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(*E*)-3-Cyanophosphoenolpyruvate, a New Inhibitor of Phosphoenolpyruvate-Dependent Enzymes[†]

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ABSTRACT: (*E*)-3-Cyanophosphoenolpyruvate has been synthesized by reacting dimethyl chlorophosphate with the potassium enolate of ethyl cyanopyruvate. The resulting trialkyl ester was deesterified with bromotrimethylsilane followed by potassium hydroxide. Subsequent treatment with Dowex-50-H^+ resin and cyclohexylamine afforded the tricyclohexylammonium salt; only the *E* geometric isomer was obtained. This compound can be photoisomerized to a 70:30 *E*:*Z* mixture. (*E*)-3-Cyanophosphoenolpyruvate is an excellent competitive inhibitor of phosphoenolpyruvate carboxylase [$K_I(Mn^{2+}) = 16\ \mu M$, $K_I(Mg^{2+}) = 1360\ \mu M$], pyruvate kinase [$K_I(Mn^{2+}) = 0.085\ \mu M$, $K_I(Mg^{2+}) = 0.76\ \mu M$], and enolase [$K_I(Mn^{2+}) = 360\ \mu M$, $K_I(Mg^{2+}) = 280\ \mu M$]. The compound is a substrate for pyruvate kinase ($V_{max} \sim 1\%$ of phosphoenolpyruvate rate), but not for the other two enzymes. No irreversible inactivation is observed with phosphoenolpyruvate carboxylase or pyruvate kinase.

Phosphoenolpyruvate is a key intermediate in a variety of metabolic processes in all living organisms (Davies, 1979). Its chemistry is mediated by enzymes that utilize either the high-energy enol phosphate bond, for example, in glycolysis and in the C-4 pathway of photosynthesis, or the ability to act as an enol pyruvyl donor, for example, in the shikimate pathway and in bacterial peptidoglycan synthesis.

In view of the central role of phosphoenolpyruvate in metabolism, enzymes that metabolize phosphoenolpyruvate are logical targets for active-site-directed (specific) and mechanism-based (suicide) inhibitors. The principal question in such studies is whether specificity of inhibition can be obtained among the variety of phosphoenolpyruvate-metabolizing enzymes that exist. Studies of the antibiotic fosfomycin indicate that such selectivity may be possible. The enzymes pyruvate kinase, phosphoenolpyruvate carboxykinase, enolase, and phosphoenolpyruvate–shikimate-5-phosphate enolpyruvoyltransferase show only competitive inhibition with this compound. However, pyruvoyltransferase is irreversibly inactivated (Cassidy & Kahan, 1973). Such specificity is not predictable at this point.

Among the other phosphoenolpyruvate analogues that have been studied, the most notable are perhaps 3-bromophosphoenolpyruvate and 3-fluorophosphoenolpyruvate. The *Z* isomer of the former compound shows very strong competitive inhibition with phosphoenolpyruvate carboxylase at short times and irreversible (suicide) inactivation at longer times (O'Leary & Diaź, 1982). Thus, the compound is presumably a substrate for phosphoenolpyruvate carboxylase. This compound is also a substrate for pyruvate kinase (Nowak & Duffy, 1984), but the enzyme is not inactivated. By contrast, this same substance is neither a substrate nor an inhibitor for enolase.

(*Z*)-3-Fluorophosphoenolpyruvate is a substrate for pyruvate kinase (Nowak & Duffy, 1984; Stubbe & Kenyon, 1972) and for phosphoenolpyruvate carboxylase (E. Diaź, unpublished results). The compound is an inhibitor and a substrate for enolase (Nowak & Duffy, 1984). The *E* isomer of this same compound is an inhibitor of pyruvate kinase and enolase and a substrate for pyruvate kinase (Nowak & Duffy, 1984).

(*E*)-3-Cyanophosphoenolpyruvate was conceived as an inhibitor of phosphoenolpyruvate-dependent enzymes. Mechanism-based inactivation by this compound would presumably proceed by way of a ketene imine intermediate (Maycock et al., 1975; Miles, 1975; Villafranca & Baldori, 1980). Here we report the synthesis of this compound and studies of its inhibitory properties against phosphoenolpyruvate carboxylase, pyruvate kinase, and enolase.

EXPERIMENTAL PROCEDURES

Materials

Phosphoenolpyruvate carboxylase was isolated from maize (Zea mays, strain W64A) by a slight modification of the method of Uedan and Sugiyama (Uedan & Sugiyama, 1976; O'Leary et al., 1981). Pyruvate kinase (type II from rabbit muscle), enolase (type III from baker's yeast), lactate dehydrogenase, malate dehydrogenase, hexokinase (from yeast), glucose-6-phosphate dehydrogenase (from *Leuconstoc mesenteroides*), phosphoenolpyruvate, ADP, ATP, NAD,[1] NADH, and Tris-HCl were purchased from Sigma Chemical Co. HEPES[1] buffer (free acid) was purchased from United States

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[1] Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; TMSP, sodium 3-(trimethylsilyl)propionate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Biochemical Corp. Dowex 50-X8-100 resin was purchased from Bio-Rad Chemical Co. and purified before use (Cleland & Dunaway-Mariano, 1980). Diethyl oxalate was purchased from Aldrich Chemical Co. Bromotrimethylsilane was purchased from Aldrich and distilled from potassium carbonate. Dimethyl chlorophosphate was prepared by methods described by Müller (1964). All solvents were purified and dried according to Perrin et al. (1980). All other reagents were of the highest quality commercially available. All aqueous solutions were made by using water that had been passed through a Millipore deionization–filtration system.

Methods

^{1}H nuclear magnetic resonance spectra were measured at 200 or 270 MHz on an IBM WP-200 or WP-270 Fourier transform instrument. Chemical shifts were measured in parts per million from tetramethylsilane or sodium 3-(trimethylsilyl)propionate. ^{13}C NMR spectra were taken at 15.1 or 50.3 MHz on a JEOL FX-60 or FX-200 Fourier transform spectrometer using tetramethylsilane or acetone as a reference. Infrared spectra were recorded on a Beckman Acculab-7, mass spectra on a Kratos MS-80. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected.

Ethyl 2-Oxo-3-cyanopropanoate (*Potassium Enolate*). This compound was synthesized by the method of von Borsche & Manteuffel (1934). The amorphous, brittle, pale yellow product was recrystallized from ethanol in the following manner. About 200 mL of absolute ethanol was brought to a boil on a hot plate; about 2 g of the powdered crude material was stirred in and boiled until it appeared that maximum dissolution had occurred (light yellow solution with some undissolved granular material); the beaker was then removed from the heat and placed on the bench top; within 1 min, a fluffy solid began to precipitate. After reaching room temperature, the beaker was cooled briefly in ice, with care being taken not to disturb the contents. The desired solid could then be poured off into a Büchner funnel, leaving the hard, granular substance behind. After being dried overnight in a drying pistol (100 °C, 1 torr), a fluffy, off-white solid was obtained. The material was only slightly hygroscopic and could be handled briefly outside a glove bag. The yield was 10 g (56%): mp 225–235 °C dec; IR (KBr disk) 2990 (s), 2920 (w), 2180 (s), 1720 (s), 1620 (s), 1580 (s), 1440 (s), 1370 (s), 1250 (s), 1030 (s) cm^{-1}.

Dimethyl 1-Carbethoxy-3-cyanovinyl Phosphate. An oven-dried 25-mL round-bottom flask containing a magnetic stirring bar was charged with the potassium enolate of ethyl cyanopyruvate (1.00 mmol, 0.18 g). A 5-mL portion of dry acetonitrile was then added via a syringe. The flask was placed in an ice bath and the suspension stirred. Dimethyl chlorophosphate (1.05 mmol, 0.17 g) was then added dropwise, and the ice bath was slowly allowed to melt and warm to room temperature. After about 6 h, during which time a tan-brown suspension was present, the reaction was stopped by diluting with about 50 mL of ether and pouring into a separatory funnel. A tan solid settled to the bottom. The ether layer was then extracted with 1 × 10 mL of 5% HCl, which removed the solid. The light brown ethereal solution was then further extracted with 4 × 10 mL of 5% $NaHCO_3$, which removed most of the color, and 1 × 10 mL of brine. The ether layer was then dried over $MgSO_4$ and filtered, and the solvent was removed with a stream of nitrogen in a 40 °C water bath. A clear, light brown liquid remained. The slightly viscous material should not be kept in the neat state. (It should be noted that an anhydrous workup did not give a better yield and the product was contaminated with the excess phosphate.) The yield was 0.192 g (77%) of a mixture of *E* and *Z* isomers: ^{1}H NMR ($CDCl_3$) for *E*, δ 1.36 (3 H, t, $^3J_{HH}$ = 7.12 Hz), 3.99 (6 H, d, $^3J_{PH}$ = 11.7 Hz), 4.33 (2 H, q, $^3J_{HH}$ = 7.12 Hz), and 6.16 (1 H, d, $^4J_{PH}$ = 1.60 Hz); ^{1}H NMR ($CDCl_3$) for *Z*, δ 1.42 (3 H, t, $^3J_{HH}$ = 7.11 Hz), 3.91 (6 H, d, $^3J_{PH}$ = 11.7 Hz), 4.47 (2 H, q, $^3J_{HH}$ = 7.11 Hz), and 6.06 (1 H, d, $^4J_{PH}$ = 2.06 Hz); ^{13}C NMR (CD_3CN) for *E*, δ 17.01, 59.19 (d, $^2J_{POC}$ = 6.6 Hz), 67.01, 98.73 (d, $^3J_{PC}$ = 4.95 Hz), 116.4 (d, $^4J_{PCN}$ = 3.3 Hz), 155.9 (d, $^2J_{PC}$ = 9.15 Hz), and 162.3 (d, $^3J_{PCO}$ = 6.6 Hz); ^{13}C NMR (CD_3CN) for *Z*, δ 17.01, 59.19 (d, $^2J_{POC}$ = 6.6 Hz), 66.84, 99.80 (d, $^3J_{PC}$ = 7.35 Hz), 116.8 (d, $^4J_{PCN}$ = 1.65 Hz), 155.4 (d, $^2J_{PC}$ = 8.25 Hz), and 163.09 (d, $^3J_{PCO}$ = 1.65 Hz); IR ($CDCl_3$) 3150 (w), 2950 (s), 2240 (s), 1735 (s), 1365 (m), 1280 (s), 1180 (m), 1040 (s), 870 (s) cm^{-1}. MS (30 eV) 249.0396 (peak match on parent ion; calculated for $C_8H_{12}NO_6P$: 249.0399). The product was approximately (by NMR) a 70:30 *E*:*Z* mixture.

(*E*)-*Tricyclohexylammonium 3-Cyanophosphoenolpyruvate*. The deesterification procedure was a slight modification of the method reported by Bartlett & Chouinard (1983). The phosphate deesterification required 6 equiv of bromotrimethylsilane and a reaction time of 15 h. The proton NMR of the product indicated the presence of the *E* isomer (~90%) with the *Z* isomer not evident. There was an ~10% "impurity" peak in the vinyl region. The yield was 0.330 g (67%): mp 111–114 °C dec; ^{1}H NMR (D_2O) for *E*, δ 1.1–2.1 (30 H, m), 3.15 (3 H, m), and 5.68 (1 H, d, $^4J_{PH}$ = 1.61 Hz); ^{1}H NMR (D_2O) for impurity, δ 5.72 (d, 0.68 Hz); IR (KBr) 3400 (broad), 2920 (s), 2880 (s), 2200 (m), 1600 (broad and s), 1450 (m), 1400 (s), 1350 (m), 1150 (s) cm^{-1}; ^{13}C NMR [D_2O; selective decoupling experiment (vinylic proton irradiated)] δ 75.6, 107.9, 152.9, and 159.5, plus numerous cyclohexyl peaks.

Photoisomerization of (*E*)-*Tricyclohexylammonium 3-Cyanophosphoenolpyruvate*. The *E* isomer was photoisomerized in a quartz NMR tube (100 mM in D_2O) with a 450-W Hanovia lamp UV source. An NMR spectrum was taken at intervals. After 15 min of irradiation, a 70:30 *E*:*Z* mixture was obtained. ^{1}H NMR (D_2O) for *E* isomer, δ 5.68 (1 H, d, $^4J_{PH}$ = 1.61 Hz); ^{1}H NMR (D_2O) for *Z* isomer, δ 5.83 (1 H, d, $^4J_{PH}$ = 1.84 Hz). No further change was noted after 0.5 h of irradiation.

Enzyme Assays. All solutions were freshly prepared and kept on ice for the duration of the experiment (unless otherwise noted). Phosphoenolpyruvate carboxylase was dialyzed for about 20 h against 100 mM HEPES buffer, pH 7.5. It was observed, during the course of this work, that one could freeze (−20 °C) and thaw pH 7.5 HEPES solutions of this enzyme and still maintain excellent activity. Steady-state rates were measured within the first 2 min on a Cary 118 spectrophotometer. Kinetic parameters were calculated by using the programs of Cleland (1979).

Phosphoenolpyruvate carboxylase was assayed by coupling it to malate dehydrogenase in the presence of NADH and observing the decrease in absorbance at 340 nm (Lane et al., 1969). Competitive inhibition of phosphoenolpyruvate carboxylase by 3-cyanophosphoenolpyruvate was studied in the presence of 5 mM $NaHCO_3$, 0.2 mM NADH, 33 mM HEPES, pH 8.0, 22 units of malate dehydrogenase, and either 0.215–2.15 mM phosphoenolpyruvate, 0–6.67 mM 3-cyanophosphoenolpyruvate, and 5 mM $MgCl_2$ or 0.209–2.09 mM phosphoenolpyruvate, 0–0.103 mM 3-cyanophosphoenolpyruvate, and 1 mM $MnCl_2$. Three-milliliter volumes of such solutions were equilibrated to 25.0 °C, and then the reaction was initiated by addition of enzyme.

For studies of the irreversible inactivation, phosphoenolpyruvate carboxylase (0.1 mg/mL) was incubated with 5 mM $NaHCO_3$, 0.2 mM dithiothreitol, 0.05 mM EDTA, 50 mM HEPES, pH 7.0, and either 5 mM $MgCl_2$ and 5.5 mM 3-cyanophosphoenolpyruvate or 1 mM $MnCl_2$ and 0.52 mM 3-cyanophosphoenolpyruvate. The same was done at pH 8.0. At pH 8.5, 0.1 mM dithiothreitol, 0.05 mM $MnCl_2$, and 50 mM Tris-HCl buffer was used with everything else the same. At pH 9.0, only the $MgCl_2$ experiment was done, using 0.5 mM dithiothreitol, 50 mM CHES buffer, and everything else the same. Aliquots were withdrawn at intervals and added directly to an assay solution containing 33 mM HEPES, pH 8.0, 0.2 mM NADH, 5 mM $NaHCO_3$, 5 mM $MgCl_2$ or 1 mM $MnCl_2$, 1 mM phosphoenolpyruvate, and 22 units of malate dehydrogenase in a volume of 1 mL. A blank solution containing no 3-cyanophosphoenolpyruvate was run in parallel.

Pyruvate kinase was assayed by coupling it to lactate dehydrogenase in the presence of NADH and observing the decrease in absorbance at 340 nm. Competitive inhibition of pyruvate kinase by 3-cyanophosphoenolpyruvate was studied in the presence of 0.3 mM ADP, 0.2 mM NADH, 33 mM HEPES, pH 7.5, 100 mM KCl, 4 units of lactate dehydrogenase, and either 0.107–1.07 mM phosphoenolpyruvate, 0–23.8 μM 3-cyanophosphoenolpyruvate, and 2 mM $MgCl_2$ or 0.11–1.1 mM phosphoenolpyruvate, 0–3.8 μM 3-cyanophosphoenolpyruvate, and 4 mM $MnCl_2$. Three-milliliter volumes of such solutions were equilibrated to 25.0 °C, and then the reaction was initiated by addition of enzyme.

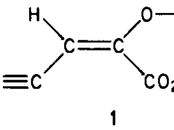
For studies of the irreversible inactivation, pyruvate kinase (7.5×10^{-3} mg/mL) was incubated with 0.3 mM ADP, 0.2 mM dithiothreitol, 0.05 mM EDTA, 50 mM HEPES, pH 7.5, 50 mM KCl, and either 2 mM $MgCl_2$ and 3.0 μM 3-cyanophosphoenolpyruvate or 4 mM $MnCl_2$ and 0.35 μM 3-cyanophosphoenolpyruvate. At pH 8.5, only the $MgCl_2$ experiment was done, using 50 mM Tris-HCl with everything else the same. Aliquots were withdrawn at intervals and added directly to an assay solution that contained 33 mM HEPES, pH 7.5, 0.3 mM ADP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 100 mM KCl, 4 mM $MnCl_2$ or 2 mM $MgCl_2$, and 4 units of lactate dehydrogenase in a volume of 1 mL. A blank solution containing no 3-cyanophosphoenolpyruvate was run in parallel.

For studies of 3-cyanophosphoenolpyruvate as a substrate for pyruvate kinase, the enzyme was assayed by coupling it to hexokinase and glucose-6-phosphate dehydrogenase in the presence of glucose and NAD and observing the increase in absorbance at 340 nm. The assay consisted of 50 mM HEPES, pH 7.5, 1 mM ADP, 100 mM KCl, 5 mM glucose, 1 mM NAD, 3 mM $MnCl_2$ or 4 mM $MgCl_2$, 6.5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, and 0.6 units of pyruvate kinase in a volume of 1 mL. This solution was premixed. The reaction was then initiated by addition of 3-cyanophosphoenolpyruvate solution to give concentrations of 1.6–20 μM when using $MgCl_2$ or 0.82–10 μM when using $MnCl_2$ in the assay solution. The same type of experiment was run in parallel with phosphoenolpyruvate as the substrate. A total of 0.12 units of pyruvate kinase was used. In the presence of $MgCl_2$ or $MnCl_2$, the phosphoenolpyruvate concentrations ranged from 7.3 to 145 μM. The reactions were run at 25.0 °C. The assay solutions were kept at room temperature.

Enolase was assayed by monitoring the decrease in phosphoenolpyruvate absorbance at 240 nm. Competitive inhibition of enolase by 3-cyanophosphoenolpyruvate was studied in the presence of 33 mM HEPES, pH 7.5, 50 mM KCl, and either 0.5 mM $MgCl_2$, 0.31–0.73 mM phosphoenolpyruvate, and 0–0.17 mM 3-cyanophosphoenolpyruvate or 0.01 mM $MnCl_2$, 0.30–0.70 mM phosphoenolpyruvate, and 0–0.18 mM 3-cyanophosphoenolpyruvate. Three-milliliter volumes of such solutions were equilibrated to 25.0 °C, and then the reaction was initiated by the addition of enzyme. It was only possible to use a narrow range of substrate and inhibitor concentrations due to the strong absorbance of 3-cyanophosphoenolpyruvate at 240 nm.

RESULTS

Synthesis. The tricyclohexylammonium salt of (*E*)-3-cyanophosphoenolpyruvate (**1**), 85–90% pure, was obtained by phosphorylation of the enolate of ethyl cyanopyruvate followed by deesterification. Photoisomerization studies (see below) provided an opportunity to obtain the NMR spectrum of the *Z* isomer. Spectra of the purified *E* isomer failed to indicate the presence of the *Z* isomer, presumably because any *Z* isomer formed during the synthesis is selectively destroyed during purification. The NMR spectrum of the purified *E* isomer consistently showed a small amount (10–15%) of an unidentified compound with a resonance just downfield of the vinylic proton resonance of the *E* isomer. The identity of the impurity remains unknown; it may be the decomposition product of the *Z* isomer present at the phosphoenolpyruvate trialkyl ester stage of the synthesis. An acid-catalyzed decomposition seems not to be occurring. The solution after Dowex treatment was allowed to sit for 90 min (instead of the usual immediate addition of cyclohexylamine). There was no change in the product.



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The exposure of (*E*)-3-cyanophosphoenolpyruvate to ultraviolet light produced, after 5 min, a 90:10 *E*:*Z* mixture and, after 15 min, a 70:30 *E*:*Z* mixture. Further irradiation produced no further change in the ratio. The final mixture was about 70% pure. During the photoisomerization, a contaminant was formed in addition to the *Z* isomer. This impurity also showed a doublet at δ 5.56 (J = 0.46 Hz) and may be the geometric isomer of the starting contaminant. The *Z* isomer has $^4J_{PH}$ = 1.84 Hz, consistent with a larger trans vs. cis H–P coupling. The purification and investigation of this mixture are in progress.

(*E*)-3-Cyanophosphoenolpyruvate is stable for several hours in D_2O at room temperature, after which time the solution turns yellow. At 0 °C, solutions are stable for 1 day, with losses estimated at 0.4%/h at pH 8.0 and 1%/h at pH 7.5. Solid (*E*)-tricyclohexylammonium 3-cyanophosphoenolpyruvate turns yellow within several hours at room temperature, but this salt can be stored for months in a desiccator at −20 °C with no apparent decomposition.

Enzyme Inhibition. Phosphoenolpyruvate carboxylase from maize is inhibited by (*E*)-3-cyanophosphoenolpyruvate. A kinetic study shows linear competitive inhibition with respect to phosphoenolpyruvate at pH 8.0 (Table I). Interestingly, the compound is 84 times more effective with Mn^{2+} than with Mg^{2+} as the metal ion.

Pyruvate kinase from rabbit muscle is strongly inhibited by (*E*)-3-cyanophosphoenolpyruvate at pH 7.5. The analogue shows linear competitive inhibition vs. phosphoenolpyruvate (Table I). The degree of inhibition is again metal ion selective with Mn^{2+}-acetivated enzyme affected 8.9 times more than

Table I: Inhibition Constants of (*E*)-3-Cyanophosphoenolpyruvate for Phosphoenolpyruvate-Utilizing Enzymes

enyzme	$K_I(\mathrm{Mn}^{2+})$ (μM)	$K_I(\mathrm{Mg}^{2+})$ (μM)
phosphoenolpyruvate carboxylase[a]	16.2	1360
pyruvate kinase[b]	0.085	0.76
enolase[c]	360	280

[a] Reactions run at pH 8.0, 25.0 °C, 5 mM Mg^{2+} or 1 mM Mn^{2+}, and 33 mM HEPES. [b] Reactions run at pH 7.5, 25.0 °C, 2 mM Mg^{2+} or 4 mM Mn^{2+}, 100 mM KCl, and 33 mM HEPES. [c] Reactions run at pH 7.5, 25.0 °C, 0.5 mM Mg^{2+} or 0.01 mM Mn^{2+}, 50 mM KCl, and 33 mM HEPES.

the Mg^{2+}-bound pyruvate kinase.

Yeast enolase shows linear competitive inhibition with respect to phosphoenolpyruvate at pH 7.5 (Table I). Of the three enzymes studied, this one is least affected by (*E*)-3-cyanophosphoenolpyruvate. Additionally, the selectivity of inhibition with regard to metal ions is the smallest with enolase and is reversed. The compound is about 1.3 times more inhibitory with Mg^{2+}-activated enzyme.

Phosphoenolpyruvate carboxylase showed no time-dependent inactivation in the presence of (*E*)-3-cyanophosphoenolpyruvate at pH 7.0, 8.0, 8.5 (Mn^{2+} or Mg^{2+} present), or 9.0 (Mg^{2+} only). At pH 7.0, both incubating and blank solutions lost no activity over a 2-h period with both metals. At pH 8.0, with Mg^{2+}, the blank and incubating solutions showed no loss in activity during 90 min, while with Mn^{2+} the incubating solution lost about 9% activity and the blank 7% in 150 min. At pH 8.5 with Mg^{2+}, the blank lost about 2% activity and the incubating solution 5% activity in 105 min; with Mn^{2+}, the blank lost 8% activity and the incubating solution 4% activity over a 105-min period. With Mg^{2+} at pH 9.0, neither the blank nor the incubating solution lost activity over 105 min.

Pyruvate kinase also showed no inactivation at pH 7.5 (Mn^{2+} or Mg^{2+}) or 8.5 (Mg^{2+}). No loss in activity was observed in 90 min with Mg^{2+} or in 200 min with Mn^{2+} at pH 7.5. Similarly, no loss in activity occurred over 90 min with Mg^{2+} at pH 8.5.

Substrate Activity. (*E*)-Tricyclohexylammonium 3-cyanophosphoenolpyruvate was found not to be a substrate for phosphoenolpyruvate carboxylase at pH 8.0 with either Mg^{2+} or Mn^{2+}. This was ascertained by observing no disappearance of NADH with 1 mM inhibitor and 5 times the amount of enzyme employed in the competitive assays, with either lactate dehydrogenase or malate dehydrogenase as coupling enzyme.

It was found in these studies that there is apparently some spontaneous hydrolysis of the compound to give cyanopyruvate, which is reduced by lactate dehydrogenase and NADH (as is found with bromo-, methyl-, and phenylphosphoenolpyruvate; Stubbe & Kenyon, 1971). At the usual coupling enzyme levels and 1 mM analogue, the rate is small (in the phosphoenolpyruvate carboxylase run, 4×10^{-4} μmol/min).

The possibility still exists that (*E*)-3-cyanophosphoenolpyruvate is a substrate for phosphoenolpyruvate carboxylase if the resultant 3-cyanooxalacetate is not a substrate for malate dehydrogenase and does not decarboxylate in solution, which seems unlikely.

(*E*)-Tricyclohexylammonium 3-cyanophosphoenolpyruvate is a substrate for pyruvate kinase at pH 7.5 with both Mg^{2+}- and Mn^{2+}-activated enzyme. The adenosine triphosphate produced was assayed with a hexokinase–glucose-6-phosphate dehydrogenase coupled assay system. The experiment was also run in parallel with phosphoenolpyruvate as substrate to allow direct comparison of rates. The kinetic parameters are shown in Table II.

Table II: Kinetic Constants for (*E*)-3-Cyanophosphoenolpyruvate as a Substrate for Pyruvate Kinase

metal	substrate[a]	K_M (μM)	V_{max} (units/mg)	V_{max}/K_M (mg^{-1} min^{-1})
Mn^{2+} [b]	PEP	100	29	290
	(*E*)-CNPEP	3.0	0.03	10
Mg^{2+} [c]	PEP	125	55	440
	(*E*)-CNPEP	4.0	0.17	42

[a] Abbrevations: PEP, phosphoenolpyruvate, (*E*)-CNPEP, (*E*)-3-cyanophosphoenolpyruvate. [b] Reactions run at pH 7.5, 25.0 °C, 1 mM ADP, 5 mM glucose, 1 mM NAD, 3 mM Mn^{2+}, 100 mM KCl, 6.5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, and 50 mM HEPES. [c] Reactions run at pH 7.5, 25.0 °C, 1 mM ADP, 5 mM glucose, 1 mM NAD, 4 mM Mg^{2+}, 100 mM KCl, 6.5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, and 50 mM HEPES.

Studies at high (*E*)-3-cyanophosphoenolpyruvate and high enzyme levels at pH 7.5 suggest that the compound is not a substrate for enolase.

DISCUSSION

A variety of compounds having a carboxyl group and a phosphate group in approximately the orientation found in phosphoenolpyruvate are inhibitors of various enzymes of phosphoenolpyruvate metabolism (Nowack & Duffy, 1984; Duffy et al., 1982; O'Leary, 1982; Stubbe & Kenyon, 1971, 1972). The fact that compounds having substituents at carbon 3 (halogen, methyl, cyano) bind well to these enzymes indicates that there is a degree of spatial tolerance at the substrate binding site. However, the fact that these compounds are at best very poor substrates for these enzymes indicates that the spatial tolerance is limited.

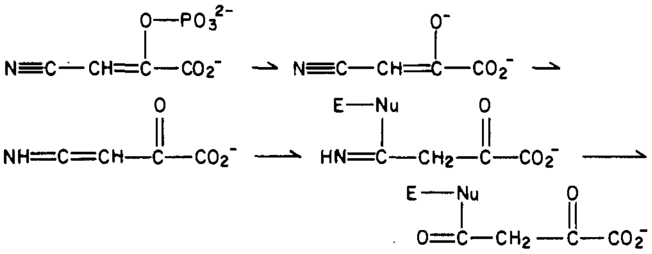
(*E*)-3-Cyanophosphoenolpyruvate is the latest addition to the known analogues of phosphoenolpyruvate. The compound is a competitive inhibitor (against phosphoenolpyruvate) of phosphoenolpyruvate carboxylase, enolase, and pyruvate kinase. The inhibition constants are similar to those observed with other phosphoenolpyruvate analogues and undoubtedly reflect a complex of geometric and electronic factors. The variation of K_I with metal ion is similar to that seen in other systems.

In the case of 3-fluorophosphoenolpyruvate and phosphoenol-α-ketobutyrate, both the *E* and the *Z* isomers have been tested against several enzymes (Nowak & Duffy, 1984; Duffy et al., 1982), and both isomers are inhibitory against pyruvate kinase, phosphoenolpyruvate carboxykinase, and enolase. To date, only the *E* isomer of 3-cyanophosphoenolpyruvate and the *Z* isomer of 3-bromophosphoenolyruvate have been tested. Preparation of the other isomers is in progress.

Like a number of other 3-substituted phosphoenolpyruvate analogues, (*E*)-3-cyanophosphoenolpyruvate is a substrate for pyruvate kinase. The striking thing about this set of compounds is that in spite of a wide range of electronic characteristics (substituents include F-, Br-, $\mathrm{CH_3}$-, and NC-), substrate activities for all these compounds are about the same (within a factor of 5) and are about a thousandfold lower than that of phosphoenolpyruvate itself. Apparently it is geometric, rather than electronic, factors that control the activities of these compounds with pyruvate kinase.

The inactivation of enzymes by 3-cyanophosphoenolpyruvate could proceed by way of a ketene imine intermediate formed by protonation of an enolate generated following phosphate transfer (Scheme I). The fact that 3-cyanophosphoenolpyruvate is a substrate for pyruvate kinase indicates that at least in this case the initial phosphate transfer envisioned in Scheme I can occur. However, either because of a low intrinsic

Scheme I



reactivity of the ketene imine intermediate or because of the high rate of protonation at carbon 3 to form 3-cyanopyruvate, inactivation of the enzyme does not occur.

In the case of phosphoenolpyruvate carboxylase, we are unable to determine whether the initial phosphate transfer occurs. This step might be precluded on either steric or electronic grounds. The enzyme does not catalyze net hydrolysis of the substrate, forming P_i and 3-cyanopyruvate, analogous to the reaction reported with phosphoenol-α-ketobutyrate (Fujita et al., 1984). Thus, if phosphate transfer occurs, it must be reversible.

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