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Analogs of Phosphoenolpyruvate. Substrate Specificities of Enolase and Pyruvate Kinase from Rabbit Muscle*

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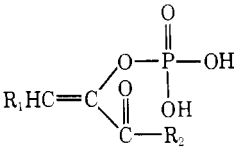
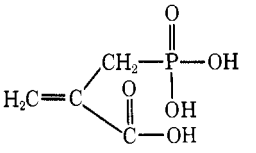
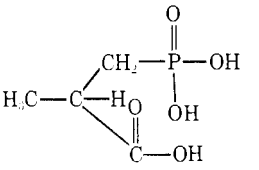
ABSTRACT: Syntheses of five analogs of phosphoenolpyruvate are described: (*Z*)-phosphoenol-3-fluoropyruvate, α-(dihydroxyphosphinylmethyl)acrylic acid, ethyl α-(dihydroxyphosphinyloxy)acrylate, α-(dihydroxyphosphinyloxy)acrylamide, and (*RS*)-α-(dihydroxyphosphinylmethyl)propionic acid. Each of these five analogs was tested as a potential substrate in the pyruvate kinase (EC 2.7.1.40) reaction. (*Z*)-Phosphoenol-3-fluoropyruvate was reactive as a substrate, but, within the limits of detection, the others were inactive. Each of the five analogs, as well as some others whose syntheses had been previously described [J. A. Stubbe and G. L. Kenyon, *Biochemistry 10*, 2669 (1971)], was also tested as a potential substrate in the enolase (EC 4.2.1.11) reaction. Both (*Z*)-phosphoenol-3-fluoropyruvate and α-(dihydroxyphosphinylmethyl)acrylic acid were shown to be substrates for this otherwise highly specific enzyme. Neither ethyl α-(dihydroxyphosphinyloxy)acrylate nor α-(dihydroxyphosphinyloxy)acrylamide nor any of the previously described analogs showed detectable reactivity. The stereochemistries of the *E* and *Z* isomers of phosphoenol-3-fluoropyruvate were assigned by comparison of nuclear magnetic resonance parameters to those of known, similar enol phosphates. Finally, modifications in the established procedure for the chemical synthesis of phosphoenolpyruvate are presented which improve the efficiency of the synthetic sequence.

Recently, we reported the syntheses of several analogs of phosphoenolpyruvate (**1**), including **2**–**4**, and presented evidence to show that both (*Z*)-phosphoenol-α-ketobutyrate (**2**) and (*Z*)-phosphoenol-3-bromopyruvate (**3**) are relatively slowly reacting substrates for pyruvate kinase from rabbit muscle (Stubbe and Kenyon, 1971). We now wish to report the syntheses of five more analogs of phosphoenolpyruvate (**1**), namely, **5**–**9**.



1, R_1 = H; R_2 = OH; phosphoenolpyruvate
2, R_1 = CH_3; R_2 = OH; phosphoenol-α-ketobutyrate [*Z* isomer]
3, R_1 = Br; R_2 = OH; phosphoenol-3-bromopyruvate [*Z* isomer]
4, R_1 = C_6H_5; R_2 = OH; phosphoenol-3-phenylpyruvate (stereochemistry not known)
5, R_1 = F; R_2 = OH; phosphoenol-3-fluoropyruvate [*Z* isomer]
6, R_1 = H; R_2 = OCH_2CH_3; ethyl α-(dihydroxyphosphinyloxy)-acrylate
7, R_1 = H; R_2 = NH_2; α-(dihydroxyphosphinyloxy)acrylamide





8, α-(dihydroxyphosphinylmethyl)acrylic acid
9, (*RS*)-α-(dihydroxyphosphinylmethyl)propionic acid

Each of these analogs was tested as a potential substrate in both the rabbit muscle pyruvate kinase (EC 2.7.1.40) and the rabbit muscle enolase (EC 4.2.1.11) reactions. Moreover, some of the analogs of phosphoenolpyruvate described previously (Stubbe and Kenyon, 1971) were also tested in the enolase reaction. Using nuclear magnetic resonance spectroscopy, stereochemical assignments were made for the *E* and *Z* isomers of phosphoenol-3-fluoropyruvate.

Materials and Methods

Most of the materials and analytical methods used were described previously (Stubbe and Kenyon, 1971). Pyruvate kinase (adenosine triphosphate:pyruvic acid phosphotransferase, EC 2.7.1.40) from rabbit muscle was purchased from Calbiochem Corp. and had a specific activity of 130 μmoles/min per mg at 25° as determined by the method of Tietz and Ochoa (1959). Enolase (phosphoenolpyruvic acid hydratase, EC 4.2.1.11) from rabbit muscle was also purchased from Calbiochem Corp. and had a specific activity of 15.0 μmoles/min per mg at 25° as determined by the method

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described by Westhead (1966). The other chemicals used as starting materials for the syntheses described below were all purchased from commercial sources in the highest purity obtainable.

Synthesis of Cyclohexylammonium Dihydrogen Phosphoenolpyruvate (**1**). Bromopyruvic acid (1.44 g, 8.6 mmoles, prepared as described previously; Stubbe and Kenyon, 1971) was dissolved in 25 ml of anhydrous ether, and this solution was added dropwise with vigorous stirring to trimethyl phosphite (1.30 g, 10.5 mmoles) at 0° over a period of 2 hr. The resulting solution was allowed to reach room temperature and left to stand for 12 hr. The ether and excess trimethyl phosphite were then removed at reduced pressure. A nuclear magnetic resonance spectrum of the product showed peaks at δ 3.90 (6 H, doublet, J_{POCH} = 11 Hz), 5.69 (1 H, apparent triplet, J = 3 Hz), 6.04 (1 H, apparent triplet, J = 3 Hz), consistent with the presence of dimethyl phosphoenolpyruvic acid. Other minor peaks appeared in the spectrum, but the product was used without further purification. Thus 25 ml of H_2O was added and the resulting homogeneous solution was left at room temperature for 3 hr. (In a pilot experiment performed in D_2O, both the methyl ester groups were shown by nuclear magnetic resonance spectroscopy to be hydrolyzed under these conditions, methanol appearing as the product.) Cyclohexylamine (1.08 g, 10.9 mmoles) was added, the solvent was removed at reduced pressure, and the product was recrystallized from methanol–ether to yield 1.44 g (63% yield based on bromopyruvic acid), mp 143–146° dec, lit. (Woods *et al.*, 1970) mp 144–146° dec. A mixture melting point with a sample prepared by the method of Clark and Kirby (1966) showed no depression.

Synthesis of Cyclohexylammonium Dihydrogen Phosphoenol-3-fluoropyruvate (**5**). For the synthesis bromofluoropyruvic acid was prepared by modifying the procedure of Bergmann and Shahak (1960). Thus diethyl oxalate, ethyl 2-fluoroacetate, and sodium ethoxide were treated using their procedure to give a 60% yield of the sodium salt of the enolate of diethyl fluorooxaloacetate, which was isolated as a white solid. This enolate salt was carefully acidified following the procedure of Nair and Busch (1958) to yield diethyl fluorooxaloacetate, bp 97–100° (1 mm), lit. (Nair and Busch, 1958) bp 98–100° (1.1 mm). The nuclear magnetic resonance spectrum ($CDCl_3$) showed peaks at δ 1.10 (3 H, triplet, J = 7 Hz), 1.13 (3 H, triplet, J = 7 Hz), 4.10 (2 H, quartet, J = 7 Hz), 4.20 (2 H, quartet, J = 7 Hz), 5.90 (1 H, doublet, J_{FCH} = 67 Hz). The diethyl fluorooxaloacetate (6.2 g, 30 mmoles) was dissolved in 15 ml of CCl_4 and 4.8 g (30 mmoles) of bromine was added dropwise with stirring. Some warming was necessary to initiate the reaction. The solvent was then removed at reduced pressure. The product, diethyl bromofluorooxaloacetate (3.8 g, 45% yield), was distilled at 86° (0.7 mm), lit. (Bergmann and Shahak, 1960) bp 105–107° (2.5 mm).

Anal. Calcd for $C_8H_{10}BrFO_5$: C, 33.68; H, 3.51; Br, 28.07. Found: C, 33.88; H, 3.57; Br, 27.82.

Alternatively, the sodium salt of the enolate form of the diethyl fluorooxaloacetate, isolated by filtration and washed with ether until colorless and then suspended in a large volume of benzene, may be brominated directly (Bergmann and Shahak, 1960).

The diethyl bromofluorooxaloacetate (28 g, 0.10 mole) was hydrolyzed by heating at 80° for 2 hr in concentrated HCl. (Bergmann and Shahak reported that this reaction proceeded to give a 35% yield of bromofluoropyruvic acid in 3 hr at room temperature. In our hands this reaction required much more vigorous conditions). After removal of the excess HCl *in vacuo*, the product was distilled at 82° (1 mm), lit. (Bergmann and Shahak, 1960) 90–92° (1.5 mm), to yield 10.1 g (54% yield) of bromofluoropyruvic acid which crystallized as a hydrate. The nuclear magnetic resonance spectrum (D_2O) showed a peak at δ 6.45 (doublet, J_{FCH} = 54 Hz).

For the preparation of dimethyl phosphoenol-3-fluoropyruvic acid a 2.3-g portion (12 mmoles) of bromofluoropyruvic acid in 25 ml of diethyl ether was added to 1.6 g (0.013 mole) of trimethyl phosphite at 0°. The solution was stirred for 3 hr while the solution warmed to room temperature. The solvent was removed at reduced pressure. A nuclear magnetic resonance spectrum (D_2O) showed peaks at δ 3.90 (6 H, doublet, J_{POCH} = 11 Hz), 7.76 (2 H, pair of doublets, J_{POCCH} = 3 Hz, J_{FCH} = 76 Hz), consistent with the presence of the Z isomer of dimethyl phosphoenol-3-fluoropyruvic acid. This product was dissolved in 25 ml of H_2O, and the progress of the hydrolysis of the methyl ester groups to form methanol and the dealkylated product was followed by nuclear magnetic resonance spectroscopy. After 8 hr at room temperature the hydrolysis was complete. Cyclohexylamine (1.38 g, 0.012 mole) was added, and the solvent was removed at reduced pressure. The resulting solid product was recrystallized from methanol–ether to give 0.82 g (15% yield) of cyclohexylammonium dihydrogen phosphoenol-3-fluoropyruvate (**5**), mp 139–141° dec.

Anal. Calcd for $C_9H_{17}FNO_6P$: C, 37.90; H, 5.98; N, 4.91; P, 10.53. Found: C, 37.68; H, 5.96; N, 5.10; P, 10.76.

In addition to the cyclohexyl peaks the nuclear magnetic resonance spectrum (D_2O) showed the vinyl proton peaks centered at δ 7.52 (1 H, pair of doublets, J_{POCCH} = 2.5 Hz, J_{FCH} = 72 Hz), and thus (see below) is presumably only the Z isomer.

For the synthesis of a mixture of the E and Z isomers of trimethyl phosphoenol-3-fluoropyruvate, methyl bromofluoropyruvate was prepared by treating the acid with diazomethane in ether–methanol as described by Bergmann and Shahak (1960) and reacted with trimethyl phosphite as described by these same authors. The product boiled at 110° (1 mm), lit. (Bergmann and Shahak, 1960) bp 135° (1.3 mm). The nuclear magnetic resonance spectrum ($CDCl_3$) showed that the product was an 80:20 mixture of the Z and E isomers. The predominant, Z isomer showed peaks at δ 3.63 (3 H, singlet), 3.73 (6 H, doublet, J_{POCH} = 11 Hz), 7.56 (1 H, pair of doublets, J_{POCCH} = 2.7 Hz, J_{FCH} = 72 Hz). The minor, E isomer showed peaks at δ 3.78 (3 H, singlet), 3.78 (6 H, doublet, J_{POCH} = 11 Hz), 7.48 (1 H, pair of doublets, J_{POCCH} = 3.5 Hz, J_{FCH} = 72 Hz).

A portion of the cyclohexylammonium dihydrogen phosphoenol-3-fluoropyruvate (**5**) was dissolved in the minimum amount of water and passed through a Dowex 50 (H^+ form) ion-exchange resin column (23 × 1.5 cm). After removal of the water, the free acid was recovered quantitatively. The free acid was dissolved in ether–methanol and treated with diazomethane until its yellow color no longer faded. After the solvent was removed, the (*Z*)-trimethyl phosphoenol-3-fluoropyruvate, free of detectable E isomer, remained, identified by its nuclear magnetic resonance spectrum in $CDCl_3$.

Synthesis of the Dicyclohexylammonium Salt of Ethyl α-(Dihydroxyphosphinyloxy)acrylate (Phosphoenolpyruvate Carboxyl Ethyl Ester, **6**). Tribenzyl phosphite was prepared from PCl_3, dimethylaniline, and benzyl alcohol by the method of Cramer and Voges (1958). To this phosphite was added

with cooling in an ice bath 1 equiv of ethyl bromopyruvate dissolved in a small amount of dry ether. The reaction mixture was stirred at room temperature for 1 hr and the solvent was removed at reduced pressure. The nuclear magnetic resonance spectrum was consistent with the structure for ethyl α-(dibenzyloxyphosphinyloxy)acrylate, showing peaks at δ 1.26 (3 H, triplet, J = 7 Hz), 4.01 (2 H, quartet, J = 7 Hz), 5.00 (4 H, doublet, J = 8 Hz), 5.33 (1 H, apparent triplet), 5.86 (1 H, apparent triplet), 7.17 (10 H, singlet).[1] The ethyl α-(dibenzyloxyphosphinyloxy)acrylate (0.39 g, 1 mmole) was dissolved in a mixture of 5 ml of CH_3OH and 5 ml of H_2O, and 0.039 g of 5% palladium on charcoal catalyst was added. After 8 hr of hydrogenation at atmospheric pressure the appropriate amount of H_2 had been absorbed. The solution was filtered to remove the catalyst, 2 mmoles of cyclohexylamine in 10 ml of H_2O was added, and the solvent was removed *in vacuo*. The residue was recrystallized from CH_3OH–Et_2O to give 0.128 g (32% yield) of the dicyclohexylammonium salt of ethyl α-(dihydroxyphosphinyloxy)acrylate (**6**), mp 184–185° dec, lit. (Benkovic and Schray, 1968) mp 170.6–171.5°.

Anal. Calcd for $C_{17}H_{35}N_2O_6P$: C, 51.78; H, 8.88; N, 7.11. Found: C, 51.47; H, 8.81; N, 7.29.

In addition to the cyclohexyl peaks the nuclear magnetic resonance spectrum (D_2O) showed peaks at δ 1.37 (3 H, triplet, J = 7 Hz), 4.27 (2 H, quartet, J = 7 Hz), 5.40 (1 H, apparent triplet), 5.78 (1 H, apparent triplet).

Synthesis of the Dicyclohexylammonium Salt of α-(Dihydroxyphosphinyloxy)acrylamide (Phosphoenolpyruvate Carboxylamide, **7**). Glycidamide was prepared from acrylonitrile and aqueous H_2O_2 at pH 7.5 by the method of Payne and Williams (1961). The nuclear magnetic resonance spectrum (D_2O) showed peaks at δ 3.00 (2 H, complex multiplet) and 3.53 (1 H, complex multiplet). The glycidamide (33 g, 0.5 mole) was added dropwise to 55 ml of 48% HBr in an ice bath. The color of the solution turned from light yellow to orange during this addition. After addition was complete, the solution was stirred in the ice bath for an additional 1 hr. The solution was then neutralized to pH 7 with solid Na_2CO_3 and extracted with three 250-ml portions of ether. Finally, the aqueous layer was extracted continuously for an additional 8 hr with 250 ml more of ether. The ether extracts were combined and dried over anhydrous $MgSO_4$, and the solvent was removed at reduced pressure to leave 10.7 g (17% yield) of α-hydroxy-β-bromopropionamide, mp 94–96°.

Anal. Calcd for $C_3H_6BrNO_2$: C, 21.43; H, 3.57; N, 8.33. Found: C, 21.35; H, 3.71; N, 8.39.

In addition to the OH and NH peaks the nuclear magnetic resonance spectrum ($CDCl_3$) showed peaks at δ 3.68 (2 H, doublet, J = 4 Hz) and 4.47 (1 H, triplet, J = 4 Hz).

The α-hydroxy-β-bromopropionamide (1.0 g, 6 mmoles) was dissolved in 100 ml of acetone and 1 molar equivalent of Jones' reagent (CrO_3 in dilute H_2SO_4) was added with cooling in an ice bath. The reaction occurred spontaneously, and the mixture was stirred at 0° for 10 min. After removal of the solvents *in vacuo*, 50 ml of $CHCl_3$ was added to the residue, and the slurry was warmed on a steam bath and filtered while hot. The $CHCl_3$ was then removed at reduced pressure to leave 0.80 g (80% yield) of bromopyruvamide, mp 80–83°.[2]

Anal. Calcd for $C_3H_4BrNO_2$: C, 21.69; H, 2.41; N, 8.43. Found: C, 21.82; H, 2.57; N, 8.46.

The nuclear magnetic resonance spectrum ($CDCl_3$) showed peaks at δ 4.43 (2 H, singlet) and 6.57 (2 H, broad hump).

Tribenzyl phosphite (6.6 g, 16 mmoles), freshly prepared as described above, was dissolved in 100 ml of dry ether, and the solution was added dropwise under N_2 to a solution of 2.65 g (16 mmoles) of bromopyruvamide in 50 ml of dry ether. The solution was stirred at room temperature for 1 hr and the solvent was removed at reduced pressure. An examination of the crude product mixture by nuclear magnetic resonance spectroscopy showed a spectrum consistent with the presence of three components: benzyl bromide, tribenzyl phosphate (presumably from air oxidation of tribenzyl phosphite), and the desired product, α-(dibenzyloxyphosphinyloxy)-acrylamide. Purification was accomplished by chromatography on a column (2 × 38 cm) of silica gel G (J. T. Baker Chemical Co.). Thus, 0.73 g of the crude mixture was placed on the column, and the column was eluted successively with 200 ml of CH_2Cl_2, 200 ml of 2% (v/v) acetone in CH_2Cl_2, 200 ml of 5% (v/v) acetone in CH_2Cl_2, 200 ml of 10% (v/v) acetone in CH_2Cl_2, and 200 ml of 50% (v/v) acetone in CH_2Cl_2. Fractions of 50 ml each were collected. The first 200 ml contained the benzyl bromide and the next 650 ml contained the tribenzyl phosphate. The desired product emerged with the last 150 ml of eluent. After removal of the solvents at reduced pressure, the product remained as an analytically pure solid, mp 48–50° (0.35 g). Later, a 3.0-g portion of the crude three component product mixture described above was dissolved in 10 ml of dry ether and left at 4° overnight. White needles (1.35 g) of the α-(dibenzyloxyphosphinyloxy)-acrylamide crystallized from the solution, mp 48–50°. Thus, the total yield was 1.70 g or 31% of the calculated amount.

Anal. Calcd for $C_{17}H_{18}NO_5P$: C, 58.79; H, 5.18; N, 4.03. Found: C, 58.59; H, 5.34; N, 3.93.

The nuclear magnetic resonance spectrum (CCl_4) showed peaks at δ 5.03 (4 H, doublet, J_{POCH} = 9 Hz), 5.26 (1 H, apparent triplet), 5.76 (1 H, apparent triplet), 7.23 (10 H, singlet).

The α-(dibenzyloxyphosphinyloxy)acrylamide (0.347 g, 1 mmole) was dissolved in a mixture of 5 ml of H_2O and 5 ml of CH_3OH and 0.035 g of 5% palladium on charcoal hydrogenation catalyst was added. After 3.5 hr of hydrogenation at atmospheric pressure, 2 equiv of H_2 had been absorbed. The solution was then separated from the catalyst by filtration, and the solvent was removed *in vacuo*. Cyclohexylamine (0.198 g, 2 mmoles) in 4 ml of H_2O was added, and the solvent was again removed *in vacuo* to leave a white solid. After recrystallization from methanol–ether, 0.20 g (54% yield) of the dicyclohexylammonium salt of α-(dihydroxyphosphinyloxy)acrylamide (**7**), mp 184–185° dec, was obtained.

Anal. Calcd for $C_{15}H_{32}N_3O_5P$: C, 49.18; H, 8.76; N, 11.24. Found: C, 48.96; H, 8.94; N, 10.98.

In addition to the broad cyclohexyl peaks the nuclear magnetic resonance spectrum (D_2O) showed peaks at δ 5.03 (1 H, apparent triplet), 5.65 (1 H, apparent triplet).

Synthesis of α-(Dihydroxyphosphinylmethyl)acrylic acid (**8**). Diethyl α,α-bis(hydroxymethyl)malonate (140 g, 0.43 mole), prepared from diethyl malonate and formaldehyde as described by Welch (1929), was heated with 500 ml of 48% HBr as described by Ferris (1955) to give 63 g of a mixture of α-(bromomethyl)acrylic acid and α,α-bis(bromomethyl)acetic acid. This mixture was not resolved into its components but was converted in approximately 38% yield by Fisher esterification to the corresponding mixture of ethyl α-(bromomethyl)-

[1] This nuclear magnetic resonance spectrum is in good agreement with that reported for this compound by Benkovic and Schray (1971).

[2] We are indebted to Mr. John Ellis for originally suggesting this synthetic approach to bromopyruvamide.

acrylate and ethyl α,α-bis(bromomethyl)acetate (Ferris, 1955).

A 22.5-g portion of this mixture of ethyl esters, containing an estimated total (ratio of esters determined by nuclear magnetic resonance spectroscopy) of 0.08 mole of material, was dissolved in 100 ml of benzene. The solution was heated to reflux and 19 g (0.16 mole) of trimethyl phosphite was added with stirring. After heating the solution at reflux for 2 hr, the solvent was removed at reduced pressure. The product, ethyl α-(dimethoxyphosphinylmethyl)acrylate, was distilled at 103–105° (1 mm). The yield was 12.5 g (71%). The nuclear magnetic resonance spectrum (neat) showed peaks at δ 1.05 (3 H, triplet, J = 7 Hz), 2.73 (2 H, doublet, J = 22 Hz), 3.73 (6 H, doublet, J = 11 Hz), 3.97 (2 H, quartet, J = 7 Hz), 5.60 (1 H, broadened doublet, J = 6 Hz), 5.98 (1 H, broadened doublet, J = 6 Hz).

A 12.5-g (56 mmoles) portion of the ethyl α-(dimethoxyphosphinylmethyl)acrylate was heated at reflux in 20 ml of 48% HBr for 1.5 hr. After removal of the solvent, a white solid crystallized from the remaining oil. The yield was 1.8 g (19%) of the α-(dihydroxyphosphinylmethyl)acrylic acid (**8**), mp 118–120°.

Anal. Calcd for $C_4H_7O_5P$: C, 28.91; H, 4.22; P, 18.67. Found: C, 28.78; H, 4.21; P, 18.65.

The nuclear magnetic resonance spectrum (D_2O) showed peaks at δ 3.00 (2 H, doublet, J = 21 Hz), 5.93 (1 H, doublet, J = 6 Hz), 6.40 (1 H, doublet, J = 6 Hz).

Synthesis of the Dicyclohexylammonium Salt of (RS)-α-(Dihydroxyphosphinylmethyl)propionic Acid (**9**). α-(Dihydroxyphosphinylmethyl)acrylic acid (**8**, 0.100 g, 0.624 mmole) was dissolved in 10 ml of methanol and 20 mg of PtO_2 catalyst was added. The solution was subjected to hydrogenation at atmospheric pressure, and 1 molar equivalent of H_2 was consumed in 20 min. The catalyst was removed by filtration and the solvent was removed at reduced pressure. Cyclohexylamine (0.059 g) was added to an aqueous solution of the product. After removal of the solvent *in vacuo*, the product was recrystallized from methanol–ether to give 0.127 g (28% yield) of the dicyclohexylammonium salt of (*RS*)-α-(dihydroxyphosphinylmethyl)propionic acid (**9**), mp 174–176°.

Anal. Calcd for $C_{16}H_{35}N_2O_5P \cdot H_2O$: C, 50.00; H, 9.63; N, 7.29. Found: C, 49.98; H, 9.57; N, 7.75.

The nuclear magnetic resonance spectrum of the free acid (D_2O) showed peaks at δ 1.27 (3 H, doublet, J = 7 Hz), 1.97 (2 H, multiplet), 2.73 (1 H, multiplet).

Experimental Section

Enzymatic Assay Procedures. Activities of the phosphoenolpyruvate analogs in the pyruvate kinase reaction were measured using the coupled-enzyme procedure with excess lactic dehydrogenase as described previously (Stubbe and Kenyon, 1971). Grassetti *et al.* (1966) and Eisman *et al.* (1965) have shown previously that fluoropyruvic acid is an excellent substrate for lactic dehydrogenase from rabbit muscle. This result was confirmed using our enzyme preparation. Ethyl pyruvate was also shown to be a substrate for this enzyme. Pyruvamide, the potential product from the reactivity of α-(dihydroxyphosphinyloxy)acrylamide (**7**) in the pyruvate kinase reaction, was not detectably reactive in the lactic dehydrogenase system. Since **7** was shown to be inactive as a substrate by polyethylenimine-cellulose thin-layer chromatography (Stubbe and Kenyon, 1971), however, no kinetic assay procedure was necessary. The observed values of V_{max} and K_m for the (*Z*)-phosphoenol-3-fluoropyruvate (**5**) in the pyruvate kinase reaction are shown in Table I. Neither α-(-dihydroxyphosphinyloxy)acrylamide (**7**), ethyl α-(dihydroxyphosphinyloxy)acrylate (**6**), α-(dihydroxyphosphinylmethyl)acrylic acid (**8**), nor (*RS*)-α-(dihyroxyphosphinylmethyl)propionic acid (**9**) showed any inhibitory effects on the normal rate of phosphoenolpyruvate reactivity as a substrate in the pyruvate kinase reaction even at 1×10^{-2} M using 1×10^{-5} M phosphoenolpyruvate, 1.7×10^{-3} M ADP, and $\sim 10^{-6}$ M enzyme. In an additional set of experiments potential inhibition by **6**, **7**, and **8** at these same concentration levels relative to phosphoenolpyruvate and ADP was reinvestigated using pyruvate kinase of higher specific activity (obtained from Miles Laboratories; specific activity 206 μmoles/min per mg at 25°), using lactic dehydrogenase which was free of ammonium sulfate and using $MgCl_2$ instead of $MgSO_4$. Once again no inhibitory effects on the normal rate of phosphoenolpyruvate reactivity as a substrate were observed.

In a control experiment the usual pyruvate kinase reaction conditions were employed using (*Z*)-phosphoenol-3-fluoropyruvate (**5**) as substrate except that the ADP was deleted. No decrease in absorption at 340 nm of the NADH was observed even after 1 hr. When ADP was added, decrease in absorption commenced immediately. This experiment showed that no phosphatase was present in the enzyme preparations used which would be capable of converting **5** to fluoropyruvate.

Assays of activities of phosphoenolpyruvate and the phosphoenolpyruvate analogs in the enolase reaction were followed spectrophotometrically by the procedure of Warburg and Christian as described by Westhead (1966), *i.e.*, one follows the decrease in absorbance of the chromophore at or near the λ_{max} of the particular enol phosphate (or analog) being tested. A list of the relevant ultraviolet spectral data for phosphoenolpyruvate and the phosphoenolpyruvate analogs investigated in the enolase reaction is given in Table II. A typical assay solution contained 0.05 M imidazole-HCl buffer (pH 6.8), 0.4 M KCl, 1×10^{-3} M $MgSO_4$, 1×10^{-5} M (ethylenedinitrilo)-tetraacetic acid, and 1×10^{-3} M phosphoenolpyruvate or phosphoenolpyruvate analog in a total volume of 3 ml. The enolase concentration was typically 1×10^{-6} M. Observed values of V_{max} and K_m for phosphoenolpyruvate and the reactive phosphoenolpyruvate analogs in the enolase reaction are shown in Table III. Since some of the phosphoenolpyruvate analogs (including phosphoenol-3-fluoropyruvate) slowly hydrolyze spontaneously in the absence of enzyme (Stubbe and Kenyon, 1971), blanks were run in the absence of enzyme and the results shown in Table III are corrected for the small decrease in absorption which was observed. Consistent with this observation, a small amount of inorganic phosphate was detected as a product by polyethylenimine-cellulose thin-layer chromatography with phosphoenol-3-fluoropyruvate (**5**) as substrate.

With (*Z*)-phosphoenol-3-fluoropyruvate (**5**) we consistently noticed an increase in absorption at 240 nm in the presence of enolase which preceded the decrease in absorption due to the enzymatic reaction (the wavelength maximum at 228 nm did not shift, however). This phenomenon was found to be concentration dependent; for example, with $\sim 10^{-6}$ M enolase and 2.5×10^{-3} M substrate the absorption increased by *ca.* 2% and 8 min was required to reach this maximum; with 1×10^{-4} M substrate the absorption increased by *ca.* 8% and 4 min was required; with 2×10^{-5} M substrate the maximum was reached in only 1 min and a *ca.* 7% increase was observed. Neither phosphoenolpyruvate itself nor α-(dihydroxyphosphinylmethyl)acrylate (**8**) showed this behavior. No obvious explanation has been found for this phenomenon.

TABLE I: Activity of Pyruvate Kinase Upon Some Phosphoenolpyruvate Analogs.

Phosphoenolpyruvate Analog Tested[a]	V_{max} [μmoles/(min mg)][b]	% Rel Rate (Phosphoenolpyruvate = 100%)	K_m[b] (M × 10^5)
Phosphoenolpyruvate (**1**)	130	(100)	2.6[c]
(*Z*)-Phosphoenol-3-fluoropyruvate (**5**)	0.33	0.23	40
Ethyl α-(dihydroxyphosphinyloxy)-acrylate (**6**)	No detectable reaction		
α-(Dihydroxyphosphinyloxy)acrylamide (**7**)			

[a] Measured using a coupled assay procedure with excess lactic dehydrogenase (see text). [b] The average of several determinations. [c] Nowak and Mildvan (1970).

TABLE II: Ultraviolet Spectral Data for Phosphoenolpyruvate and Some Phosphoenolpyruvate Analogs.

Compound	λ_{max}[a] (nm)	ε
Phosphoenolpyruvate (**1**)	232	3.02 × 10^3 (ε 2.91 × 10^3 at 230 nm)[b]
(*Z*)-Phosphoenol-α-ketobutyrate (**2**)	231	2.82 × 10^3
(*Z*)-Phosphoenol-3-bromopyruvate (**3**)	231	5.33 × 10^2
Phosphoenol-3-phenylpyruvate[c] (**4**)	229[d]	1.10 × 10^4
(*Z*)-Phosphoenol-3-fluoropyruvate (**5**)	228	1.37 × 10^3 (ε 1.28 × 10^3 at 240 nm)
Ethyl α-(dihydroxyphosphinyloxy) acrylate (**6**)	230	5.00 × 10^3 (ε 3.09 × 10^3 at 240 nm)
α-(Dihydroxyphosphinyloxy)acrylamide (**7**)	228	3.11 × 10^3
α-(Dihydroxyphosphinylmethyl)acrylate (**8**)	230.5	9.91 × 10^2 (ε 5.52 × 10^2 at 240 nm)

[a] Measured in 0.05 M imidazole-HCl buffer (pH 6.8) which was 0.4 M in KCl, 1 × 10^{-3} M in $MgSO_4$, and 1 × 10^{-5} M in (ethylenedinitrilo)tetraacetic acid. [b] Wold and Ballou (1957); measured in 0.05 M phosphate buffer (pH 7.0). [c] The stereochemistry of this analog was not established. [d] This product has another maximum at 274 nm (ε 1.12 × 10^5).

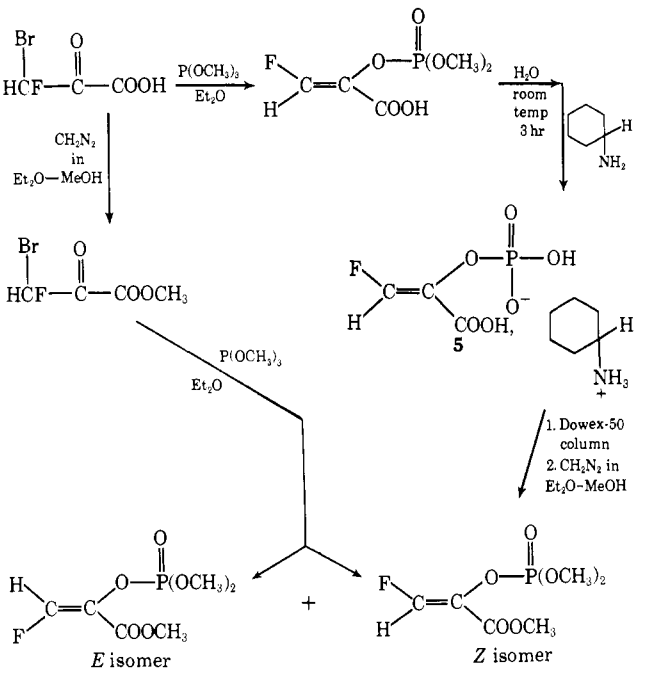
TABLE III: Activity of Enolase Upon Some Phosphoenolpyruvate Analogs.

Phosphoenolpyruvate Analog Tested[a]	V_{max}[b] [μmoles/(min mg)]	% Rel Rate (Phosphoenolpyruvate = 100%)	K_m[b] (M × 10^5)
Phosphoenolpyruvate (**1**)	15	(100)	9.2[c]
(*Z*)-Phosphoenol-3-fluoropyruvate (**5**)	0.14	0.9	2.0
α-(Dihydroxyphosphinylmethyl)acrylate (**8**)	0.24	1.6	25
(*Z*)-Phosphoenol-α-ketobutyrate (**2**)	No detectable reaction		
(*Z*)-Phosphoenol-3-bromopyruvate (**3**)			
Phosphoenol-3-phenylpyruvate (**4**)			
Ethyl α-(dihydroxyphosphinyloxy)acrylate (**6**)			
α-(Dihydroxyphosphinyloxy)acrylamide (**7**)			

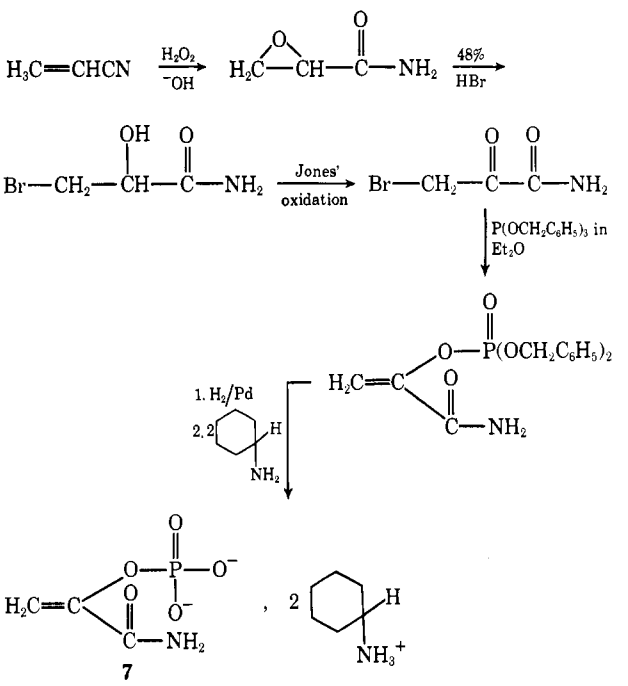
[a] Measured spectrophotometrically (see text). [b] The average of several determinations. [c] Czok and Bücher (1960).

Product Studies Using Polyethylenimine-Cellulose Thin-layer Chromatography. As the pyruvate kinase reaction with (*Z*)-phosphoenol-3-fluoropyruvate as substrate proceeded, samples of the reaction mixture were spotted on polyethylenimine-cellulose thin-layer chromatograms, and were developed and visualized as described previously (Stubbe and Kenyon, 1971). The observed R_F value for (*Z*)-phosphoenol-3-fluoropyruvate (**5**) using 1.2 N LiCl as eluent was 0.60. As observed for phosphoenolpyruvate and the other reactive phosphoenolpyruvate analogs (Stubbe and Kenyon, 1971), when (*Z*)-phosphoenol-3-fluoropyruvate (**5**) was used as a substrate, concomitant decreases in the intensities of the spots for this analog and that for ADP were observed to accompany an increase in the intensity of the spot for ATP. In the absence of active

SCHEME I



SCHEME II



enzyme none of these changes were observed. Also, neither α-(dihydroxyphosphinyloxy)acrylamide (**7**) nor ethyl α-(dihydroxyphosphinyloxy)acrylate (**6**) displayed this behavior.

The progress of the enolase reactions were also followed by polyethylenimine thin-layer chromatography. When phosphoenolpyruvate or (*Z*)-phosphoenol-3-fluoropyruvate (**5**) was used as the substrate, 0.8 N NaCl was used as the eluent. The observed R_F values for phosphoenolpyruvate, (*Z*)-phosphoenol-3-fluoropyruvate (**5**), 2-phosphoglyceric acid, and the product from the enolase reaction with **5** (see Discussion) were 0.36, 0.35, 0.48, and 0.58, respectively. When α-(dihydroxyphosphinylmethyl)acrylate (**8**) was used as a substrate in the enolase reaction, 0.5 N NaCl was used as the eluent. Using the usual molybdate spray the α-(dihydroxyphosphinylmethyl)-acrylate (**8**) was visualized as a red-blue spot[3] (R_F 0.82), and the product, presumably β-hydroxy-α-(dihydroxyphosphinylmethyl)propionate, was visualized as a blue-green spot[3] (R_F 0.57). Usually two chromatograms had to be prepared for each sample taken for these latter investigations since **8** had to be spotted at about 5 times the concentration of its hydrated product in order to be easily detected.

For phosphoenolpyruvate and the two reactive analogs a decrease in intensity of the spot for phosphoenolpyruvate (or phosphoenolpyruvate analog) was observed to be accompanied by a concomitant increase in the intensity of the spot corresponding to the product. In the absence of active enzyme no such behavior was observed.

Stereochemical Assignments for the E and Z Isomers of Phosphoenol-3-fluoropyruvate. The assignment of the *Z* configuration to the isomer isolated from the synthesis of phosphoenol-3-fluoropyruvate parallels the similar assignments made for phosphoenol-α-ketobutyrate (**2**) and phosphoenol-3-bromopyruvate (**3**) (Stubbe and Kenyon, 1971). As in the case of the other pairs of isomers (Stubbe and Kenyon, 1971) the (*Z*)-trimethylphosphoenol-3-fluoropyruvate vinyl proton absorbed at a lower field chemical shift (δ 7.56) than the *E* isomer (δ 7.48) and also had a smaller $^1HCCO^{31}P$ coupling constant (J = 2.7 Hz) than the *E* isomer (J = 3.5 Hz). Scheme I shows the chemical transformations that were performed to demonstrate the stereochemical relationships among the esterified and nonesterified phosphoenol-3-fluoropyruvates.

Chemical Syntheses. Synthetic approaches to the phosphoenol-3-fluoropyruvates, the α-(dihydroxyphosphinyloxy)-acrylamide (**7**) and the α-(dihydroxyphosphinylmethyl)acrylic acid (**8**) are shown in Schemes I, II, and III, respectively.

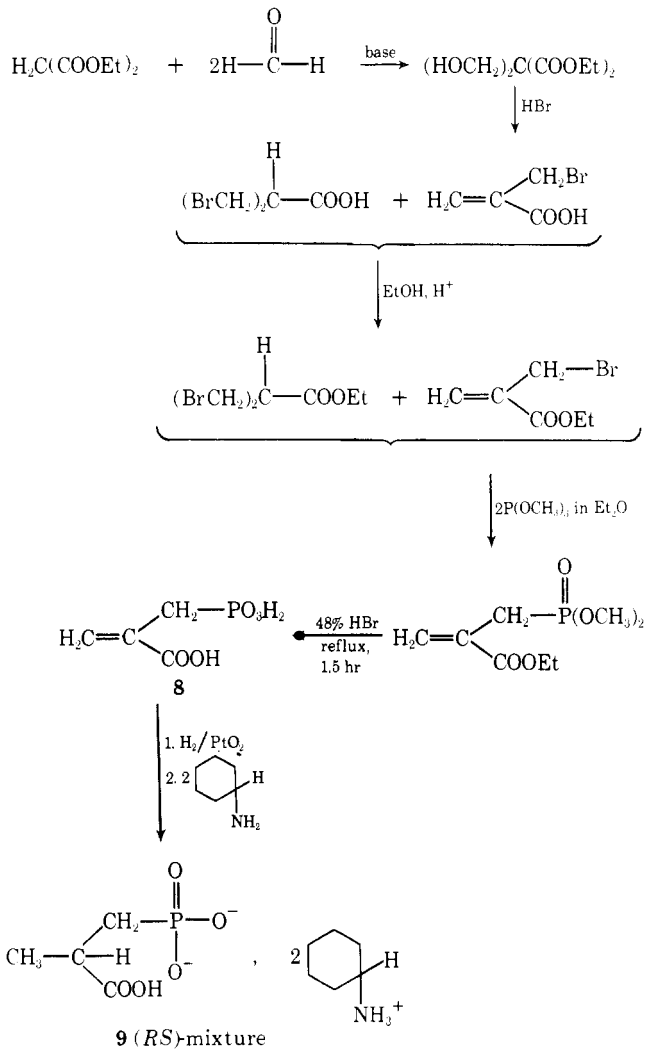
Discussion

The discovery that dimethyl phosphoenol-3-bromopyruvic acid undergoes spontaneous hydrolysis of both methyl ester groups in water in a few hours at room temperature to give a 73% isolated yield of phosphoenol-3-bromopyruvic acid (**3**) (Stubbe and Kenyon, 1971) has led us to extend this rather remarkable reaction to the syntheses of the free acids of other enol phosphates, including the generation of phosphoenolpyruvate itself from its dimethyl ester and the generation of phosphoenol-3-fluoropyruvic acid (**5**) from its dimethyl ester. Using this new procedure the pure cyclohexylammonium dihydrogen phosphoenolpyruvate (**1**) can be prepared from bromopyruvic acid and trimethyl phosphite in a matter of hours rather than in days as previously described (Clark and Kirby, 1966). With the 3-fluoro and 3-bromo analogs, moreover, the procedure of Clark and Kirby (*i.e.*, adding 1 molar equivalent of cyclohexylamine to an aqueous solution of the dimethyl ester and waiting 3 days) failed in our hands to give any detectable desired nonesterified product.

The (*Z*)-phosphoenol-3-fluoropyruvate (**5**), although still a very slowly reacting substrate for pyruvate kinase, had a somewhat greater V_{max} than either the (*Z*)-phosphoenol-3-methylpyruvate (phosphoenol-α-ketobutyrate, **2**) or the (*Z*)-phosphoenol-3-bromopyruvate (**3**). This is consistent with the idea presented earlier (Sutbbe and Kenyon, 1971) that bulk toler-

[3] Other phosphonates have been visualized with molybdate spray; the spots observed are not necessarily blue (Trowbridge and Kenyon, 1970; Christensen *et al.*, 1969).

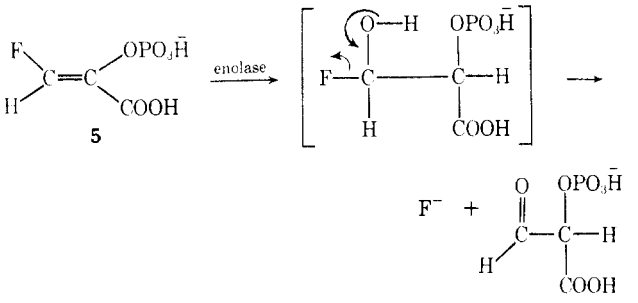
SCHEME III



ance by the enzyme for substituents on the 3 position of phosphoenolpyruvate is rather limited. Since a fluoro group is less bulky than either a methyl or a bromo substituent, it is not surprising that it reacts with a greater V_{max}. That neither α-(dihydroxyphosphinyloxy)acrylamide (**7**) nor the ethyl α-(dihydroxyphosphinyloxy)acrylate (**6**) are pseudosubstrates for pyruvate kinase nor are they very good competitive inhibitors with respect to phosphoenolpyruvate in the pyruvate kinase reaction indicates that the ionized carboxylate group is necessary for good binding and reactivity in the enzymatic reaction. Nowak and Mildvan (1971) have recently suggested that the ionized carboxylate may be bridging to the enzyme *via* the K^+ ion required for optimal activity. That the carboxylate anion binds to a specific site on the enzyme had been suggested earlier (see Mildvan *et al.*, 1967; Suelter, 1970). The negative result with phosphoenolpyruvate carboxylamide (**7**) is especially significant, we believe, since sterically it possesses a structure only slightly different from that of phosphoenolpyruvate itself. That the α-(dihydroxyphosphinylmethyl)acrylate (**8**) is not very inhibitory toward pyruvate kinase provides yet another example where replacement of an oxygen bonded to a phosphorus by a methylene group in a phosphate ester of a normal substrate can dramatically decrease the enzyme's affinity for the analog (see Larsen *et al.*, 1969).

This analog, α-(dihydroxyphosphinylmethyl)acrylate (**8**), however, does apparently act as a slowly reacting substrate in the enolase reaction. Although the presumed product of this reaction, β-hydroxy-α-(dihydroxyphosphinylmethyl)propionate, was not isolated and characterized, we were able to detect its formation by following its appearance using polyethylenimine-cellulose thin-layer chromatography; concomitantly, (**8**) was observed to disappear both by ultraviolet spectroscopic measurements and by polyethylenimine-cellulose thin-layer chromatography. To our knowledge the only other known pseudosubstrate in the rabbit muscle enolase reaction is D-erythronic acid 3-phosphate [HOOCCH(OH)CH(OPO_3H_2)CH_2OH] which was treated by Wold and Barker (1964) as an analog of 2-phosphoglycerate in the reverse reaction from the one being considered here. They followed the reaction by monitoring the increase in absorbance at 240 nm and reported a V_{max} equal to that for 2-phosphoglycerate itself.

The action of rabbit muscle enolase upon phosphoenol-3-fluoropyruvate (**5**) as a substrate is more complicated since both expected products, fluoride ion (Lohman and Meyerhof, 1934) and tartronic acid semialdehyde phosphate (Spring and Wold, 1970) are reported to be inhibitors of the rabbit muscle enolase



Again, in this case the phosphorylated product was not isolated and characterized, although evidence for its formation included the detection of a new spot, visualized by molybdate spray, by polyethylenimine-cellulose thin-layer chromatography.

None of the other phosphoenolpyruvate analogs substituted by more bulky groups on the 3 position (*e.g.*, 3-phenyl, 3-bromo, or 3-methyl) was detectably reactive as a substrate for the enolase reaction. That the (*Z*)-phosphoenol-α-ketobutyrate (**2**) was inactive is consistent with the earlier finding of Wold and Ballou (1957) that (2*R*,3*R*)-2,3-dihydroxybutyric acid 2-phosphate (D-*erythro*-2,3-dihydroxybutyric acid 2-phosphate) was inactive as a substrate in the rabbit muscle enolase reaction. They did find that this compound was a competitive inhibitor, however. Using the stereochemical results of Cohn *et al.* (1970) one can show that (Z)-phosphoenol-α-ketobutyrate (**2**) would be the expected product from the action of rabbit muscle enolase on the (2*R*,3*R*)-2,3-dihydroxybutyric acid 2-phosphate and *vice versa*.

That neither ethyl α-(dihydroxyphosphinyloxy)acrylate (**6**) nor α-(dihydroxyphosphinyloxy)acrylamide (**7**) react as substrates in the rabbit muscle enolase reaction is consistent with the predictions of Wold and Barker (1964) who felt that the ionized carboxylate group of phosphoenolpyruvate is necessary for activity with this enzyme.

Finally, the stereochemical assignments for the *E* and *Z* isomers of phosphoenol-3-fluoropyruvate appear to be on as firm a basis as the corresponding assignments for those of the isomers of phosphoenol-3-bromopyruvate (Stubbe and Kenyon, 1971). It is encouraging to note that for the 3-

methyl, 3-bromo, and 3-fluoro analogs the predominant product in the Perkow reaction of the β-halo-α-ketoacid with trimethyl phosphite consistently has its $|J_{^{31}POCCH}|$ coupling constant greater than that for the stereoisomer and that the vinyl proton of the predominant product always has a chemical shift at lower field than that for the stereoisomer.

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Formyltetrahydrofolate Synthetase. Substrate Binding to Monomeric Subunits*

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ABSTRACT: Formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum* has a tetrameric structure and contains four nucleotide and four folate binding sites. The enzyme can be dissociated into catalytically inactive monomers by dialysis to remove monovalent cations. Substrate binding experiments were performed with the monomeric enzyme. Monomers that can be reassociated and reactivated bind nucleotides with an affinity equivalent to native tetramer. Therefore, the nucleotide site is not altered by dissociation or association processes and is intrinsic to the monomeric subunit. However, the monomeric enzyme does not bind tetrahydroteroyl triglutamate even though the tetrameric form of the enzyme has a high binding affinity for this form of the coenzyme. This suggests that the folate binding site is created by the association of monomers to produce the active tetrameric enzyme. Alteration of the folate binding site during the dissociation process is sufficient to explain the catalytic inactivity of the monomeric enzyme.

Formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum* has a tetrameric structure and a molecular weight of 240,000 (Scott and Rabinowitz, 1967). MacKenzie and Rabinowitz (1971) have obtained evidence that the four subunits consist of identical or very nearly identical polypeptide chains. The enzyme behaves as a single species of 60,000 molecular weight during polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate and it gives a single band by isoelectric focusing in the presence of 8 M urea. Previously described substrate binding experiments indicate that there are four nucleotide and four folate binding sites per mole of tetrameric enzyme (Curthoys and Rabinowitz, 1971a,b). This is consistent with the evidence that the subunits are identical and its suggests that the binding sites for both substrates are intrinsic to the monomeric subunit.

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