyruvate formed during the reaction, inactivated the enzyme only to a slight extent (Figure 1).

DISCUSSION

S-PEP has been synthesized by an indirect route involving halide elimination from a thiolactate derivative, a route analogous to an early route used for the synthesis of PEP itself (Baer & Fischer, 1949). This reaction has now fallen out of favor because of the convenience of the Perkow reaction for synthesis of PEP (Cramer & Voges, 1959; Clark & Kirby, 1966), but the latter reaction would not be expected to be useful for synthesis of the thio derivative.

Hydrolysis of S-PEP. S-PEP is stable for at least several hours in aqueous solution at neutral pH but hydrolyzes more rapidly than PEP itself (Benkovic & Schray, 1968). Like other thiophosphates, it rapidly decomposes at low pH. In the presence of Mn^{2+}, the compound hydrolyzes quite rapidly, perhaps initiated by complexing of the metal ion with sulfur. A similar effect is not observed with Mg^{2+}.

The product of the hydrolysis of S-PEP is apparently thiopyruvate. This compound can exist in two tautomeric forms, a thio keto form and an enethiol form:



The initial product of spontaneous or alkaline phosphatase catalyzed hydrolysis is expected to be the enethiol form. Unlike the case of ketones, where the keto form is by far the predominant form, thio ketones exist to a significant degree in the enethiol form (Duus, 1979). Equilibration between the two forms is expected to occur within minutes (perhaps within seconds). Evidence from this study indicates that this equilibrium is facile. First, the compound reacts rapidly with 5,5′-dithiobis(2-nitrobenzoic acid) and with 4,4′-dipyridyl disulfide, which are expected to react only with the enethiol form. Second, the compound also reacts readily with lactate dehydrogenase and NADH (see below), which are expected to react only with the keto form.

The keto form of thiopyruvate is expected to react with water at some (unknown) rate to form H_2S and pyruvate. The latter compound is, of course, a substrate for lactate dehydrogenase, so the observed oxidation of NADH to NAD^+ in the presence of S–PEP, alkaline phosphatase, and lactate dehydrogenase must be carefully considered before it can be concluded that thiopyruvate, rather than pyruvate, is being reduced. This was demonstated in two different ways. First, the stoichiometry of the lactate dehydrogenase reduction has been compared to that of the reaction of the sulfur-containing product with sulfhydryl reagents. H_2S is expected to react with 2 equiv of thiol reagent, whereas the enethiol is expected to react with one (cf. Scheme II; Svenson, 1980; Nashef et al., 1977). We demonstrated stoichiometric equivalence between NADH oxidized and thiol reagent reduced. Second, we demonstrated chromatographically that thiolactate is formed as a result of the reduction.

To our knowledge, dehydrogenases have not previously been demonstrated to reduce thio ketones. However, this is more likely due to the paucity of thio ketones for use as substrates than due to the lack of reactivity. The one analogous case is the nonenzymatic reduction of thiobenzophenone and thiopivalophenone by 1-benzyl-1,4-dihydronicotinamide (Abeles et al., 1956; Ohno et al., 1978a,b; Yasui et al., 1981).

Binding of Phosphates and Thiophosphates to Enzymes. Phosphoglycolate is an analogue of PEP that binds tightly to a number of enzymes which act on PEP (Duffy & Nowak, 1984). Phosphothioglycolate, the corresponding sulfur compound, is a poor inhibitor of pyruvate kinase and PEP carboxylase, in all cases being bound 10–100-fold less tightly than the oxygen analogue. It is not clear whether this effect is due to changes in geometry or changes in electronic structure or whether it reflects coordination of the sulfur to the metal.

For pyruvate kinase, comparison of PEP with S-PEP is made difficult by the fact that the K_m for PEP ordinarily is not a true binding constant. Nonetheless, it is interesting that the K_m values for the two compounds are quite similar (the second-order rate constants V_{max}/K_m are, of course, quite different).

For PEP carboxylase, the observed K_m values approximate binding constants, and it appears that the thio and oxygen compounds bind to the enzyme with similar strength when Mg^{2+} is used. Values obtained in the presence of Mn^{2+} are made problematical by the metal-induced decomposition of the sulfur-containing substrate.

Pyruvate Kinase. S-PEP is the first bridging thiophosphate demonstrated to be a substrate for pyruvate kinase, although numerous phosphate esters are substrates. Activity of pyruvate kinase toward this compound was demonstrated both by reduction of the three-carbon product with lactate dehydrogenase and NADH and by coupling of the ATP produced to hexokinase. The thio substrate is several thousandfold less reactive than the natural substrate, presumably reflecting the lower thermodynamic activity of the enethiol phosphate.

PEP Carboxylase. In the presence of HCO_3^-, PEP carboxylase converts S-PEP into thiopyruvate and P_i. The reaction is 10–50-fold slower than the carboxylation of PEP. No evidence for carboxylation was ever seen. This includes (a)

absence of products that could be reduced by malate dehydrogenase, (b) stoichiometric equivalence between amount of substrate added and amount of NADH consumed in the presence of lactate dehydrogenase, and (c) lack of an induction period in formation of NADH-reducible material. Thiooxalacetate is not a known compound, and it is possible that if formed, it would decarboxylate rapidly. However, it is unlikely that this is the principal product in this case. Thus, this compound can be added to a growing list of alternate substrates of PEP carboxylase that are in essence hydrolyzed rather than carboxylated.

S-PEP slowly inactivates PEP carboxylase. Inactivation is not due to formation of H_2S in the solution, because reaction of the enzyme with H_2S is severalfold slower than inactivation. The kinetics of inactivation do not appear to be first order. The inactivation mechanism might involve a reaction of either thiopyruvate or its enol, but this has not been pursued.

Enolase. S-PEP is also a substrate for enolase. In preliminary experiments, an attempt was made to synthesize S-PEP via enolase-catalyzed elimination of water from 2-phospho-2-mercapto-3-hydroxypropanoate. The ^{31}P NMR spectrum of the reaction product indicated that S-PEP was formed. However, the equilibrium in this reaction may be more unfavorable than in the case of phosphoglycerate, and the reaction is probably not of use for the synthesis of S-PEP because of the difficulty of purifying S-PEP from the reaction mixture. Nonetheless, it follows from these observations that S-PEP is a substrate for enolase.

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