Stereochemical Studies of the Pyruvate Kinase Reaction with (*Z*)- and (*E*)-Phosphoenol-α-ketobutyrate†

Mella Adlersberg, Jean Dayan, William E. Bondinell,‡ and David B. Sprinson*

ABSTRACT: (*Z*)- and (*E*)-phosphoenol-2-ketobutyrate were synthesized. [3-^{2}H]-2-Ketobutyrates were formed from both isomers in the pyruvate kinase reaction in ^2H_2O and were converted to chiral propionates. Authentic (2*S*)-[2-^{2}H]propionic acid was also prepared, and the optical rotatory dispersion curves of the propionates were compared. The rotation compared with standard propionate at 240 nm of sodium (2*R*)-[2-^{2}H]propionate from the *Z* isomer was 47% (i.e., 53% was *RS*), and of (2*S*)-[2-^{2}H]propionate from the *E* isomer was 29% (i.e., 71% was *RS*). Protonation at C-3 of the 2 *si*, 3 *re* face of the pseudosubstrates would have yielded (2*R*)- and (2*S*)-[2-^{2}H]propionates from the *Z* and *E* analogues, respectively. An explanation offered for the nonstereoselective protonation that occurred is dissociation of the enol from the enzyme and subsequent random protonation in solution.

The stereochemical course of the pyruvate kinase reaction has been investigated by two methods. In one method [3-^{2}H,^{3}H]pyruvate resulting from isotopically asymmetric [3-^{2}H,^{3}H]phosphoenolpyruvate was converted to acetyl CoA and then to malate which was analyzed by ^{3}H release in the fumarase reaction (Rose, 1970). It was found that protonation at C-3 occurred at the *si* face of PEP[1] [designated by a counterclockwise sequence of phosphate, carboxyl, and vinyl groups (Hanson, 1966)]. In the other method [3-^{2}H]-2-ketobutyrate resulting from the action of pyruvate kinase on the pseudosubstrate (*Z*)-phosphoenol-2-ketobutyrate in D_2O was converted to [2-^{2}H]propionate (Bondinell and Sprinson, 1970; Stubbe and Kenyon, 1971) (Figure 1). The ORD curves of the sodium propionates indicated largely 2*R* configuration, and hence proton addition mostly at C-3 of the 2 *si*, 3 *re* face (Hanson, 1966). However, the rotations were low. The present report is concerned with an investigation of the synthesis and substrate properties of both (*Z*)- and (*E*)-phosphoenol-2-ketobutyrate. The preparation of authentic chiral [2-^{2}H]-propionate was reinvestigated in order to provide a standard material for comparison with the biologically obtained propionates.

Experimental Section

Methods. Unless otherwise indicated, melting points were taken on a Fischer-Johns block and are uncorrected. Solvents were removed by evaporation and solids were dried at reduced pressure. NMR spectra were taken at 60 MHz, unless otherwise indicated, and chemical shifts were in parts per million downfield from an internal standard of sodium 4,4-dimethyl-4-silapentane-1-sulfonate or tetramethylsilane. Solutions of cyclohexylammonium or barium salts were passed through Dowex 50 H^+ form, neutralized with 1.0 N KOH, and evaporated to dryness. The resulting tripotassium salts were analyzed by NMR in ^2H_2O. Unless otherwise stated, methyl esters were prepared from the potassium salts by conversion with a sixfold excess of $AgNO_3$ to insoluble silver salts, which were collected by filtration, washed, dried, and stirred with excess Ag_2O in refluxing methyl iodide. AgI and solvent were removed, and the residual methyl ester was analyzed by NMR in C^2HCl_3 solution. ORD measurements were taken on a Jasco J20 spectropolarimeter. Mass spectra were taken on a JEOL-JMS-07 mass spectrometer. Total phosphate was determined by the method of Ames and Dubin (1960) and enol phosphate by hydrolysis with mercuric acetate (Reynard et al., 1961). Protein was determined by the method of Lowry et al. (1951). Deuterium analyses were carried out with the falling drop method of measuring ^{2}H in ^2H_2O by Mr. J. Nemeth, Urbana, Ill.

Materials. Rabbit muscle pyruvate kinase and lactate dehydrogenase and yeast hexokinase were from Boehringer. (2*R*,3*R*)-Butanediol was a generous gift from Dr. F. J. Simpson and was also purchased from Burdick and Jackson, Muskegon, Mich. (2*R*,3*S*)-[3-^{2}H]-2-Acetoxybutane was a generous gift from Dr. G. K. Helmkamp. Silica gel thin-layer plates were from Analtech, Newark, Del. Chemicals were the best grade available from commercial sources.

Synthesis of 3-Bromo-2-ketobutyric Acid. 2-Ketobutyric acid was brominated as described previously (Sprinson and Chargaff, 1946): mp 61 °C (sealed tube), reported mp 60 °C (sealed tube); NMR δ 1.8 (d, J = 6.5 Hz, 3 H), 5.2 (q, J = 6.5 Hz, 1 H).

Synthesis of Diethyl (1-Hydroxy-1-carboxy-2-bromopropyl)phosphonate. 3-Bromo-2-ketobutyric acid (14 g, 78 mmol) and diethylphosphonate (10.7 g, 78 mmol) were dissolved in anhydrous ether (10 mL) and refluxed for 24 h (Cramer and Voges, 1959). The crystalline solid was removed by filtration and washed with ether (yield 18 g, 71%): mp 161–162 °C. Recrystallization from benzene gave: mp 162–163 °C; NMR (C^2HCl_3), δ 1.37, 1.39 (2 t, J = 7 Hz, CH_3–C–O, 6 H), 1.82 (broad d, J = 7 Hz, CH_3–CBr, 3 H), 4.34, 4.42 (2 dq, J_{H-H} and J_{P-H} = 7 Hz, C–CH_2–O, 4 H), 4.74 (dq, J_{P-H} = 1.5 and J_{H-H} = 7 Hz, CHBr, 1 H).

Anal. Calcd for $C_8H_{16}BrO_6P$: C, 30.11; H, 5.05; Br, 25.04; P, 9.71. Found: C, 29.85; H, 5.13; Br, 24.81; P, 9.68.

NMR of methyl ester (diazomethane) in C^2HCl_3 was: δ 1.36

† From the Department of Biochemistry, Columbia University, New York, New York 10032. *Received April 7, 1977.* Supported by grants from the American Cancer Society, the American Heart Association, the National Institutes of Health of the Public Health Service, and the National Science Foundation. This work was done during the tenure by D.B.S. as a Career Investigator from the American Heart Association. W.E.B. was a Career Investigator Fellow of the American Heart Association, 1969–1971.

‡ Present address: Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101.

[1] Abbreviations used are: PEP, phosphoenolpyruvate; ORD, optical rotatory dispersion; NMR, nuclear magnetic resonance.

(t, J = 7 Hz, CH_3-C-O, 6 H), 1.87 (dd, J_{P-H} = 1 and J_{H-H} = 7 Hz, CH_3-CBr, 3 H), 3.92 (s, CO_2CH_3, 3 H), 4.26 (dq, J_{H-H} and J_{P-H} = 7 Hz, C-CH_2-O, 4 H), 4.8 (dq, J_{P-H} = 1.5 and J_{H-H} = 7 Hz, CHBr, 1 H).

Synthesis of Cyclohexylammonium Dihydrogen (Z)-Phosphoenol-2-ketobutyrate. Diethyl (1-hydroxy-1-carboxy-2-bromopropyl)phosphonate (2.1 g, 6 mmol) in water (30 mL) was treated with 1.0 N aqueous sodium hydroxide (12 mL). The solution was allowed to stand for 3 days at 25 °C, adjusted to pH 7.0, diluted to 500 mL, and loaded on a column (25 × 2.5 cm) of Dowex 1-X8 chloride (Bartlett, 1959). Elution with 0.02 N HCl removed a phosphorus containing compound which did not release inorganic phosphate with aqueous mercuric acetate, and elution with 0.04 N HCl gave (*Z*)-phosphoenol-α-ketobutyric acid in 50% yield as determined by enol phosphate assay. Combined fractions of desired compound were concentrated, and the residue was dissolved in water and treated with cyclohexylamine (300 mg, 3 mmol). Water was evaporated and the residue was crystallized from methanol–ether to give 562 mg (37%) of cyclohexylammonium dihydrogen (*Z*)-phosphoenol-α-ketobutyrate: mp 148–148.5 °C. Stubbe and Kenyon (1971) report mp 147–149 °C. An analytical sample was prepared by recrystallization from the same solvent. NMR of tripotassium salt: δ 1.7 (dd, J = 2.5 and 7 Hz, CH_3-C=, 3 H), 6.0 (dq, J = 2.5 and 7 Hz, HC=, 1 H). NMR analysis of trimethyl ester was in agreement with that reported by Stubbe and Kenyon (1971).

Anal. Calcd for $C_{10}H_{20}NO_6P$: C, 42.70; H, 7.17; N, 4.98; P, 11.01. Found: C, 42.86; H, 7.18; N, 4.83; P, 11.01.

Synthesis of Cyclohexylammonium Dihydrogen (E)- and (Z)-Phosphoenol-2-ketobutyrate. 3-Bromo-2-ketobutyric acid (1.6 g, 8.7 mmol) was added to a solution of trimethyl phosphite (1.15 g, 9.2 mmol) in anhydrous ether (25 mL) and stirred for 15 min as described by Clark and Kirby (1966) for the synthesis of PEP. Evaporation of ether left an oil which was by NMR mainly (*E*)- and (*Z*)-dimethylphosphoenol-α-ketobutyrate (isomer ratio 2 to 9). The oil was dissolved in water (10 mL) and treated with cyclohexylamine (0.9 g, 8.7 mmol), and the solution was allowed to stand for 3 days. Water was removed by evaporation and the crystalline residue was recrystallized three times from methanol–ether to yield 1.1 g (44%) of cyclohexylammonium dihydrogen (*E*)- and (*Z*)-phosphenol-α-ketobutyrate (ratio 2 to 9): mp 148–148.5 °C. Our previously reported mp 154–155 °C (Bondinell and Sprinson, 1970) was in error. Woods et al. (1972) have reported that cyclohexylammonium phosphoenol-α-ketobutyrate was thermally unstable. Analysis and NMR were reported previously (Bondinell and Sprinson, 1970).

Photoisomerization of (E)- and (Z)-Phosphoenol-α-ketobutyrate. Tripotassium (*E*) and (*Z*)-phosphoenol-α-ketobutyrate (isomer ratio 2:9) (1 mmol) in water (30 mL) was stirred and irradiated with a 15-W ultraviolet light source for 24 h. The *E* and *Z* isomer ratio was changed to 1:3 (by NMR analysis). Continued irradiation resulted in total conversion to other materials.

Preparation of Cyclohexylammonium Dihydrogen (E)-Phosphoenol-2-ketobutyrate. Method 1. Removal of Z Analogue from EZ Mixture with Pyruvate Kinase. A solution (95 mL) of tripotassium phosphoenol-α-ketobutyrate (20 mmol, *E* and *Z* isomer ratio 1 to 3, prepared by photoisomerization as described above), Na_2ADP (1 mmol), D-glucose (16 mmol), KCl (100 mmol), $MgSO_4$ (12 mmol), and Tris buffer, pH 7.5 (50 mmol), was adjusted to pH 7.5 with 1.0 N HCl. The solution was warmed to 37 °C, treated with pyruvate kinase (20 mg) and hexokinase (4 mg), and kept at 37 °C for 48 h. Hydrogen peroxide (5 mL, 28%) was added to decompose

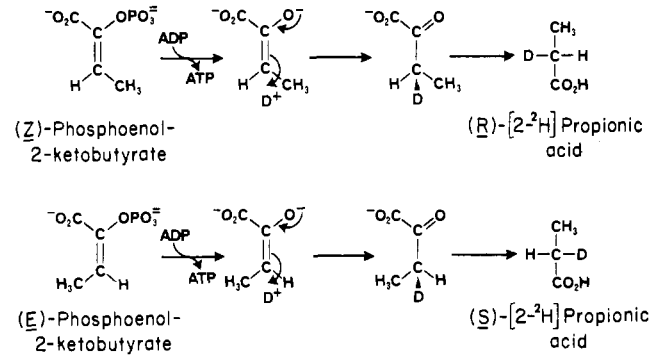

FIGURE 1: Protonation by *si* attack of (*Z*)- and (*E*)-phosphoenol-2-ketobutyrate in the pyruvate kinase reaction.

α-ketobutyrate and, 1 h later, catalase (2.7 mg) to destroy the remaining H_2O_2. After 1 h the solution was heated to 85 °C for 10 min, cooled, filtered, adjusted to pH 7.9 with 5.0 N KOH, diluted to 8 L, and loaded on a column (24 × 2.5 cm) of Dowex 1-X8 chloride. Elution with 0.02 N and then 0.06 N HCl gave the desired compound (measured by absorption at 240 nm) in 0.06 N HCl. Fractions of the latter were pooled, adjusted to pH 7.5 with 1.0 N KOH, and evaporated to dryness. The residue was dissolved in water (200 mL), adjusted to pH 8.2 with KOH, and treated with a sixfold excess of aqueous barium acetate (final volume 300 mL) and 95% ethanol (600 mL). The suspension was chilled, and the barium salt was collected by centrifugation and dried. The crushed solid was extracted four times with water (20 mL), and the pooled extracts were diluted to 300 mL and treated with 95% ethanol (700 mL). The suspension was stored at 4 °C, and the salt was collected by decantation and centrifugation, and dried. Purification was repeated once more. Barium (*E*)-phosphoenol-α-ketobutyrate from three preparations (3.4 mmol, 23% on basis of *E* isomer originally present) was converted to tripotassium salt: NMR δ 1.79 (dd, J = 1.2 and 7.5 Hz, CH_3—C=, 3 H), 5.65 (dq, J = 2 and 7.5 Hz, CH=, 1 H). The monocyclohexylammonium salt was prepared as described for the *Z* isomer: mp 148.5 °C.

Anal. Calcd for $C_{10}H_{20}NO_6P$: C, 42.70; H, 7.17; N, 4.98; P, 11.01. Found: C, 42.78; H, 7.27; N, 4.83; P, 10.97.

Method 2. Chemical Synthesis. trans-Epoxybutyric acid (mp 84 °C) was prepared by epoxidation of *trans*-crotonic acid with hydrogen peroxide and sodium tungstate catalyst (Payne and Williams, 1959): NMR (Me_2SO-d_6, 100 MHz): δ 1.29 (d, J = 5 Hz, CH_3-C, 3 H), 3.12 (dq, J = 2 and 5 Hz, H on C-3), 3.17 (d, J = 2 Hz, H on C-2).

Concentrated HCl (50 mL, 37%) at −15 °C was saturated with HCl gas (12 g) and treated with small portions of *trans*-epoxybutyric acid (10 g, 0.10 mol) with efficient stirring and chilling (ice-salt bath) while maintaining the very exothermic reaction at 3–5 °C. HCl gas was passed through the cold reaction mixture for a few minutes, and the flask was stoppered and allowed to stand overnight at room temperature. Excess HCl was removed by passing a stream of air through the solution kept at 30 °C, and it was cooled and extracted six times with 75-mL portions of ether. The ether solution was dried over $CaCl_2$, solvent was removed, and the residue (12 g, 89%) was recrystallized from anhydrous benzene (75 mL), yielding 7.6 g (56%) of *erythro*-3-chloro-2-hydroxybutyric acid: mp 81 °C. An analytical sample was prepared by another recrystallization from anhydrous benzene: mp 82–83 °C [reported 85–86 °C, Melikoff, 1886]; NMR (Me_2SO-d_6, 100 MHz), δ 1.43 (d, J = 7 Hz, CH_3-C, 3 H), 4.07 (d, J = 5 Hz, H on C-2), 4.29 (dq, J = 5 and 7 Hz, H on C-3).

Anal. Calcd for $C_4H_7ClO_3$: C, 34.17; H, 5.09; Cl, 25.6. Found: C, 34.8; H, 4.91; Cl, 25.6.

(*E*)-Phosphoenol-2-ketobutyrate was prepared by a modification of the methods of Cramer and Weimann (1961) and Lauppe et al. (1972). The chlorohydroxybutyric acid (4.15 g, 30 mmol) in dry acetonitrile (10 mL) was treated with 98% orthophosphoric acid (196 mg, 2 mmol) and the mixture was concentrated twice to dryness from solution in acetonitrile. The residue was dissolved in acetonitrile (10 mL), chilled in an ice bath, and treated dropwise with stirring with triethylamine (18.2 g, 180 mmol) followed by trichloroacetonitrile (3.47 g, 24 mmol). The solution was heated for 2 h at 40 °C under moisture exclusion, and Et_3NHCl was removed by filtration. The filtrate was treated with water (1 mL), and concentrated to a brown syrup which was taken up in H_2O (50 mL). The solution was extracted three times with ether (50 mL) to remove trichloroacetamide, and the ether extracts were washed with H_2O (50 mL). The combined aqueous layers were stirred for a few minutes with an excess of Dowex 50 H^+ form (50 mL, 85 mequiv), and the resin was removed by filtration. The filtrate was adjusted to pH 3 with cyclohexylamine, extracted four times with ether (100 mL) to remove unreacted starting material, and neutralized with 1 N KOH to pH 7.1. The solution was freed of ether, diluted to 2.1 L and loaded on a column (24 × 1.5 cm) of Dowex 1-X8 chloride. Elution with 0.02 N and 0.04 N HCl gave the desired compound (measured by absorption at 240 nm) in 0.04 N HCl. The solution was concentrated to dryness, and the residue was dissolved in H_2O (25 mL) and brought to pH 3 with cyclohexylamine (130 mg, 1.3 mmol). The solution was evaporated to dryness, and the residue was recrystallized from methanol–ether to yield 137 mg of cyclohexylammonium dihydrogen (*E*)-phosphoenol-α-ketobutyrate: mp 154 °C (capillary); NMR spectrum of the tripotassium salt was identical with that reported above for *E* isomer. Mother liquors were concentrated to dryness, and the residue, recrystallized as before, yielded a second crop (100 mg), mp 152–153 °C, NMR, *E* isomer 85%. Total yield: 237 mg (48% based on phosphoric acid).

Phosphorylation of *erythro*-3-chloro-2-hydroxybutyric acid with $POCl_3$ in dimethylaniline as described for the synthesis of PEP from 3-chlorolactic acid (Baer, 1952) gave a mixture of *E* and *Z* isomers in a ratio of 1:1 as determined by NMR.

[2-^2H]Propionate from Pyruvate Kinase Reaction with (Z)- and (E)-Phosphoenol-2-ketobutyrate. A solution (10 mL) containing 0.1 M monocyclohexylammonium (*Z*)- or (*E*)-phosphoenol-α-ketobutyrate, 0.1 M Na_2ADP, 2 M KCl, 0.24 M $MgSO_4$, and 1 M Tris-Cl buffer, pH 7.5, was adjusted to pH 7.7 with 0.1 N KOH, and evaporated to dryness. The residue was exchanged three times with $^2H^+$ by adding and evaporating 10 mL of ^2H_2O, dissolved in ^2H_2O (200 mL, 99.8 mol %), and warmed to 37 °C. A suspension of pyruvate kinase (10 mg per mL, 5.0 mL for *Z* isomer and 10.0 mL for *E* isomer) was centrifuged for 10 min at 17 000*g*. The supernatant solution was discarded, and the enzyme was dissolved in 20 mL of ^2H_2O and added to the reaction mixture. A 2-mL aliquot was removed and kept at 37 °C in order to measure progress of the reaction by formation of α-ketobutyrate (Friedemann and Haugen, 1943). The remainder of the incubation mixture at 37 °C was treated with 25% hydrogen peroxide (4.4 mmol) to convert α-ketobutyrate as it was formed to propionate. The reaction was complete after 3.5 h, and catalase (16.8 mg) was added. After 15 min the incubation mixture was heated at 100 °C for 2 min, cooled, and filtered through glass wool. The solution was adjusted to pH 10 and evaporated to dryness.

The residue was dissolved in water (10 mL) and brought to pH 1.0 with concentrated H_2SO_4. The solution was steam distilled, and 100 mL of distillate was collected, neutralized to pH 10 with 5 N NaOH, and evaporated to dryness. A stirred solution of the white solid in water (0.5 mL) was treated slowly with acetone (30 mL) and the precipitate was collected by filtration. The moist product was dissolved in minimum hot 95% ethanol and the solution was treated with acetone to slight turbidity, clarified with hot 95% ethanol, and cooled slowly to give large, white, flat crystals of sodium propionate. NMR (^2H_2O) of sodium (2*R*)-[2-^2H]propionate from (*Z*)-phosphoenol-α-ketobutyrate was: δ 1.15 (d, J = 7 Hz, CH_3-, 3 H), 2.17 (m, H on C-2).

Anal. Calcd for $C_3H_4{}^2HO_2Na$: C, 37.1; H + ^2H, 6.2; apparent H (as reported in elementary analysis, i.e., calcd for $C_3H_5O_2Na$), 5.16. Found: for sodium (2*R*)- and (2*S*)-[2-^2H]propionate, respectively: C, 36.6; H, 5.36; and C, 37.4; H, 5.61.

Preparation of (2S)-[2-^2H]Propionate from (2R,3S)-[3-^2H]Butanol-2. (2*R*,3*S*)-[3-^2H]Butanol-2 was prepared from (2*R*,3*R*) butanediol as described previously (Leroux and Lucas, 1951; Helmkamp et al., 1956): $[\alpha]^{25°C}_D$ −13.45; reported $[\alpha]^{25°C}_D$ −13.59. Calcd for $C_4H_9{}^2HO$: ^2H, 10.0 atom % excess. Found: 9.73.

The deuteriobutanol (10.1 g, 0.135 mol) was oxidized with potassium hypobromite as described by Retey and Lynen (1965) except that the ethereal solution of propionic acid was carefully dried and distilled at atmospheric pressure on a Vigreux column until the volume was reduced by two-thirds. The residue was dried again overnight with Na_2SO_4 and ether removed as described above. The residue (approximately 10 mL) was fractionated on a vacuum jacketed Vigreux column, and a forerun (3.8 g) collected at 90–139 °C (propionic acid–H_2O azeotrope has bp 99 °C). Propionic acid (2.1 g) was collected at 139–140 °C and redistilled at the same temperature (yield, 1.0 g). A solution of binary azeotrope (3.8 g) in 4.7 mL of benzene was fractionally distilled to remove water and yielded 0.84 g of propionic acid.

Anal. Calcd for $C_3H_5{}^2HO_2$: C, 48.0%; apparent H (as reported in elementary analysis), 8.00. Found: C, 47.3; H, 8.09.

A solution of 0.4 mL of twice distilled propionic acid in 30 mL of H_2O was adjusted to pH 10 with 5 N NaOH, and the solution was evaporated to dryness. The crystalline residue was dissolved in a small amount of water and crystallized by addition of acetone. Recrystallization as described above from 95% ethanol–acetone and drying the product at 100 °C gave 207 mg of sodium propionate: NMR (^2H_2O) δ 1.15 (d, J = 7 Hz, 3 H), 2.17 (m, 1 H).

(2*S*)-[2-^2H]Propionic acid was also prepared from (2*R*,3*S*)-[3-^2H]2-acetoxybutane obtained from Dr. G. K. Helmkamp (bp 110 °C (375 mm)).

Anal. Calcd for $C_6H_{11}{}^2HO_2$: C, 61.5; apparent H (as reported in elementary analysis), 10.3; ^2H (atom % excess), 8.33. Found: C, 61.4; H, 10.6; ^2H, 8.31.

A solution of the ester (1.65 g, 14.1 mmol) in 11.3 mL of 2.5 N NaOH in 50% methanol was stirred overnight at room temperature, diluted with H_2O (15 mL), saturated with NaCl, and extracted six times with ether (15 mL). Combined ether extracts were dried over K_2CO_3, and ether and methanol were removed slowly at atmospheric pressure. The residue (1.3 mL) was oxidized as described above, and the ethereal solution of product (9.5 mequiv of acid) was extracted with a slight excess of NaOH. Sodium propionate (670 mg, 50%) was precipitated from 0.5 mL of aqueous solution with acetone (30 mL), purified twice by partition chromatography on silicic acid (Varner, 1957), and crystallized as described above.

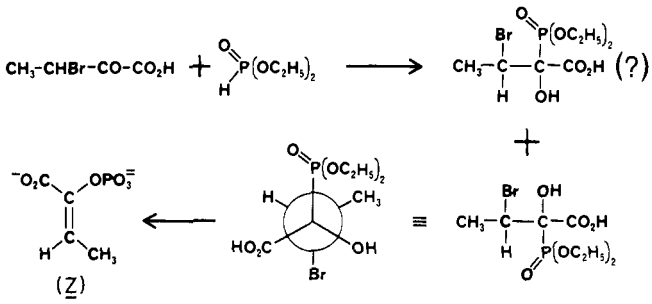

FIGURE 2: Condensation of diethyl phosphonate and 3-bromo-2-ketobutyric acid, and structure of adduct leading to (*Z*)-phosphoenol-2-ketobutyrate.

TABLE I: Kinetic Data for (*Z*)- and (*E*)-Phosphoenol-2-ketobutyrate in the Pyruvate Kinase Reaction.

Compound	V_{max} (μmol min^{-1} mg^{-1})	K_m (M × 10^5)
(*Z*)-Phosphoenol-2-ketobutyrate	0.09[a]	1.7[a]
(*E*)-Phosphoenol-2-ketobutyrate	0.31	16
Phosphoenolpyruvate	130[b]	2.6[c]

[a] Stubbe and Kenyon (1971) reported V_{max} of 0.10 and K_m of 2.5 × 10^{-5}. [b] Stubbe and Kenyon (1972). [c] Nowak and Mildvan (1970).

Synthesis of 4-Phenylphenacyl Propionate. A solution of sodium propionate (9.6 mg, 0.1 mmol) in water (0.2 mL) was adjusted to pH 7 with 0.02 N HCl, treated with 4-phenylphenacyl bromide (27.5 mg, 0.1 mmol) in ethanol (1.3 mL), and refluxed for 1 h. The reaction mixture was filtered hot through glass wool and concentrated to dryness. The residue was dissolved in acetone (0.5 mL), centrifuged to remove NaBr, and the supernatant solution was concentrated to dryness. The residue was dissolved in a small volume of acetone and applied on a preparative silica gel plate (20 × 20 cm, 2 mm) which was developed with toluene. The product (visualized by UV) was eluted with acetone, solvent was removed, and the residue was dissolved in ether (2 mL). Insoluble material was removed by filtration and the filtrate concentrated to dryness: yield 16.6 mg (62%); mp 101–102 °C (reported mp 102 °C (Drake and Bronitsky, 1930)).

Initial Velocities of Pyruvate Kinase with (Z)- and (E)-Phosphoenol-2-ketobutyrate. Rate measurements in triplicate were carried out according to Mildvan and Leigh (1964) in 2.0 mL containing bovine serum albumin (1 mg), $MgSO_4$ (8 μmol), KCl (120 μmol), NADH (0.1 μmol), Tris-Cl, pH 7.5 (100 μmol), rabbit muscle lactate dehydrogenase (0.6 mg), *E* or *Z* analogue (2 × 10^{-4} to 1.5 × 10^{-5} M), and ADP (2 × 10^{-4} to 8 × 10^{-4} M). The reaction was initiated by adding 25 μL of appropriately diluted pyruvate kinase, and decrease in absorption of NADH at 340 nm was followed with a recording Gilford spectrophotometer. The slope of the tangent to the recorded curve at the extrapolated starting point was taken as a measure of initial velocity.

Sodium (2R)-[2-^{2}H]Propionate from Homoserine. Sodium propionate was prepared from homoserine by the action of cystathionase exactly as described by Krongelb et al. (1968). It was further purified by steam distillation as described above.

Results and Discussion

Synthesis of Substrates. Treatment of 3-bromo-2-ketobutyric acid with trimethyl phosphite in a Perkow reaction at room temperature, according to the procedure of Clark and Kirby (1966) for the synthesis of PEP, afforded a mixture of about 80% (*Z*)- and 20% (*E*)-phosphoenol-2-ketobutyrates (Bondinell and Sprinson, 1970). Stubbe and Kenyon (1971) found that a change in reaction conditions from room temperature to 0 °C resulted in isolation of pure *Z* analogue. In a related reaction diethyl phosphonate and 3-bromo-2-ketobutyric acid in boiling ether solution, according to Cramer and Voges (1959), gave high yields of adduct from which we obtained pure *Z* isomer with aqueous base at room temperature. In contrast to the acid the methyl ester of 3-bromo-2-ketobutyric acid and trimethyl phosphite at 0 °C gave a mixture of 75% *Z* and 25% *E* analogues (Stubbe and Kenyon, 1971). In view of the complex nature of the Perkow reaction (Choppard et al., 1965; Borowitz et al., 1973), it is difficult to rationalize these results. Our assignments of *E* and *Z* stereochemistry on the basis of proton NMR were confirmed by the measurement of Stubbe and Kenyon (1971) of the $^{13}CC{=}C^1H$ coupling constants of a mixture of *E* and *Z* [1-^{13}C]-enriched methyl esters. The adduct obtained from diethyl phosphonate and 3-bromo-2-ketobutyric acid probably represented only one of two possible diastereoisomers (Figure 2), as suggested by its sharp melting point, and NMR of the free acid and its methyl ester. The structure assigned to the adduct is based on a presumed anti orientation of the C–Br and C–P bonds during the rearrangement–elimination leading to (*Z*)-phosphoenol-2-ketobutyrate.

Pure *E* isomer was prepared from *erythro*-3-chloro-2-hydroxybutyric acid according to the method of Cramer and Weimann (1961) and Lauppe et al. (1972) for preparing PEP. This stereospecific result could be expected from anti elimination of halide during the procedure. The *E* isomer was also prepared from *E* and *Z* mixtures by removing the *Z* isomer which reacted more rapidly in the pyruvate kinase reaction, and isolating residual *E* isomer in pure form. An attempt to prepare the *E* isomer by phosphorylation of *erythro*-3-chloro-2-hydroxybutyric acid with $POCl_3$ in a manner analogous to the synthesis of PEP from 3-chlorolactic acid (Baer, 1952) gave a mixture of *E* and *Z* isomers in a ratio of 1:1.

Kinetic Properties of (E)- and (Z)-Phosphenol-2-ketobutyrates. Maximum velocity and K_m values were obtained for the *E* and *Z* isomers as pseudosubstrates in the pyruvate kinase reaction (Table I). The K_m for the *Z* isomer is close to that of PEP, whereas that of the *E* isomer is 10-fold larger. On the other hand, V_{max} for *E* and *Z* isomers are of the same order of magnitude, approximately 1000-fold lower than V_{max} for PEP.

Formation of Chiral Propionates. [3-^{2}H]-2-Ketobutyrates, formed enzymically from the PEP analogues, were converted to propionate in the presence of an excess of H_2O_2 in order to prevent possible nonenzymic exchange of keto acid with ^2H_2O during prolonged incubation with enzyme. It was found that propionic acid from these incubations was best purified by steam distillation from the reaction mixture, isolation as sodium propionate, and recrystallization from ethanol–acetone, as indicated in Experimental Section. Abundance of various ^{2}H species was calculated from mass spectral analysis of 4-phenylphenacyl propionates (Table II). Carbon and hydrogen analyses of the sodium salts gave acceptable values.

Synthetic (2*S*)-[2-^{2}H]propionic acid was prepared on a large scale by the reactions shown in Figure 3. (2*R*,3*S*)-[3-^{2}H]-Butanol-2, which gave the reported rotation (Helmkamp et al., 1956), was oxidized with hypobromite according to Retey and Lynen (1965) to propionic acid which was distilled

TABLE II: Analytical Data of Chiral Sodium [2-^{2}H]Propionates.

Source	% abundance of ^{2}H species[a]				Elementary anal.[b]		^{2}H[c] (atom % excess)
	d_0	d_1	d_2	d_3	C	H	
(*Z*)-Phosphoenol-2-ketobutyrate	9.8	90.2			36.6	5.36	
(*E*)-Phosphoenol-2-ketobutyrate	8.9	86.0	5.1		37.4	5.61	
L-Homoserine[d]		5.6	56.0	38.4	37.6	5.41	
(2*R*,3*S*)-[3-^{2}H]Butanol-2	1.8	98.2			36.4	5.03	18.5
(2*R*,3*S*)-[3-^{2}H]-2-Acetoxybutane					36.6	5.05	18.9

[a] Calculated from parent ions of 4-phenylphenacyl propionate mass spectra (Caprioli, 1972). [b] Calculated for $C_3H_4{}^2HO_2Na$: C, 37.1; apparent H as reported in elementary analysis, 5.16. [c] Deuterium analysis by falling drop method on ^2H_2O obtained by combustion. Calculated: ^{2}H, 20.0 atom % excess. [d] From 2-ketobutyrate formed in cystathionase reaction.

TABLE III: Optical Rotatory Dispersion Values for [2-^{2}H]Propionates.

Source:	Phosphoenol-2-ketobutyrate[a]		Homoserine[b]	(2*R*,3*S*)-[3-^{2}H]-2-Acetoxybutane	(2*R*,3*S*)-[3-^{2}H]-Butanol-2	(2*R*,3*S*)-[3-^{2}H]-Butanol-2
Compound:[c]	*Z*	*E*	Sodium propionate			Propionic acid
Chirality:	(−)*R*[d]	(+)*S*	(−)*R*	(+)*S*[d]	(+)*S*	(+)*S*
330[f]				3.6	4.0	5.7
320				3.6	4.1	6.2
310				4.3	4.5	6.7
300	2.0[g]	2.0[g]	1.3	4.8	5.4	9.2
290	2.1	1.8	2.3	5.6	6.1	10.4
280	2.8	2.3	2.4	6.7	7.4	11.9
270	3.2	2.7	3.0	8.3	8.7	15.1
260	4.5	3.0	3.8	9.7	10.9	19.4
250	5.7	3.4	5.1	13.1	14.2	25.8
245	6.9	4.5	6.0	15.1	17.0	31.6
240	8.9	5.0	7.7	18.4	20.3	41.2
235	11.7		9.1	22.9	25.2	55.7
230	15.2	7.2	11.2			71.0
240[e]	9.9	5.8			20.7	

[a] Three independent experiments were carried out with the *Z* isomer and two with the *E* isomer. Rotations agreed within 10%. [b] Propionate from 2-ketobutyrate formed in cystathionase reaction. [c] Concentration, 14–16 mg and 4.1 mg of sodium propionate and propionic acid, respectively, per mL of H_2O in a 3-mL cell of 1-cm light path. [d] Similar rotations were obtained on these samples with a Cary 60 spectropolarimeter in Dr. G. Fasman's laboratory, Brandeis University. [e] Corrected for presence of d_0 and d_2 species (Table II). [f] Wavelength, nm. [g] $[\alpha]_{nm}^{25}$.

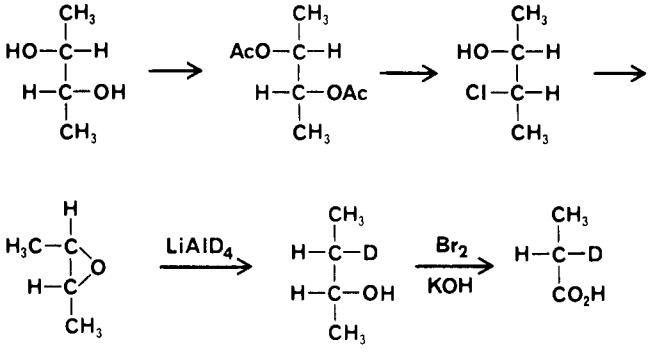

FIGURE 3: Synthesis of (2*S*)-[2-^{2}H]propionic acid from (2*R*,3*R*)-butanediol.

twice within a one degree range and converted to the sodium salt. Sodium (2*S*)-[2-^{2}H]propionate was also prepared from (2*R*,3*S*)-[3-^{2}H]-2-acetoxybutane. ORD spectra were taken on the free acid and the sodium salts (Table III). The values for (2*S*)-[2-^{2}H]propionic acid were reasonably close to those reported at 250 and 230 nm by Retey et al. (1966), i.e., +27.5 and +80 °C. Depending on wavelength, our data for synthetic sodium (2*S*)-[2-^{2}H]propionate were 65 to 90% higher than those reported for the *R* isomer by Zagalak et al. (1966) who used a different reaction scheme for its synthesis. We are unable to explain the discrepancy between our ORD values and those of Zagalak et al. (1966). However, since we obtained essentially identical values from two independently synthesized sodium [2-^{2}H]propionates, and the values for propionic acid were in agreement with those of Retey et al. (1966), we considered it justified to compare the rotations of the sodium propionates from the (*Z*)- and (*E*)-phosphoenol-2-ketobutyrates with those of the sample from the carefully distilled propionic acid.

Rotations of Chiral Propionates. The propionates from (*Z*)- and (*E*)-phosphoenol-2-ketobutyrate contained 90.2% and 86.0%, respectively, of the theoretical amount of deuterium at C-2 (Table II). The rotation at 240 nm of propionates from PEP analogues and of the synthetic propionate was therefore corrected to a value which would correspond to 100% optically active species, and this is recorded in the last line of Table III. The rotation of the (2*R*)-[2-^{2}H]propionate, derived from (*Z*)-phosphoenol-2-ketobutyrate (Figure 1), is 47% of that of the synthetic material (i.e., 53% is *RS*), and the rotation of the (2*S*)-[2-^{2}H]propionate, from the *E* isomer, is 29% of that shown by synthetic propionate (i.e., 71% is *RS*). Sodium (*S*)-[2-^{2}H]propionate derived from L-homoserine in the cystathionase reaction (Greenberg, 1957) had only 5% d_1 species. Hence the substantial rotation of +7.7 at 240 nm must be due to a large fraction of chiral molecules labeled on both C-2 and C-3. This sample of propionate was prepared as a potential standard since it was reported to have rotations close to those of synthetic propionate (Krongelb et al., 1968). A low value

of $[8.5]°_{240}$ was also reported by Posner and Flavin (1972) for a sample of propionate isolated from the cystathionine γ-synthase reaction.

Protonation of the pseudosubstrates or of their enol intermediates in the pyruvate kinase reaction occurred only partially stereospecifically. Thus protonation at C-3 of the 2 *si*, 3 *re* face was 47% in (*Z*)-phosphoenol-2-ketobutyrate (Figure 1) and only 29% in the *E* isomer. Since 0.9 atom of ^2H was incorporated on C-3 of 2-ketobutyrate from either analogue, it would appear that considerable nonstereoselective protonation had occurred. Our results are therefore not analogous to the stereospecific protonation reported by Rose (1970) with isotopically asymmetric [3-^{2}H,^{3}H]phosphoenolpyruvate. An explanation for the unexpected stereochemistry of protonation is that distortion of the analogues in the active site of the enzyme resulted in release of the enol and consequent random protonation in solution to give rather large fractions of *RS* species. Presumably the enzyme binds *E*-enol less effectively than *Z*-enol, resulting in greater random protonation of *E*-enol. Pyruvate kinase ionizes the protons on C-3 of pyruvate in the presence of ATP, Mg^{2+}, and K^+, but substitution of a methyl group on C-3, as in 2-ketobutyrate, greatly diminishes this enolization process (Rose, 1960).

A less likely explanation for our results is that the bulky methyl group in the substrate sufficiently distorts the ternary enzyme complex so as to promote partial protonation at C-3 of the 2 *re*, 3 *si* face, about 25% for the *Z* isomer, and about 35% for the *E* isomer. Distortion owing to the methyl group may be greater with (*E*)-phosphoenol-2-ketobutyrate since its K_m is considerably higher than that of the *Z* analogue (Table I), and therefore its enol may fit the active site more poorly. We have assumed that the natural substrate, PEP, yields stereospecifically pure pyruvate, and essentially completely chiral acetyl CoA and malate in the reactions used by Rose (1970) to determine the stereochemical course of the pyruvate kinase reaction. However, a certain amount of racemization may also occur in pyruvate owing to release of enol from enzyme. It is not clear whether the technique of isotope discrimination is sufficiently sensitive to detect such racemization.

A small fraction (5%) of the propionate from (*E*)-phosphoenol-2-ketobutyrate was doubly labeled with ^2H, and we made the reasonable assumption that it is on C-2 (C-3 of 2-ketobutyrate). This small incorporation may be due to proton exchange by reversal of the forward reaction as observed by Robinson and Rose (1972) for PEP.[2]

Acknowledgments

We are most grateful to Dr. E. A. Kabat, Dr. Florence Maisonrouge-McAuliffe, and Dr. S. Beychok for their patient help with the optical rotatory dispersion measurements, and instruction in the use of the spectropolarimeter. Thanks are due to Dr. Kabat for the hospitality of his laboratory in using the instrument. We also thank Dr. G. D. Fasman for the use of his Cary 60 spectropolarimeter. We thank Dr. G. K. Helmkamp for a generous gift of (2*R*,3*S*)-[3-^{2}H]-2-acetoxybutane, and Dr. F. J. Simpson for a generous gift of (2*R*,3*R*)-butanediol. It is a pleasure to acknowledge many stimulating discussions with Dr. I. A. Rose, Dr. A. S. Mildvan, and Dr. Mildred Cohn.

[2] Note Added in Proof: The presence of only one diastereomer in diethyl (1-hydroxy-1-carboxy-2-bromopropyl) phosphonate was also shown by ^{13}C NMR. The trans configuration of the Br and P atoms was established in a single-crystal x-ray diffraction structure determination by Dr. Lawrence S. Rosen.

References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235,* 769.
Baer, E. (1952), *Biochem. Prep. 2,* 24.
Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 459.
Bondinell, W. E., and Sprinson, D. B. (1970), *Biochem. Biophys. Res. Commun. 40,* 1464–1467.
Borowitz, E. J., Yee, K. C., and Crouch, R. K. (1973), *J. Org. Chem. 38,* 1713.
Caprioli, R. M. (1972), in Biochemical Applications of Mass Spectrometry, Waller, G. R., Ed., New York, N.Y., Wiley-Interscience, p 751.
Choppard, P. A., Clark, V. M., Hudson, R. F., and Kirby, A. J. (1965), *Tetrahedron 21,* 1961.
Clark, V. M., and Kirby, A. J. (1966), *Biochem. Prep. 11,* 101.
Cramer, F., and Voges, D. (1959), *Chem. Ber. 92,* 952.
Cramer, F., and Weimann, G. (1961), *Chem. Ber. 94,* 996.
Drake, N. L., and Bronitsky, J. (1930), *J. Am. Chem. Soc. 52,* 3715.
Friedemann, T. E., and Haugen, G. E. (1943), *J. Biol. Chem. 147,* 415.
Greenberg, D. M. (1957), *Methods Enzymol. 5,* 936.
Hanson, K. R. (1966), *J. Am. Chem. Soc. 88,* 2731.
Helmkamp, G. K., Joel, C. D., and Sharman, H. (1956), *J. Org. Chem. 21,* 844.
Krongelb, M., Smith, T. A., and Abeles, R. H. (1968), *Biochim. Biophys. Acta 167,* 473.
Lauppe, H. F., Rau, G., and Hengstenberg, W. (1972), *FEBS Lett. 25,* 357.
Leroux, P. J., and Lucas, H. J. (1951), *J. Am. Chem. Soc. 73,* 41.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Melikoff, P. v. (1886), *Ann. Chem. 234,* 205.
Mildvan, A. S., and Leigh, R. A. (1964), *Biochim. Biophys. Acta 89,* 393.
Nowak, T., and Mildvan, A. S. (1970), *J. Biol. Chem. 245,* 6057.
Payne, G. B., and Williams, P. H. (1959), *J. Org. Chem. 24,* 54.
Posner, B. I., and Flavin, M. (1972), *J. Biol. Chem. 247,* 6412.
Reed, G. H., and Cohn, M. (1973), *J. Biol. Chem. 248,* 6436.
Retey, J., and Lynen, F. (1965), *Biochem. Z. 342,* 256.
Retey, J., Umani-Ronchi, A., and Arigoni, D. (1966), *Experientia 22,* 72.
Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. (1961), *J. Biol. Chem. 236,* 2277.
Robinson, J. L., and Rose, I. A. (1972), *J. Biol. Chem. 247,* 1096.
Rose, I. A. (1960), *J. Biol. Chem. 235,* 1170.
Rose, I. A. (1970), *J. Biol. Chem. 245,* 6052.
Sprinson, D. B., and Chargaff, E. (1946), *J. Biol. Chem. 164,* 417.
Stubbe, J. A., and Kenyon, G. L. (1971), *Biochemistry 10,* 2669.
Stubbe, J. A., and Kenyon, G. L. (1972), *Biochemistry 11,* 338.
Woods, A. E., Chatman, V. B., and Clark, R. A. (1972), *Biochem. Biophys. Res. Commun. 46,* 1.
Varner, J. E. (1957), *Methods Enzymol. 3,* 397.
Zagalak, B., Frey, P. A., Karabatsos, G. L., and Abeles R. H. (1966), *J. Biol. Chem. 241,* 3028.