Proc. Nat. Acad. Sci. U. S. 44, 140.
Konev, S. V. (1967), Fluorescence and Phosphorescence of Proteins and Nucleic Acids, New York, N. Y., Plenum Press, p 39.
Kurtin, W. E., and Song, P. S. (1968), *Photochem. Photobiol. 7*, 263.
Labeyrie, F., di Franco, A., Iwatsubo, M., and Baudras, A. (1967), *Biochemistry 6*, 1791.
Longworth, J. W. (1966), *Biopolymers 4*, 1131.
Mével-Ninio, M., Pajot, P., and Labeyrie, F. (1971), *Biochimie 53*, 35.
Monteilhet, C., and Risler, J. L. (1970), *Eur. J. Biochem. 12*, 165.
Nag-Chaudhuri, J., and Augenstein, L. (1964), *Biopolymers Symp. 1*, 441.
Pajot, P., and Groudinsky, O. (1970), *Eur. J. Biochem. 12*, 158.
Spyridakis, A., Naslin, L., and Labeyrie, F. (1971), *Biochimie 53* (in press).
Steiner, R. F., and Kolinsky, R. (1968), *Biochemistry 7*, 1014.
Stryer, L. (1960), *Radiat. Res., Suppl. 2*, 432.
Symons, R. H. (1965), *Biochim. Biophys. Acta 103*, 298.
Truong, T., Bersohn, R., Brumer, P., Luk, C. K., and Tao, T. (1967), *J. Biol. Chem. 242*, 2979.
Weber, G., and Teale, F. J. W. (1959), *Discuss. Faraday Soc. 27*, 134.
Weber, G., and Teale, F. J. W. (1965), *Proteins 3*, 485.
Weinryb, I., and Steiner, R. F. (1968), *Biochemistry 7*, 2488.
Weinryb, I., and Steiner, R. F. (1970), *Biochemistry 9*, 135.

Analogs of Phosphoenolpyruvate. On the Specificity of Pyruvate Kinase from Rabbit Muscle*

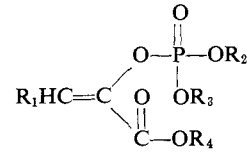
Jo Anne Stubbe† and George L. Kenyon‡

ABSTRACT: Several compounds with structures analogous to phosphoenolpyruvate were synthesized for the purpose of investigating the specificity of pyruvate kinase (EC 2.7.1.40). Contrary to the report of A. E. Woods *et al.* [*Biochemistry 9*, 2334 (1970)], it has been found that phosphoenol-α-ketobutyrate is a pseudosubstrate in the pyruvate kinase reaction. Phosphoenol-3-bromopyruvate was also shown to be a pseudosubstrate, but, within the limits of detection, phosphoenol-3-phenylpyruvate was inactive. The stereochemistries of the *E* and *Z* isomers of both phosphoenol-α-ketobutyrate and phosphoenol-3-bromopyruvate were assigned using nuclear magnetic resonance spectroscopy. (*Z*)-Phosphoenol-α-ketobutyrate has been shown to yield (3*R*)-[3-^{2}H]α-ketobutyrate stereospecifically when the enzymatic reaction was carried out in D_2O.

This corresponds to the addition of deuterium at C-3 on the 2-*si*, 3-*re* face of the (*Z*)-phosphoenol-α-ketobutyrate or its mechanistic equivalent in the enzyme-catalyzed reaction. This result confirms recent similar findings of Bondinell and Sprinson [*Biochem. Biophys. Res. Commun. 40*, 1464 (1970)].

In a recent paper Woods *et al.* (1970) reported the synthesis of several new homologs of phosphoenolpyruvate (PEP)[1] and presented evidence to show that none of these homologs, including phosphoenol-α-ketobutyrate, was active as a pseudosubstrate in the pyruvate kinase (EC 2.7.1.40) reaction. We have synthesized several analogs of PEP (**1**), including **2**–**9**.

We wish to present evidence to show that phosphoenol-α-ketobutyrate (**4**) is a relatively slowly reacting pseudosubstrate for pyruvate kinase, that phosphoenol-3-bromopyruvate (**7**) is even more reactive, but that no detectable reaction was observed with phosphoenol-3-phenylpyruvate (**9**) or the other analogs listed. We have also made stereochemical assignments for the *E* and *Z* isomers of both phosphoenol-α-ketobutyrate and phosphoenol-3-bromopyruvate using nmr spectroscopy and have shown that *Z*-phosphoenol-α-ketobutyrate gives (3*R*)-[3-^{2}H]α-ketobutyric acid stereospecifically[2] when the enzymatic conversion is carried out in D_2O. This confirms the stereochemical course for the protonation of PEP itself in the enzymatic conversion recently found by Rose (1970) using tritium–deuterium-labeling techniques.



1, R_1, R_2, R_3, R_4 = H
2, R_1, R_2 = H; R_3 = CH_3; R_4 = C_2H_5
3, R_1 = H; R_2, R_3 = CH_3; R_4 = C_2H_5
4, R_1 = CH_3; R_2, R_3, R_4 = H
5, R_1, R_2, R_3 = CH_3; R_4 = H
6, R_1, R_2, R_3, R_4 = CH_3
7, R_1 = Br; R_2, R_3, R_4 = H
8, R_1 = Br; R_2, R_3 = CH_3; R_4 = H
9, R_1 = C_6H_5; R_2, R_3, R_4 = H

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[1] Abbreviation used is: PEP, phosphoenolpyruvate.

[2] A high degree of stereoselectivity for this reaction has obviously not been established in this study since the specific rotation of our isolated sodium (*R*)-α-[^{2}H]propionate is considerably lower than the highest reported value (see Results). In light of the results of Rose (1970) and Bondinell and Sprinson (1970), we believe, however, that the reaction is a stereospecific one and that we partially racemized the asymmetric center during our oxidation and isolation procedures.

Recently, a communication by Bondinell and Sprinson (1970) has appeared which in part duplicates and confirms our work reported here on the phosphoenol-α-ketobutyrate.

Materials and Methods

Infrared spectra were measured on a Perkin-Elmer Infracord spectrometer (Model 237). Ultraviolet spectra were taken on either a Cary 14 or Gilford recording spectrophotometer. Proton nuclear magnetic resonance spectra were determined on either a Varian Model T-60 or HA-100 nuclear magnetic resonance spectrometer using tetramethylsilane as standard. The ^{13}C nuclear magnetic resonance spectrometer has been described previously (Sternlicht *et al.*, 1971). Melting points are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. High-resolution mass spectra were taken on a Consolidated Electrodynamics Corp. Model 21-110B spectrometer. Optical rotatory dispersion measurements were made using a Cary 60 spectropolarimeter.

Two batches of pyruvate kinase (adenosine triphosphate: pyruvic acid phosphotransferase, EC 2.7.1.40) from rabbit skeletal muscle were purchased from Calbiochem Corp. and had specific activities of 140 and 154 μmoles per min per mg, respectively, as determined by the method of Tietz and Ochoa (1959). Lactic acid dehydrogenase from rabbit muscle (EC 1.1.1.27) was also supplied by Calbiochem Corp. Luciferin was the product of Sigma Chemical Co. Firefly luciferase (Sigma Chemical Co.) was purified and recrystallized twice according to the procedure of McElroy (1963). Bovine serum albumin was also the product of Sigma Chemical Co.

α-Ketobutyric acid was prepared by the procedure of Moureu *et al.* (1952), mp 33–36°. Phenylpyruvic acid was prepared by the same procedure: mp 139–142°, lit. (Moureu *et al.*, 1952) mp 149°. Trimethylphosphite, pyruvic acid, and ethyl pyruvate were all supplied by Aldrich Chemical Co. Tris, phosphoenolpyruvic acid (K^+ salt), ADP, ATP, and NADH were the products of Calbiochem Corp. Ethyl bromopyruvate was prepared from ethyl pyruvate by the procedure of Kuhn and Drury (1949).

Bromination of α-Keto Acids. A simplified procedure for the preparation of both bromopyruvic acid and β-bromo-α-ketobutyric acid is as follows.

The acid, either neat or in CCl_4 solution, is treated with a slight excess of bromine (Sprinson and Chargaff, 1946; Woods *et al.*, 1970). Unlike Woods *et al.* (1970), we found that no special drying of the bromine was necessary. After bromine uptake has ceased, the solvent is removed *in vacuo*, and the oily product is dissolved in excess 1,2-dichloroethane which is then removed at reduced pressure. This procedure is repeated several times. The product, by this time free of HBr, crystallizes in the flask in nearly quantitative yield and may be conveniently recrystallized from $CHCl_3$ to which a drop or two of cyclohexene has been added (Clark and Kirby, 1966). The β-bromo-α-ketobutyric acid (Sprinson and Chargaff, 1946) obtained in this manner showed the following nuclear magnetic resonance spectrum ($CDCl_3$): δ 1.75 (3 H, doublet, J = 6 Hz) and 5.20 (1 H, quartet, J = 6 Hz).

3-Bromo-3-phenylpyruvic acid was best prepared by bromination in acetic acid. Thus phenylpyruvic acid (3.7 g) was reacted with bromine (3.5 g) in 15 ml of glacial acetic acid. After removal of the acetic acid and HBr at reduced pressure, the product was recrystallized from $CHCl_3$ to give 2.3 g (43% yield) of the bromoacid, mp 91–94°.

Anal. Calcd for $C_9H_7BrO_3$: C, 44.44; H, 3.13; Br, 32.92. Found: C, 44.19; H, 3.00; Br, 33.06.

The nuclear magnetic resonance spectrum (D_2O) showed peaks at δ 5.79 (1 H, singlet) and 6.85 (5 H, singlet).

3,3-Dibromopyruvic acid (mp 82–84°) was prepared by the method of Cooper and Owen (1966), who reported a melting point range of 82–86°. The nuclear magnetic resonance spectrum (D_2O) showed a peak at δ 6.10 (singlet).

Synthesis of Cyclohexylammonium Dihydrogen (Z)-Phosphoenol-α-ketobutyrate. β-Bromo-α-ketobutyric acid (0.80 g, 4.4 mmoles) was dissolved in 15 ml of anhydrous ether, and the resulting solution was added to trimethyl phosphite (0.76 g, 6.8 mmoles) in an ice bath. If this mixture were allowed to warm to room temperature, a white, crystalline product identified as (*Z*)-dimethylphosphoenol-α-ketobutyric acid (mp 84–86°) separated from the solution.

Anal. Calcd for $C_6H_{11}O_6P$: C, 34.28; H, 5.24; P, 14.76. Found: C, 33.99; H, 5.46; P, 14.52.

A nuclear magnetic resonance spectrum of this product (Me_2SO-d_6) showed peaks at δ 1.78 (3 H, quartet, $J_{HCH} \cong 8$ Hz, J_{POCCH} = 3 Hz), 3.76 (6 H, doublet, J_{POCH} = 11 Hz), and 6.56 (1 H, octet, $J_{HCH} \cong 8$ Hz, J_{POCCH} = 1.5 Hz). For the preparation of the desired nonesterified product, however, this intermediate was not isolated. The ether was removed and 0.43 g (4.4 mmoles) of cyclohexylamine in 20 ml of H_2O was added. The reaction mixture was allowed to stand at room temperature for 3 days and was purified as described by Clark and Kirby (1966). The yield of cyclohexylammonium dihydrogen (*Z*)-phosphoenol-α-ketobutyrate was 0.48 g (39%), mp 147–149°. For mixtures of the *E* and *Z* isomers of the same salt Woods *et al.* (1970) reported a melting point range of 139–140.5° and Bondinell and Sprinson (1970) reported a melting point range of 154–155°.

Anal. Calcd for $C_{10}H_{20}NO_6P$: C, 42.70; H, 7.17; P, 11.01. Found: C, 42.59; H, 7.16; P, 10.92.

The nuclear magnetic resonance spectrum (D_2O) showed peaks at δ 1.10–2.33 (broad multiplet), 1.96 (3 H, quartet, J_{HCH} = 7 Hz, J_{POCCH} = 3 Hz), and 6.63 (1 H, octet, J_{HCH} = 7 Hz, J_{POCCH} = 1.5 Hz).

Synthesis of (E)- and (Z)-Trimethyl Phosphoenol-α-ketobutyrate. Freshly distilled α-ketobutyric acid (4.3 g, 42 mmoles), anhydrous methanol (4.8 g, 150 mmoles), and 2 drops of ethanesulfonic acid were dissolved in 25 ml of 1,2-dichloroethane and heated at reflux for 18 hr (Clinton and Laskowski, 1948). The resulting solution was cooled and washed successively with 10-ml portions of water, dilute $NaHCO_3$, and water. After drying over anhydrous $MgSO_4$, the 1,2-dichloroethane layer was distilled to give 2.6 g (53% yield) of *methyl α-ketobutyrate*, bp 76° (20 mm). The nuclear magnetic resonance spectrum (CCl_4) showed peaks at δ 1.06 (3 H, triplet, J = 7 Hz), 2.70 (2 H, quartet, J = 7 Hz), and 3.70 (3 H, singlet). The methyl α-ketobutyrate (2.6 g, 22 mmoles) was dissolved in 10 ml of CCl_4 and a solution of 3.5 g (22 mmoles) of bromine in 15 ml of CCl_4 was added. The solvent was removed at reduced pressure, and the product was distilled at 72° (1 mm) to give 3.4 g (78% yield) of *methyl β-bromo-α-ketobutyrate*. The nuclear magnetic resonance spectrum (neat) showed peaks at δ 1.70 (3 H, doublet, J = 6 Hz), 3.73 (3 H, singlet), and 4.96 (1 H, quartet, J = 6 Hz). The methyl β-bromo-α-ketobutyrate (3.4 g, 17 mmoles) was dissolved in 10 ml of benzene, and the solution was cooled in an ice bath. This solution was then added to an ice-cold solution of 2.5 g (22 mmoles) of trimethyl phosphite in 10 ml of benzene. The addition required 15 min, and the mixture was stirred for an additional 30 min. After the benzene was removed *in vacuo*, the product

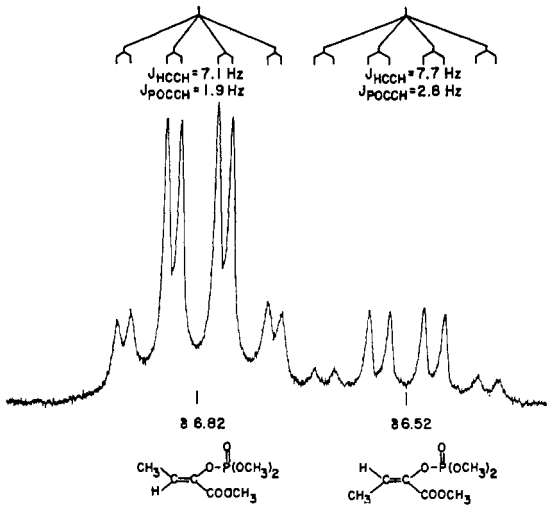


FIGURE 1: The proton nuclear magnetic resonance spectrum (neat) at 100 MHz showing the vinyl proton region of a mixture of 75% (*Z*)-trimethyl phosphoenol-α-ketobutyrate and 25% (*E*)-trimethyl phosphoenol-α-ketobutyrate.

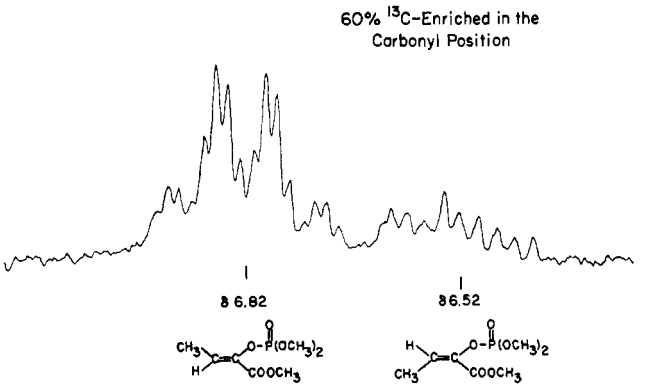


FIGURE 2: The proton nuclear magnetic resonance spectrum (CCl_4) at 100 MHz showing the vinyl proton region of a mixture of 75% (*Z*)-trimethyl phosphoenol-α-ketobutyrate and 25% (*E*)-trimethyl phosphoenol-α-ketobutyrate, both 60% ^{13}C enriched in the carbonyl position. This spectrum was obtained using a computer of average transients (CAT).

was distilled at 85° (1 mm) to give 3.0 g (77% yield) of the mixture of *E* and *Z* isomers to *trimethyl phosphoenol-α-ketobutyrate*. A portion of this liquid was analyzed by high-resolution mass spectrometry, and the parent ion was accurately mass measured.

Anal. Calcd for $C_7H_{13}O_6P$: 224.0450. Found: 224.0457.

The nuclear magnetic resonance spectrum (neat) showed that it was a 75:25 mixture of the two stereoisomers. The *Z* isomer (major component) showed peaks at δ 2.12 (3 H, quartet, J_{HCH} = 8 Hz, J_{POCCCH} = 3 Hz), 4.08 (3 H, singlet), 4.15 (6 H, doublet, J_{POCH} = 11 Hz), and 6.82 (1 H, octet, J_{HCH} = 8 Hz, J_{POCCH} = 2 Hz). The *E* isomer showed peaks at δ 2.32 (3 H, quartet, J_{HCH} = 8 Hz, J_{POCCH} = 3 Hz), 4.10 (3 H, singlet), 4.12 (6 H, doublet, J_{POCH} = 11 Hz), and 6.52 (1 H, octet, J_{HCH} = 8 Hz, J_{POCCH} = 2.5 Hz). The vinyl proton region of a nuclear magnetic resonance spectrum taken at 100 MHz is shown in Figure 1.

The pure (*Z*)-trimethyl phosphoenol-α-ketobutyrate was obtained from cyclohexylammonium dihydrogen (*Z*)-phosphoenol-α-ketobutyrate in the following way. The cyclohexylammonium salt (100 mg, 0.35 mmole) was passed through a Dowex 50 (H^+ form) ion-exchange resin column (23 × 1.5 cm) and, after removal of the water, the free triacid was recovered quantitatively. A portion of this acid was dissolved in 20 ml of anhydrous ether and 5 ml of dry methanol. Diazomethane was added until its yellow color no longer faded, and the solvents were removed to leave the pure (*Z*)-trimethyl phosphoenol-α-ketobutyrate, identified by its nuclear magnetic resonance spectrum in D_2O.

In another experiment a portion of (*Z*)-dimethyl phosphoenol-α-ketobutyric acid was also converted into (*Z*)-trimethyl phosphoenol-α-ketobutyrate by a similar reaction with diazomethane in ether.

For the synthesis of a sample of (*E*)- and (*Z*)-trimethyl phosphoenol-α-ketobutyrate which was 60% ^{13}C enriched in the carbonyl position the following modifications of the above described synthesis were employed. Potassium cyanide (2.0 g, Prochem Inc., 60 atom % ^{13}C) was converted to [^{13}C]CuCN (1.01 g, 75% yield) by the method of Supniewski and Salzberg (1941). The [^{13}C]CuCN was converted to [^{13}C]CH_3CH_2-(C=O)CN by reaction with propionyl bromide by a procedure analogous to the known synthesis of labeled pyruvonitrile (Calvin *et al.*, 1949). [1-^{13}C]α-Ketobutyric acid was obtained in 86% yield by heating the nitrile at reflux in concentrated HCl for 1 hr. The [1-^{13}C]α-ketobutyric acid was then esterified, brominated and reacted with trimethyl phosphite as described above. The nuclear magnetic resonance spectrum of the vinyl proton region at 100 MHz for the resulting mixture of these ^{13}C-enriched products is shown in Figure 2.

Synthesis of Cyclohexylammonium Dihydrogen Phosphoenol-3-phenylpyruvate. α-Bromo-α-phenylpyruvic acid (2.00 g, 8.2 mmoles) in 15 ml of ether was added dropwise to a solution of 1.12 g (9 mmoles) of trimethyl phosphite in 20 ml of ether over a period of 1.75 hr. The ether and excess trimethyl phosphite were then removed *in vacuo* and 0.89 g (9.0 mmoles) of cyclohexylamine in 25 ml of H_2O was added to the crude product mixture. After 3 days the solvent was removed under reduced pressure, and the product was recrystallized by the procedure for purifying cyclohexylammonium dihydrogen phosphoenolpyruvate described by Clark and Kirby (1966). The yield of crystalline product was 0.88 g (31%), mp 142–145° dec.

Anal. Calcd for $C_{15}H_{22}NO_6P$: C, 52.45; H, 6.35; N, 4.08. Found: C, 52.73; H, 6.30; N, 4.30.

The nuclear magnetic resonance spectrum (Me_2SO-d_6) showed peaks at δ 1.20–2.20 (11 H, multiplet), 6.78 (1 H, singlet), 7.23, and 7.40 (5 H, multiplets).

Synthesis of Dimethyl Phosphoenol-3-bromopyruvic Acid. α,α-Dibromopyruvic acid (5 g, 20 mmoles) was added all at once to 2.5 g (20 mmoles) of trimethyl phosphite in 10 ml of anhydrous ether in an ice bath. The solution was allowed to warm to room temperature and after about 3 hr a white solid crystallized from the solution. This product was removed by filtration, the filtrate was concentrated, and more product was obtained. The total yield was 3.2 g (58%) of dimethyl phosphoenol-3-bromopyruvic acid (mp 117–120°) which required no further purification.

Anal. Calcd for $C_5H_8BrO_6P$: C, 21.82, 2.91; Br, 29.09; P, 11.27. Found C, 22.04; H, 3.12; Br, 29.16; P, 11.15.

The nuclear magnetic resonance spectrum (D_2O) showed the presence of both the *Z* (major) isomer and *E* (minor) isomer. The *Z* isomer showed peaks at δ 3.64 (6 H, doublet, J_{POCH} = 11 Hz) and 7.27 (1 H, doublet, J_{POCCH} = 1.6 Hz). The *E* isomer showed peaks at δ 3.47 (6 H, doublet, J_{POCH} = 11 Hz) and 7.03 (1 H, doublet, J = 2.4 Hz).

TABLE I: Activity of Pyruvate Kinase upon PEP Analogs Substituted in the Vinyl (3) Position.

PEP Analog Tested[a]	Substituent in the 3 Position	V_{max} (μmoles/(min mg))	% Rel Rate (PEP = 100%)	K_m[b] (M × 10^5)
Phosphoenolpyruvate (PEP)	3-Hydrogen	154[c]	(100)	2.6[d]
(*Z*)-Phosphoenol-3-bromopyruvate	3-Bromo	0.30[b,e]	0.19	4.4
(*Z*)-Phosphoenol-α-ketobutyrate	3-Methyl	0.10[b]	0.065	2.5[f]
Phosphoenol-3-phenylpyruvate[g]	3-Phenyl	No detectable reaction[e]		

[a] A coupled assay procedure using excess lactic dehydrogenase was employed (see Methods). In each case the expected substituted pyruvic acid product was shown in a separate experiment to be a pseudosubstrate for the lactic dehydrogenase. [b] The average of several determinations. [c] Reynard *et al.* (1961) reported V_{max} = 260 for a highly purified pyruvate kinase preparation. [d] Nowak and Mildvan (1970). [e] Both the 3-bromo and 3-phenyl analogs hydrolyze under the reaction conditions in the absence of active enzyme to give bromopyruvic acid and phenylpyruvic acid, respectively. With the 3-bromo analog this reaction is relatively slow in the absence of the enzyme and did not interfere with our V_{max} or K_m value measurements. [f] Woods *et al.* (1970) reported a K_I value of 6.5×10^{-5}. [g] The stereochemistry of this analog was not established.

Synthesis of Cyclohexylammonium Dihydrogen Phosphoenol-3-bromopyruvate. Attempts to prepare this compound by the method described for the synthesis of the corresponding 3-methyl and 3-phenyl analogs failed. Accordingly, the following procedure was used. Dimethyl phosphoenol-3-bromopyruvic acid (0.4 g, mixture of *E* and *Z* isomers) was dissolved in 20 ml of H_2O, and the solution was left at room temperature for 0.5 hr. The solvent was then removed at 25° in a high vacuum to give crude phosphoenol-3-bromopyruvic acid. A nuclear magnetic resonance spectrum of this material indicated that hydrolysis of the phosphate ester groups was complete. A solution of 0.16 g of cyclohexylamine in 10 ml of H_2O was then added, and after several minutes the water was removed at 25° in a high vacuum. The solid residue was recrystallized from CH_3OH–ether to give 0.37 g (73% yield) of cyclohexylammonium dihydrogen phosphoenol-3-bromopyruvate, mp 125–127° dec.

Anal. Calcd for $C_9H_{17}BrNO_6P$: C, 31.21; H, 4.91; Br, 23.12; N, 4.04. Found: C, 31.40; H, 5.16; Br, 23.20; N, 3.99.

In addition to the broad cyclohexyl peaks the nuclear magnetic resonance spectrum (Me_2SO-d_6) showed a peak at δ 6.80 (1 H, doublet, J_{POCCH} = 2.2 Hz), and thus is presumably only the *Z* isomer.

Synthesis of Dimethyl 1-Carbethoxyvinylphosphate. Trimethyl phosphite (31.9 g, 0.257 mole) in 50 ml of benzene was added dropwise over a period of 2 hr to a solution of 50 g (0.257 mole) of ethyl bromopyruvate in 60 ml of benzene cooled in an ice bath. The solvent was removed *in vacuo* and the dimethyl 1-carbethoxyvinylphosphate was distilled at 99–102° (0.9 mm), lit. (Chopard *et al.*, 1965) bp 106–109° (0.5 mm). The yield was 40.1 g (70%). The nuclear magnetic resonance spectrum (neat) showed peaks at δ 1.18 (3 H, triplet, *J* = 7 Hz), 3.70 (6 H, doublet, *J* = 11 Hz), 4.10 (2 H, quartet, *J* = 7 Hz), 5.40 (1 H, apparent triplet, *J* = 3 Hz), and 5.75 (1 H, apparent triplet, *J* = 3 Hz).

Synthesis of S-Methylthiuronium Methyl 1-Carbethoxyvinylphosphate. Dimethyl 1-carbethoxyvinylphosphate (17 g, 76 mmoles) was dissolved in 130 ml of acetonitrile in a 250-ml flask. Thiourea (5.8 g, 76 mmoles) was added, and the solution was heated at reflux for 12 hr. The hot solution was filtered to remove unreacted thiourea and then allowed to cool. The product which precipitated was removed by filtration and recrystallized from fresh acetonitrile. The yield of *S*-methylthiuronium methyl 1-carbethoxyvinylphosphate was 13.2 g (58%), mp 81–82.5°. Further recrystallization did not raise the melting point.

Anal. Calcd for $C_8H_{17}N_2O_6PS$: C, 32.00; H, 5.67; P, 10.33; S, 10.67. Found: C, 31.86; H, 5.60; P, 10.39; S, 10.88.

The nuclear magnetic resonance spectrum showed peaks at δ 1.25 (3 H, triplet, *J* = 7 Hz), 2.70 (3 H, singlet), 3.55 (3 H, doublet, *J* = 11 Hz), 4.20 (2 H, quartet, *J* = 7 Hz), 5.40 (1 H, apparent triplet, *J* = 2 Hz), and 5.80 (1 H, apparent triplet, *J* = 2 Hz).

Experimental Section and Results

Coupled-Assay Procedure. Assays of activities of PEP analogs in the pyruvate kinase reaction were performed using the coupled-enzyme procedure of Mildvan and Leigh (1964) employing lactic dehydrogenase from rabbit muscle. In the amounts used in our assay method, this preparation was found to be free of detectable pyruvate kinase activity. The concentrations of the reactants were typically 1.7×10^{-3} M ADP, 3.4×10^{-3} M $MgSO_4$, 1×10^{-4} M NADH, 0.1 M KCl, 0.05 M Tris buffer (pH 7.5), and 2×10^{-3} M PEP or PEP analog in a total volume of 3 ml. The enzyme concentrations were 1×10^{-5} M lactic dehydrogenase and 1×10^{-9} M pyruvate kinase. The decrease in absorption of NADH at 340 nm was followed using a recording ultraviolet spectrophotometer. Meister (1950) has previously shown that lactic dehydrogenase from rabbit muscle can use α-ketobutyric acid and phenylpyruvic acid as pseudosubstrates. These results were confirmed with our enzyme preparation, and bromopyruvic acid was also shown to be a pseudosubstrate. A compilation of observed values of V_{max} and K_m for PEP and the reactive analogs as substrates in the coupled-enzyme reaction are shown in Table I.

In a control experiment the above reaction conditions were repeated with (*Z*)-phosphoenol-α-ketobutyrate as substrate except that the ADP was deleted and the enzyme concentrations were fourfold greater than usual. No change in the base line on the ultraviolet spectrophotometer was observed even after 1.5 hr. Upon addition of ADP, change in absorbance at

340 nm commenced immediately. This experiment indicated that neither enzyme preparation used contained detectable phosphatase activity capable of converting (*Z*)-phosphoenol-α-ketobutyrate into α-ketobutyrate.

Product Studies Using Polyethylenimine Cellulose Thin-Layer Chromatography. As the pyruvate kinase reaction proceeded, samples of the reaction mixture were spotted on polyethylenimine cellulose thin-layer plates (Randerath and Randerath, 1964). Ascending chromatography was used with 1.2 N LiCl or NaCl as the eluent, 20 min being the approximate elution time. The PEP (or PEP analog), ATP, and ADP spots were visualized using molybdate spray (Hanes and Isherwood, 1949). The ATP, ADP, and phosphoenol-3-phenylpyruvate spots were also visualized directly under ultraviolet light. All of the spots detected in a given experiment were cleanly separated from one another. The observed R_F values for PEP, (*Z*)-phosphoenol-α-ketobutyrate, (*Z*)-phosphoenol-3-bromopyruvate, phosphoenol-3-phenylpyruvate, ATP, and ADP were 0.53, 0.63, 0.59, 0.17, 0.12, and 0.41, respectively. Using freshly prepared plates, as little as 5×10^{-10} mole of these products could be detected.

When PEP, phosphoenol-α-ketobutyrate, or phosphoenol-3-bromopyruvate was used as substrate, concomitant decreases in the PEP (or PEP analog) and ADP spots were observed to accompany a corresponding increase in the intensity of the ATP spot. None of the other analogs **2**–**9** tested displayed this behavior. Also, in the absence of active pyruvate kinase, neither PEP nor the 3-methyl nor 3-bromo analog showed this behavior.

In an additional control experiment the usual coupled enzyme assay mixture was prepared (see above) except that neither PEP nor one of the reactive PEP analogs was added, and the solution was made 1×10^{-3} M in K_2HPO_4. Samples of this reaction mixture were removed at 0, 0.25, 1.5, and 2.5 hr and chromatographed in the usual manner. No ATP formation was detectable, even after 2.5 hr, and no change in intensity of the ADP or P_i spots was detectable during this same period.

Detection of ATP as Product from the Reaction of (Z)-Phosphoenol-α-ketobutyrate in the Pyruvate Kinase Reaction by a Coupled-Enzyme Procedure with Luciferase. The usual pyruvate kinase reaction mixture, using (*Z*)-phosphoenol-α-ketobutyrate as substrate, was prepared (see above). A luciferase reaction mixture, except for the ATP, was also prepared by slight modification of the procedure of Johnson *et al.* (1970); *i.e.*, dithiothreitol was not added and $NaH_2A_8O_4$–$Na_2HA_8O_4$ buffer (pH 7.45) was used in place of the glycylglycine buffer. Observation of luminescence required the presence of all the components of both enzyme reaction mixtures. That is, if either ADP or the (*Z*)-phosphoenol-α-ketobutyrate, for example, were deleted, no luminescence was observed.

Isolation of α-Ketobutyric Acid from the Reaction of (Z)-Phosphoenol-α-ketobutyrate in the Pyruvate Kinase Reaction. A 0.05 M solution of Tris–Tris·HCl buffer (150 ml) was prepared which contained the following concentrations of reactants: 1.7×10^{-3} M ADP, 2×10^{-3} M cyclohexylammonium dihydrogen (*Z*)-phosphoenol-α-ketobutyrate, 3.4×10^{-3} M $MgSO_4$, and 0.1 M KCl. To this solution was added 0.5 mg (~153 IU of activity) of pyruvate kinase. The reaction was allowed to proceed at room temperature, and the reaction was followed by polyethylenimine cellulose thin-layer chromatography. After 24 hr, the solution was acidified by the addition of several drops of concentrated H_2SO_4. The solution was then extracted continuously with ether for 8 hr. The ether extracts were dried over anhydrous $MgSO_4$, and the ether was removed at reduced pressure to leave ~10 mg of product (~38% yield), which had a nuclear magnetic resonance spectrum identical with that of known α-ketobutyric acid.

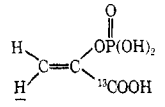
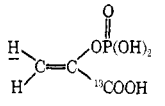
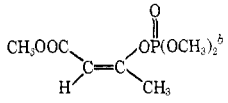
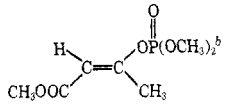
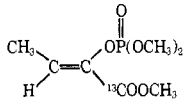
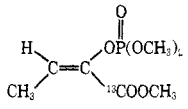
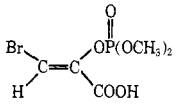
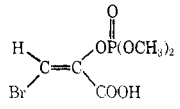
In a control experiment the (*Z*)-phosphoenol-α-ketobutyrate was shown to be stable under the acidic conditions used in the work up.

Reaction of (Z)-Phosphoenol-α-ketobutyrate in the Pyruvate Kinase Reaction in D_2O. A 0.05 M solution of Tris–Tris·DCl buffer was prepared by dissolving 2.73 g of Tris in 450 ml of D_2O (Bio-Rad Laboratories, 99.98% deuterium) and acidifying with DCl to pD 7.49. Various components of the reaction mixture were then added. The solution contained: 3.4×10^{-3} M $MgSO_4$, 0.1 M KCl, 2×10^{-3} M cyclohexylammonium dihydrogen (*Z*)-phosphoenol-α-ketobutyrate, 1.7×10^{-3} M ADP, and 2×10^{-3} M NADH. Lactic dehydrogenase (40 μl, 1×10^{-9} M, 242 IU of activity) and pyruvate kinase (150 μl, 1×10^{-9} M, 214 IU of activity) were added to the solution. The reaction was allowed to proceed at room temperature. Examination of the products by thin-layer chromatography (see above) indicated that the reaction was complete after 24 hr. The reaction mixture was then made strongly acidic by the addition of several drops of concentrated D_2SO_4, and the α-hydroxy-β-deuteriobutyric acid was isolated by continuous ether extraction over a period of 7 hr. The ether extracts were dried over anhydrous $MgSO_4$, and the ether was removed at reduced pressure. The nuclear magnetic resonance spectrum (D_2O) of the product showed peaks at δ 0.77 (3 H, doublet, J = 7 Hz), 1.53 (1 H, multiplet), and 3.97 (1 H, doublet, J = 7 Hz). The α-hydroxy-β-deuteriobutyric acid was dissolved in a mixture of 10 ml of H_2O and 1 ml of 1 M potassium phosphate buffer (pH 7.5). The solution was cooled in an ice bath, and 6 ml of 6% $KMnO_4$ solution was added. This mixture was stirred for 20 min at 0°, and then the excess $KMnO_4$ was destroyed by the addition of solid $NaHSO_3$ until the purple color disappeared. The MnO_2 which formed was separated by centrifugation, was washed a few times with more water, and the water washings were combined. The combined extracts were then made strongly basic by the addition of 3 N NaOH solution, and this solution was filtered to remove more MnO_2. The water was then removed from the filtrate to leave a white solid. The nuclear magnetic resonance spectrum (D_2O) showed that it was a mixture of sodium [α-^{2}H]propionate, showing peaks at δ 0.80 (3 H, doublet, J = 8 Hz), 1.92 (1 H, multiplet), and sodium acetate, showing a peak at δ 1.67 (singlet); the mixture also contained inorganic salts. Removal of these inorganic salts was accomplished by chromatography through a silicic acid column (12.5 × 1.5 cm) prepared by the method of Varner (1957). The concentrations of sodium acetate and sodium [α-^{2}H]propionate were determined by nuclear magnetic resonance spectroscopy. The specific rotation for the sodium [α-^{2}H]propionate was determined on the spectropolarimeter to be −4° at 240 nm and at 25°.

In another experiment, the above reaction was repeated with reactants which had been exhaustively exhanged in D_2O prior to use. The $[\alpha]_{240}^{25}$ was again −4°.

A somewhat higher specific rotation for this product was obtained using the oxidation method described by Rose (1970) and Bondinell and Sprinson (1970). Thus the reaction was performed as described above except that neither lactic dehydrogenase nor NADH was added. Upon completion of the reaction, 1 molar equiv of H_2O_2 was added to the solution, and the mixture was incubated at 37° for 3 hr. After heating for several minutes at 100°, the solution was brought to pH 10.5 with dilute NaOH. The solvent was removed at reduced pressure and the [α-^{2}H]propionate was purified by the method

TABLE II: Comparison of Nuclear Magnetic Resonance Parameters for Some Related Enol Phosphates.

Compound	Chemical Shift (δ, ppm from Me_4Si)		Coupling Constant ($\|J\|$ in Hz)		
	HC=	CH_3C=	$^1HC{=}CO^{31}P$	$C^1H_3C{=}CO^{31}P$	$^1HC{=}C^{13}C$
	5.33[a]		1.45[a]		3.1[a]
	5.15[a]		1.15[a]		9.2[a]
	5.47[c]	2.15[c]	0.9[c]		*d*
	5.76[c]	2.36[c]	1.8[c]		*d*
	6.82	2.12	1.9	3.0	2.9
	6.52	2.32	2.8	3.3	9.5 ± 0.5
	7.27		1.7		*d*
	7.03		2.4		*d*

[a] Cohn *et al.* (1970). [b] The assignments of the configurations for this pair of isomers have been confirmed independently by measurement of the ^{13}C nuclear magnetic resonance chemical shifts for the allylic methyl carbons (see Results). [c] Stiles *et al.* (1961); see also Fukuto *et al.* (1961). [d] Not measured.

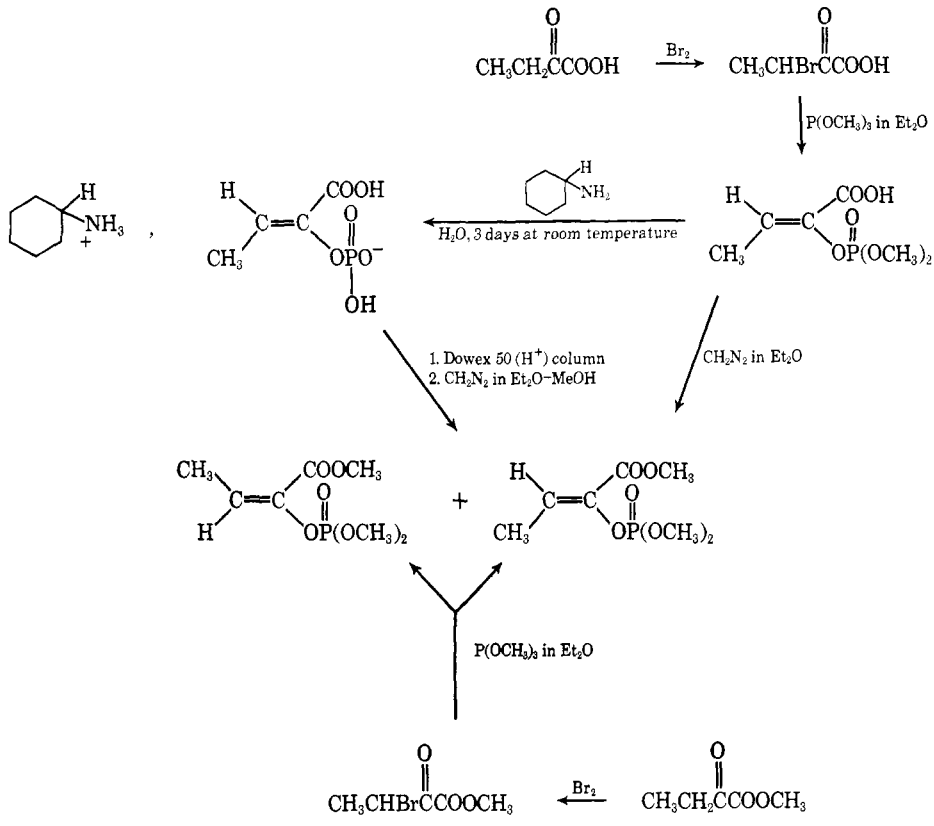
of Varner (1957). A low yield of [α-^{2}H]propionate ($[\alpha]_{240}^{25}$ −6°) was obtained.

Stereochemical Assignments for the E and Z Isomers of Phosphoenol-α-ketobutyrate and Phosphoenol-3-bromopyruvate. The stereochemical assignments for the *E* and *Z* isomers of trimethyl phosphoenol-α-ketobutyrate are based on comparison of nuclear magnetic resonance parameters to those of other similar known enol phosphates. Table II lists the relevant nuclear magnetic resonance data. The vinyl proton regions for the nuclear magnetic resonance spectra taken at 100 MHz for both a 75:25 mixture of (*E*)- and (*Z*)-trimethyl phosphoenol-α-ketobutyrate and a similar mixture 60% ^{13}C enriched in the carbonyl position are shown in Figures 1 and 2, respectively. The additional splitting due to the ^{13}C enrichment is apparent in Figure 2. By a comparison of the observed spectrum to one calculated assuming values of $J_{^{13}CCCH}$ (cis) = 2.9 Hz and $J_{^{13}CCCH}$ (trans) = 9.5 ± 0.5 Hz, a reasonable agreement was obtained. In an additional experiment the ^{31}P nucleus was decoupled (irradiation at 40.5 MHz, NMR Specialties heteronuclear decoupler) and again a reasonable agreement between observed and calculated spectra was obtained by assuming the above [^{13}C]H coupling constants. Scheme I shows the chemical transformations which were performed to show the stereochemical relationships among the esterified and nonesterified phosphoenol-α-ketobutyrates.

The stereochemical assignments for the *E* and *Z* isomers of phosphoenol-3-bromopyruvate are less certain, and are based on the relative chemical shifts and [^{31}P]H coupling constants for the vinyl protons for the pair of isomers when compared to the same parameters for the isomers of phosphoenol-α-ketobutyrate (Table II). It is also reasonable to expect that the same isomer (the *Z* isomer) would be the predominant product in the Perkow reaction in the syntheses of both the phosphoenol-α-ketobutyrate and the phosphoenol-3-bromopyruvate.

In an additional experiment samples of the *E* and *Z* isomers

SCHEME I



of methyl 3-(dimethoxyphosphinyloxy)crotonate (α- and β-phosdrins, donated by Shell Development Co., Modesto, Calif.; Stiles *et al.*, 1961; Fukuto *et al.*, 1961) were examined by natural abundance ^{13}C nuclear magnetic resonance spectroscopy (1 M solutions in $CDCl_3$). As expected (Grant and Cheney, 1967; Jautelat *et al.*, 1970; Bhalerao and Rapoport, 1971) the *Z* isomer (β-phosdrin, allylic methyl cis to the vinyl hydrogen) showed a spectrum in which the allylic methyl carbon resonated at lower field (chemical shift + 105.9 ppm from benzene) than the corresponding methyl carbon of the *E* isomer (α-phosdrin, allylic methyl trans to the vinyl hydrogen; chemical shift + 109.3 ppm from benzene).

Discussion

In agreement with the earlier study by Rose (1960) we have found that none of the partially or fully esterified derivatives of PEP or the PEP analogs were detectably reactive as pseudosubstrates for the enzyme. Some tolerance of relatively small substituents (*e.g.*, methyl and bromo) on the 3 position of the PEP, however, was found. In a similar study, Woods *et al.* (1970) did not detect reactivity for the 3-methyl analog, phosphoenol-α-ketobutyrate, in the rabbit muscle pyruvate kinase reaction, probably because of the relative insensitivity of their assay procedure. We have employed the coupled-assay procedure of Mildvan and Leigh (1964) using lactic dehydrogenase, and had no difficulty detecting reaction rates for both phosphoenol-α-ketobutyrate and phosphoenol-3-bromopyruvate. Using this procedure our kinetic measurements indicated that neither the 3-methyl nor 3-bromo substituent had much effect on the observed K_m value, but both adversely affected the maximal velocity. The negative effect of bulky substituents on the 3 position was pointed out by Woods *et al.* (1970) who found that whereas the 3-methyl and 3-ethyl analogs of PEP were effective as competitive inhibitors of pyruvate kinase, homologs with more bulky substituents in this position were not. The fact that our 3-phenyl analog was not detectably reactive as a pseudosubstrate also supports this conclusion.

The most unambiguous single experiment that we found for screening for pseudosubstrates of pyruvate kinase was to follow the reaction by polyethylenimine cellulose ion-exchange thin-layer chromatography. For PEP and the reactive analogs the observed intensity of the spot for ATP increased concomitantly with observed decreases in the intensities of spots for both ADP and PEP or the PEP analog. At the same time, no P_i was observed to be formed. This method has the advantage of extreme sensitivity while avoiding a coupled enzymatic assay procedure. For example, on properly prepared, fresh thin-layer plates as little as 5×10^{-10} mole of chromatographed P_i could be easily detected.

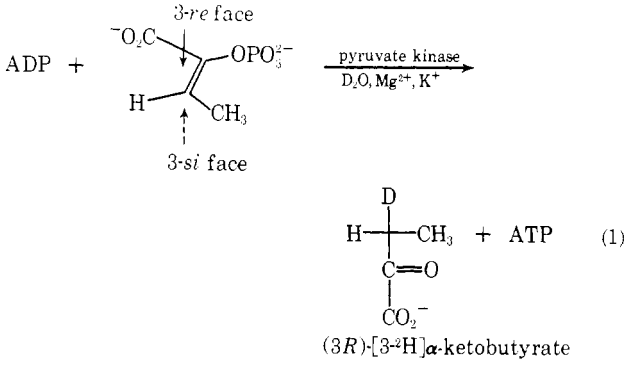
In our experiments only the pure (*Z*)-phosphoenol-α-ketobutyrate was tested as a pseudosubstrate. We have so far been unable to obtain a pure sample of the *E* isomer, although we have obtained a 75:25 mixture of the trimethyl phosphoenol-α-ketobutyrate stereoisomers.

Our stereochemical assignments for the *E* and *Z* isomers of the phosphoenol-α-ketobutyrate are based on a comparison of certain nuclear magnetic resonance parameters with those of known, related enol phosphates. The most compelling evidence for these assignments came from measurement of the $^{13}CC{=}C^{1}H$ coupling constants for a mixture of the *E* and *Z* isomers 60 atom % ^{13}C enriched in the carbonyl position. The *Z* isomer had $J_{^{13}CC=C^{1}H}$ (cis) = 2.9 Hz, and the *E* isomer had $J_{^{13}CC=C^{1}H}$ (trans) = 9.5 ± 0.5 Hz. These values compare favorably with values found by Cohn *et al.* (1970)

for PEP itself, $J_{^{13}CC=CH}$ (cis) = 3.1 Hz and $J_{^{13}CC=CH}$ (trans) = 9.0 Hz. No known exceptions have been found to the generalization that for $^{13}CC{=}C^{1}H$ coupling constants $|J|$ (trans) is greater than $|J|$ (cis) (Cohn *et al.*, 1970). This generalization has been found to apply to homonuclear and heteronuclear coupling constants between several pairs of elements directly attached to a carbon–carbon double bond (Bovey, 1969). Confirmatory evidence for these stereochemical assignments comes from the fact that the relative chemical shifts for the vinyl protons cis and trans to the phosphate group are the same as those found for PEP itself (Cohn *et al.*, 1970).

Our corresponding stereochemical assignments for the *E* and *Z* isomers of dimethyl phosphoenol-3-bromopyruvic acid[3] were made on the following bases: the relative chemical shifts for the vinyl protons in the nuclear magnetic resonance spectrum, the similarities between the $J_{^{31}POC=CH}$ coupling constants with those of the (*E*)- and (*Z*)-trimethyl phosphoenol-α-ketobutyrates,[4] and the expectation that, since bromo and methyl groups are very similar in size, the same stereoisomer should dominate in the product mixture obtained from synthesis by the Perkow reaction.

Since we have found that (*Z*)-phosphoenol-α-ketobutyrate was converted into sodium (*R*)-(−)-[α-^{2}H]propionate by our series of enzymatic and chemical conversions, the deuterium must have added stereospecifically[2] at C-3 on the 2-*si*, 3-*re* face (Hanson, 1966) of the PEP analog or its mechanistic equivalent in the pyruvate kinase catalyzed portion of the reaction sequence, *i.e.*, eq 1. Our calculations indicate that the



(1)

sodium [α-^{2}H]propionate we obtained had a specific rotation of $[\alpha]_{240} = -4°$ in one case and $-6°$ in another reaction. Bondinell and Sprinson (1970), starting with an 80:20 mixture of the *Z* and *E* isomers of phosphoenol-α-ketobutyrate in a similar experiment with pyruvate kinase, reported a value of $[\alpha]_{240} = -7°$. Generating the sodium (*R*)-(−)-[α-^{2}H]-propionate in a different manner, Krongelb *et al.* (1968) reported $[\alpha]_{240} = -12.3°$. The reason for the discrepancy between our specific rotation and that of Bondinell and Sprinson is not known, although the oxidation conditions we used for conversion of the product of the enzymatic reaction into the sodium [α-^{2}H]propionate may have led to some racemization.

The finding that (*Z*)-phosphoenol-α-ketobutyrate or its mechanistic equivalent is deuterated stereospecifically at the C-3 position on its 2-*si*, 3-*re* face is in accord with the finding of Rose (1970) for PEP itself who employed tritium–deuterium-labeling techniques in a similar study, and serves to reinforce the validity of the interlocking stereochemical assignments which he has made for various enzyme reactions. Rose and his coworkers have also recently established the stereochemical course of the addition of CO_2 to PEP in several enzyme systems (Rose *et al.*, 1969).

We are currently investigating the interactions of our analogs with other enzymes which function with PEP.

Acknowledgments

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References

Benkovic, S. J., and Shray, K. J. (1969), *J. Amer. Chem. Soc. 91*, 5653.

Bhalerao, V. T., and Rapoport, H. (1971), *J. Amer. Chem. Soc. 93*, 105.

Bondinell, W. E., and Sprinson, D. B. (1970), *Biochem. Biophys. Res. Commun. 40*, 1464.

Bovey, F. A. (1969), Nuclear Magnetic Resonance Spectroscopy, New York, N. Y., Academic Press, p 226.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankovich, P. F. (1949), Isotopic Carbon, New York, N. Y., Wiley, p 210.

Chopard, P. A., Clark, V. M., Hudson, R. F., and Kirby A. J. (1965), *Tetrahedron 21*, 1961.

Clark, V. M., and Kirby, A. J. (1963), *J. Amer. Chem. Soc. 85*, 3705.

Clark, V. M., and Kirby, A. J. (1966), *Biochem. Prepn. 11*, 101.

Clinton, R. O., and Laskowski, S. C. (1948), *J. Amer. Chem. Soc. 70*, 3135.

Cohn, M., Pearson, J. E., O'Connell, E. L., and Rose, I. A. (1970), *J. Amer. Chem. Soc. 92*, 4095.

Cooper, D. J., and Owen, L. N. (1966), *J. Chem. Soc. C*, 533.

Fukuto, T. R., Hornig, E. O., Metcalf, R. L., and Winton, M. Y. (1961), *J. Org. Chem. 26*, 4620.

Grant, D. M., and Cheney, B. V. (1967), *J. Amer. Chem. Soc. 89*, 5315.

Hanes, C. S., and Isherwood, F. A. (1949), *Nature (London) 164*, 1107.

[3] When the usual hydrolytic conditions (*i.e.*, cyclohexylamine, pH 7, 3 days, room temperature) for removal of the phosphate methyl ester groups in the attempted preparation of phosphoenol-3-bromopyruvate from this intermediate were employed, no product containing vinyl hydrogens in the nuclear magnetic resonance spectrum was obtained. If the free acid of the dimethyl ester was dissolved in water and left for a brief time without the addition of cyclohexylamine, however, the ester groups were hydrolyzed with remarkable rapidity (see Results). This indicates that a hydrolysis mechanism involving acid catalysis as well as neighboring group participation (Clark and Kirby, 1963), pentacovalent phosphorus intermediates and pseudorotational conversions (Benkovic and Shray, 1969) may be operating.

[4] In contrast to the reliability of stereochemical assignments made on the basis of assuming $|J|_{^{31}POC^{1}H}$ (trans) being greater than $|J|_{^{31}POC^{1}H}$ (cis) (Kenyon and Westheimer, 1966; Martin *et al.*, 1967), the generalization does not hold if the ^{31}P nucleus is not directly attached to one of the carbons of the double bond (Cohn *et al.*, 1970). For example, for phosphoenolpyruvate $|J|_{^{31}POCC^{1}H}$ (trans) > $|J|_{^{31}POCC^{1}H}$ (cis), but for both the (*E*)- and (*Z*)-methyl 3-(dimethoxyphosphinyloxy)crotonates and the [*E*]- and (*Z*)-trimethyl phosphoenol-α-ketobutyrates $|J|_{^{31}POCC^{1}H}$ (cis) > $|J|_{^{31}POCC^{1}H}$ (trans) (see Table II).

Hanson, K. R. (1966), *J. Amer. Chem. Soc. 88*, 2731.
Jautelat, M., Grutzner, J. B., and Roberts, J. D. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 288.
Johnson, R. A., Hardman, J. G., Broadus, A. E., and Sutherland, E. W. (1970), *Anal. Biochem. 35*, 91.
Kenyon, G. L., and Westheimer, F. H. (1966), *J. Amer. Chem. Soc. 88*, 3557.
Krongelb, M., Smith, T. A., and Abeles, R. H. (1968), *Biochim. Biophys. Acta 167*, 473.
Kuhn, R., and Drury, K. (1949), *Ann. 564*, 32.
Martin, D. J., Gordon, M., and Griffin, C. E. (1967), *Tetrahedron 23*, 1831.
McElroy, W. D. (1963), *Methods Enzymol. 6*, 445.
Meister, A. (1950), *J. Biol. Chem. 184*, 117.
Mildvan, A. S., and Leigh, R. A. (1964), *Biochim. Biophys. Acta 89*, 393.
Moureu, H., Chovin, P., Garein, M., and Ventrillard, J. (1952), *Bull. Soc. Chim. France*, 296.
Nowak, T., and Mildvan, A. S. (1970), *J. Biol. Chem. 245*, 6057.
Randerath, K., and Randerath, E. (1964), *J. Chromatog. 16*, 111.
Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. (1961), *J. Biol. Chem. 236*, 2277.
Rose, I. A. (1960), *J. Biol. Chem. 235*, 1170.
Rose, I. A. (1970), *J. Biol. Chem. 245*, 6052.
Rose, I. A., O'Connell, E. L., Noce, P., Utter, M. F., Wood, H. G., Willard, J. M., Cooper, T. G., and Benjiman, M. (1969), *J. Biol. Chem. 244*, 6130.
Sprinson, D. B., and Chargaff, E. (1946), *J. Biol. Chem. 164*, 417.
Sternlicht, H., Kenyon, G. L., Packer, E. L., and Sinclair, J. (1971), *J. Amer. Chem. Soc. 93*, 199.
Stiles, A. R., Reilley, C. A., Pollard, G. R., Tieman, C. H., Ward, L. F., Phillips, D. D., Soloway, S. B., and Whetstone, R. R. (1961), *J. Org. Chem. 26*, 3960.
Supniewski, J. V., and Salzberg, P. L. (1941), *in* Organic Syntheses, Gilman, H., and Blatt, A. H., Ed., Collect. Vol. I, New York, N. Y., Wiley, p 46.
Tietz, A., and Ochoa, S. (1959), *Arch. Biochem. Biophys. 78*, 477.
Varner, J. E. (1957), *Methods Enzymol. 3*, 397.
Woods, A. E., O'Bryan, J. M., Mu, P. T. K., and Crowder, R. D. (1970), *Biochemistry 9*, 2334.

Interaction of Nicotinamide–Adenine Dinucleotide and Its Analogs with Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: Several structural analogs of NAD were tested for their effects on the properties of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Of the 13 analogs tested only three were effective substrates: acetylpyridine-AD, thionicotinamide-AD, and deamino-NAD. Several other analogs including ADP-ribose, ADP, AMP, NMN, and pyridine carbaldehyde-AD were inhibitors competitive with NAD. The latter analog was also competitive with the substrate phosphoglyceraldehyde. Two of the analogs gave a spectrum with the enzyme similar to that observed with β-NAD. These compounds, acetylpyridine-AD and deamino-NAD, also showed anticooperative binding to the enzyme. Several analogs produced partial stabilization of the enzyme at 45°. In decreasing order of stabilization these are: β-NAD, ADP-ribose, thionicotinamide-AD, ADP, NMN. From these studies as well as determinations of the kinetic and binding constants of the analogs it is possible to deduce a probable conformation of the bound coenzyme and to specify the major points of contact between the coenzyme and the enzyme. A model of the enzyme–coenzyme complex is presented.

In recent years there has been interest in the unusual kinetic and binding properties of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (GPDH)[1] and the corresponding enzyme obtained from yeast. Kirschner *et al.* (1966) have reported that the yeast GPDH exhibits positive cooperative binding of the coenzyme NAD under some conditions while the muscle enzyme does not give cooperative binding. On the other hand, Conway and Koshland (1968) have shown that there is anticooperative binding of NAD to the muscle enzyme. In a more recent study Cook and Koshland (1970) have reexamined the binding of NAD to the yeast enzyme and have shown that there is positive cooperativity in the first half of the curve and negative or no cooperativity in the second half of the curve.

These obvious functional differences between the yeast and muscle enzymes are particularly interesting because of the high degree of homology in the primary structures of GPDHs from different species (Harris and Perham, 1968). One possible explanation for the functional differences is that the NAD

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[1] Abbreviations used are: GPDH, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); ADP-ribose, adenosine diphosphoribose; acetylpyridine-AD, acetylpyridine-adenine dinucleotide; thionicotinamide-AD, thionicotinamide–adenine dinucleotide; deamino-NAD, nicotinamide–hypoxanthine dinucleotide; deaminoacetylpyridine-AD, acetylpyridine–hypoxanthine dinucleotide; DTT, dithiothreitol; IAA, iodoacetic acid.