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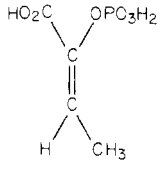
Stereospecificity of (*E*)- and (*Z*)-Phosphoenol-α-ketobutyrate with Chicken Liver Phosphoenolpyruvate Carboxykinase and Related Phosphoenolpyruvate-Utilizing Enzymes†

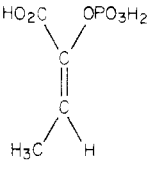
Thomas H. Duffy, Howard J. Saz, and Thomas Nowak*,‡

ABSTRACT: The diastereomers (*E*)- and (*Z*)-phosphoenol-α-ketobutyrate (PEB) were synthesized and purified as analogues of the substrate phosphoenolpyruvate. The two isomers are distinguishable by their differences in ^1H chemical shifts. These analogues have been shown to have no substrate activity with phosphoenolpyruvate carboxykinase, enolase, or pyruvate phosphate dikinase. These isomers do exhibit substrate activity with pyruvate kinase, however. The Mn^{2+}-activated pyruvate kinase shows no significant differences in K_m or V_{max} values between *E*- and *Z*-PEB. The Mg^{2+} enzyme also shows no substantial differences in V_{max} for the diastereomers but shows a dramatic difference between the K_m of *Z*-PEB (K_m = 5 μM) and of *E*-PEB (K_m = 500 μM). The cation thus appears to affect the specificity of the enzyme for these ligands. These analogues showed specificity of inhibition of Mn^{2+}-activated pyruvate kinase. The *Z* isomer (K_I = 0.45 μM) is more effective than the *E* isomer (K_I = 15 μM) in causing inhibition. PRR titrations demonstrate that the dissociation constants, K_3, of these ligands from the enzyme-Mn complex agree with the K_I values and that the *Z* isomer is more like phosphoenolpyruvate than is *E*-PEB with respect to ligand binding to the enzyme. The enzyme phosphoenolpyruvate carboxykinase demonstrates the opposite stereospecificity of inhibition. The *E* isomer (K_I = 1.5 μM) is a more potent inhibitor than is the *Z* isomer (K_I = 32 μM). PRR titration studies demonstrate agreement between K_3 and K_I values. The titration of the enzyme-Mn with the *Z* isomer resembles the titration of the enzyme-Mn complex with phosphoenolpyruvate. The Mn^{2+}-activated enolase exhibits absolute stereospecificity of inhibition with K_I (*Z*-PEB) = 11 μM and K_I (*E*-PEB) > 3 mM. The Mg^{2+}-activated enzyme shows no change in inhibition by *Z*-PEB (K_I = 8 μM) but is susceptible to inhibition by *E*-PEB (K_I = 80 μM). The cation appears to affect the type of interaction which occurs between *E*-PEB and enolase. PRR studies of enolase-Mn, however, demonstrate that not only does *Z*-PEB show a strong interaction with the enzyme (K_3 = 4 μM) but *E*-PEB does also (K_3 = 8 μM). The nature of this interaction and failure to elicit inhibition is still unclear.

The synthesis of both the *E* and the *Z* isomers of phosphoenolbutyrate (PEB)[1] has been previously reported by Sprinson and his colleagues (Adlersberg et al., 1977).



Z-PEB



E-PEB

Initial reports, using a *Z*,*E* (80:20) mixture, showed competitive inhibition vs. P-enolpyruvate with rabbit muscle pyruvate kinase (Woods et al., 1970). Subsequent studies have demonstrated that the *E*,*Z* mixture (Bondinell & Sprinson, 1970) and the pure *Z* isomer (Stubbe & Kenyon, 1971) also serve as substrates for pyruvate kinase. These preliminary studies demonstrated a stereospecific protonation of PEB by pyruvate kinase (Bondinell & Sprinson, 1970; Stubbe & Kenyon, 1971). The stereospecificity was identical with that demonstrated by Rose (1970), who used the isotopically asymmetric [3-^2H, ^3H]P-enolpyruvate. These experiments showed that the enolate of pyruvate, formed during the catalytic reactions, is specifically protonated by the enzyme on the *si* face of C-3 to yield pyruvate. Subsequent studies with pure *Z* and *E* isomers of PEB demonstrated that although they served as substrates for pyruvate kinase, absolute stereospecific protonation was not observed (Adlersberg et al., 1977). These results suggest that some population of the isomeric enolates of α-ketobutyrate dissociates from the enzyme prior to protonation.

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[1] Abbreviations: PEB, phosphoenol-α-ketobutyrate; P-enolpyruvate, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; 2-PGA, D(+)-2-phosphoglycerate; OAA, oxalacetate; PRR, solvent proton longitudinal relaxation rate.

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The homologous enzyme P-enolpyruvate carboxykinase (PEPCK) from pigeon liver has the same stereospecificity as does pyruvate kinase. P-enolpyruvate has been shown to be carboxylated at the *si* face of C-3 to yield oxalacetate (Rose et al., 1969). Preliminary studies with PEB (no stereochemical assignment made) showed strong competitive inhibition against P-enolpyruvate with pigeon liver (Söling et al., 1971) and hog liver (Silverstein et al., 1980) PEPCK. The enzymes pyruvate kinase and PEPCK are stereospecifically inhibited by D- and by L-2-phospholactate with opposite stereospecificites for these two enzymes (Nowak & Mildvan, 1970).

The enzyme enolase catalyzes a trans hydration of P-enolpyruvate to yield D-2-PGA (Cohn et al., 1970). Although several substrate analogues act as substrates for enolase (Wold, 1971), *Z*-PEB was demonstrated not to serve as a substrate for this enzyme (Stubbe & Kenyon, 1972) but acts as a competitive inhibitor (Söling et al., 1971). Enolase was shown to catalyze the irreversible isomerization of 2-phospho-3-butenoic acid to specifically yield *Z*-PEB (Appelbaum & Stubbe, 1975). Little stereospecificity of inhibition by D- and L-phospholactates was observed with enolase (Nowak & Mildvan, 1970). Stereospecificity in the vertical plane of the P-enolpyruvate site has been investigated with these enzymes; however, stereospecificity in the horizontal plane has not been adequately addressed.

The present paper describes a modified synthesis and purification of the *Z* and *E* diastereomers of PEB. These isomers have been characterized by high-resolution ^1H NMR. So that insight into the stereoselectivity of various P-enolpyruvate-utilizing enzymes could be obtained, these analogues have been tested kinetically as possible substrates and inhibitors of PEPCK from chicken liver and from *Ascaris*, rabbit muscle pyruvate kinase, yeast enolase, and pyruvate phosphate dikinase from *Bacteroides symbiosus*. PRR titrations were performed with several of the enzymes to determine the binding constants and to obtain structural information as to the nature of the binding of the analogues to the respective enzyme–Mn complexes.

Experimental Procedures

Materials

PEPCK was purified from chicken liver as previously described (C. Hebda and T. Nowak, unpublished results). PEPCK from *Ascaris* muscle was partially purified via the methods previously described (Bueding & Saz, 1968). Yeast enolase was purified by the procedure of Westhead & McLain (1964). Lactate dehydrogenase, malate dehydrogenase, hexokinase, catalase, and pyruvate kinase were purchased from Boehringer Mannheim Corp. *B. Symbiosus* pyruvate phosphate dikinase, purified by the method of Milner et al. (1975), was a generous gift from Drs. N. Goss and H. G. Wood (Case Western Reserve University). P-enolpyruvate, IDP, ADP, AMP, and NADH were purchased from Sigma. $NaH^{14}CO_3$ was purchased from Amersham/Searle Co. All other reagents were of the highest purity commercially available. All solutions were made by using distilled water which was passed through a mixed-bed deionizing column.

Methods

Kinetic Assays. Initial velocity studies of PEPCK were performed by measuring the rate of formation of OAA from P-enolpyruvate with a continuous assay coupled with malate dehydrogenase as previously described (C. Hebda and T. Nowak, unpublished results). The reaction mixture consisted of Tris-HCl, pH 7.4 (65 μmol), KCl (100 μmol), $MnCl_2$ (4 μmol), P-enolpyruvate (2 μmol), IDP (2 μmol), β-mercaptoethanol (143 μmol), $KHCO_3$ (200 μmol), NADH (0.14 μmol), and 22 eu of malate dehydrogenase in a 1-mL volume. The reaction was initiated by the addition of an appropriate amount of PEPCK. The decrease in absorption of NADH was followed at 340 nm as a function of time by using a Gilford 240 or 250 spectrophotometer. The temperature was controlled at 25 °C. The direct assay which measures $^{14}CO_2$ incorporation into P-enolpyruvate has been previously described (C. Hebda and Nowak, unpublished results).

Initial velocity studies of pyruvate kinase were performed by monitoring the decrease of absorption of NADH at 340 nm using a coupled pyruvate kinase–lactate dehydrogenase assay. The assay consisted of Tris-HCl, pH 7.4 (50 μmol), KCl (100 μmol), $MnCl_2$ (4 μmol), ADP (1 μmol), NADH (0.14 μmol), P-enolpyruvate (2 μmol), and lactate dehydrogenase (20 μg) in a volume of 1 mL. The reaction was initiated by the addition of pyruvate kinase (Mildvan & Leigh, 1964). The temperature was kept at 25 °C.

Initial velocity studies of enolase were performed by monitoring the increase of absorption at 240 nm due to the formation of P-enolpyruvate as a function of time. The assay consisted of Tris-HCl, pH 7.5 (65 μmol), KCl (50 μmol), $MnCl_2$ (0.01 μmol), and 2-PGA (1 μmol) in a total volume of 1 mL. The reaction was initiated with enzyme. Assays performed with Mg^{2+} as the cation activator were done by using 0.5 μmol of $MgCl_2$ in place of the $MnCl_2$. The temperature was controlled at 25 °C.

Initial velocity studies of pyruvate,phosphate dikinase were performed by monitoring the decrease of absorption of NADH at 340 nm using a pyruvate,phosphate dikinase–lactate dehydrogenase coupled assay. The assay consisted of imidazole chloride, pH 6.8 (50 μmol), NH_4Cl (20 μmol), $MgCl_2$ (20 μmol), PP_i (0.04 μmol), P-enolpyruvate (1 μmol), AMP (0.4 μmol), NADH (0.14 μmol), lactate dehydrogenase (1 eu) and pyruvate,phosphate dikinase in 1 mL (South & Reeves, 1975). The temperature was maintained at 25 °C.

Proton Relaxation Rate Studies (PRR). PRR measurements were performed on a Seimco pulsed NMR spectrometer operating at 24.3 MHz using the Carr–Purcell 180–τ–90 pulse sequence (Carr & Purcell, 1954). Measurements were taken at room temperature (22 ± 1 °C). Prior to the experiments all enzymes were passed through a Bio-Rad P-2 column (1.1 × 25 cm) having a 2-cm layer of Chelex-100 on the top. The column was preequilibrated with 0.065 M Tris-HCl buffer, pH 7.4. The enzyme was concentrated by using a collodion bag, vacuum filtration apparatus. The enzyme and Mn^{2+} were combined with the proper buffer and salt, as used in the respective kinetic assays, in a final volume of 0.05 mL. Unless indicated otherwise, the concentration of Mn^{2+} was 40 μM, and the concentration of enzyme sites was in excess of Mn^{2+} (60–150 μM sites). The titrations were performed with an identical sample which also contained the ligand to be titrated. Increments of the second sample were then titrated into the first so that no change in enzyme or Mn^{2+} concentration occurred, and the PRR was then measured.

The dissociation constants and enhancements of the ternary complexes, ϵ_T, were determined by a computer fit to the PRR titration data which minimized the percent standard deviation of ϵ_T (Reed et al., 1970). The equilibria and their respective constants which were used are

$$K_1 = \frac{[M][S]}{[MS]} \qquad K_3 = \frac{[E\text{-}M][S]}{[EMS]} \qquad K_s = \frac{[E][S]}{[ES]}$$

$$K_D = \frac{[E][M]}{[EM]}$$

Values for K_I and K_D were independently evaluated. The values for K_s and K_3 were varied to obtain the "best fit" to the data. The values reported are the averages obtained from several titrations.

Synthesis of (Z)-Tripotassium Phosphoenolbutyrate (Z-PEB). Z-PEB was prepared by a modification of the methods set forth by Adlersberg et al. (1977). Preparation of diethyl 1-hydroxy-1-carboxy-2-bromopropanephosphonate–3-bromo-2-ketobutyric acid was synthesized by the method of Stubbe & Kenyon (1971). A 4-g sample of 3-bromo-2-ketobutyric acid and a 3.06-g sample of diethyl phosphonate (freshly distilled under reduced pressure before use) were dissolved in 3 mL of ether and refluxed for 24 h. A long column packed with glass beads was used as the reflux condenser, and periodic addition of ether was made to the reaction (Cramer & Voges, 1959): NMR ($CDCl_3$) δ 1.37, 1.39 (2 t, J = 7 Hz, CH_3CO, 6 H), 1.82 (br d, J = 7 Hz, CH_3CBr, 3 H), 4.34, 4.42 (2 dq, J_{H-H} = 7 Hz and J_{P-H} = 1.8 Hz, CCH_2O, 4 H), 4.74 (dq, J_{P-H} = 1.5 and J_{H-H} = 7 Hz, CHBr, 1 H). Approximate yield of 70% was obtained.

Diethyl 1-hydroxy-1-carboxy-2-bromopropanephosphonate (2.1 g, 6 mmol) in H_2O (30 mL) was treated with 1.0 N aqueous NaOH (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 (1.5 mL/min) (Bartlett, 1959). For maximization of recovery, a loading rate of ≤1.5 mL/min was used. The NaOH treatment and column separation were kept in the dark to avoid unwanted photochemical reactions. Elution with 0.06 N HCl gave unwanted products, and elution with 0.1 N HCl gave pure Z-PEB (monitored by A_{240}). The fractions which absorb at 240 nm were pooled and neutralized with KOH, and the solvent was removed under reduced pressure. Concentrations were calculated from phosphate tests (Ames, 1966).

The NMR spectrum (D_2O) showed a peak at δ 2.44 (dd, J = 2.5 and 7 Hz) and a peak at δ 6.75 (dq, J = 2.5 and 7 Hz). (Figure 1B).

Synthesis of (E)-Tripotassium Phosphoenolbutyrate (E-PEB). β-Bromo-α-ketobutyric acid was synthesized by the method of Stubbe & Kenyon (1971). The NMR spectrum ($CDCl_3$) showed a peak at δ 1.75 (3 H doublet, J = 6 Hz) and a peak at δ 5.20 (1 H, quartet, J = 6 Hz). An E,Z (15:85) mixture of cyclohexylammonium dihydrogen phosphoenolbutyrate was synthesized by the methods described by Adlersberg et al. (1977). This resultant E,Z mixture of PEB was photoisomerized by a modification of the previously described method (Adlersberg et al., 1977). A 4-g sample of an E,Z mixture of the cyclohexylammonium salt was desalted and the solution made 50 mM. By use of quartz cuvettes and a 450-W (medium pressure mercury lamp) UV source, a 15:85 E,Z mixture was photoisomerized (1.5 h) to obtain a 45:55 E,Z mixture of isomers (Figure 2).

The E isomer was obtained pure by a modification of the enzymatic treatment set forth by Adlersberg et al. (1977). The photoisomerized mixture (20 mM) in H_2O (95 mL) which contained Na_2ADP (1 mmol), D-glucose (16 mmol), KCl (100 mmol), $MgSO_4$ (12 mmol), and Tris buffer (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 16 h. Hydrogen peroxide (5 mL, 28%) was added to decompose α-ketobutyrate, and 1 h later, catalase (2.7 mg) was added to destroy any 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

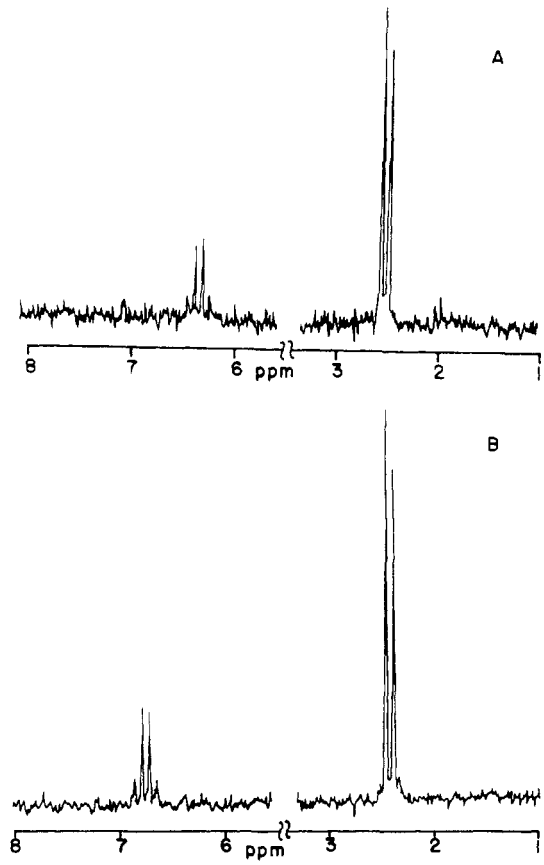

FIGURE 1: Phosphorus-decoupled proton nuclear magnetic resonance spectrum at 100 MHz (in D_2O) of (A) E-tripotassium phosphoenol-α-ketobutyrate and (B) Z-tripotassium phosphoenol-α-ketobutyrate. The pH values for both samples were identical (pD = 7.0).

(1.5 mL/min) (Bartlett, 1959). Stepwise elution with 0.02 N and 0.06 N HCl washed unwanted material from the column (monitored by A_{240}). Elution with 0.1 N HCl gave pure E-PEB. The fractions which absorb at 240 nm were pooled and neutralized with KOH, and the solvent was removed under reduced pressure. Concentrations were calculated from phosphate tests as described previously. The NMR spectrum (D_2O) showed a peak at δ 2.55 (dd, J = 1.9 and 7.4 Hz) and a peak at δ 6.35 (dq, J = 1.9 and 7.4 Hz) (Figure 1A).

Results

Synthesis of E- and Z-PEB. The pure Z isomer of PEB was obtained by modification of the methods set forth by Adlersberg et al. (1977). The Z isomer was shown to be greater than 95% free of E isomer by ^1H NMR (Figure 1B). The procedures used by Stubbe & Kenyon (1971) failed, in our hands, to yield pure Z isomer; 5–10% of contaminant of the E isomer was always present. Pure E isomer was obtained by preferential removal of the Z isomer from an E,Z mixture using the enzymes pyruvate kinase and hexokinase. The E and Z isomers have different ^1H NMR chemical shifts and therefore provide a means of differentiation of the two isomers (Figure 2). The E isomer was shown to be greater than 94% free of Z isomer by NMR (Figure 1A). No contaminants of the opposite isomer were detected. The limits of purity are based on the signal to noise of the resultant ^1H spectra obtained.

PEB Isomers as Substrates. Both the E and the Z isomers of PEB were tested as substrates for chicken liver P-enolpyruvate carboxykinase. Neither isomer showed substrate activity with the continuous assay. The limits of detection for

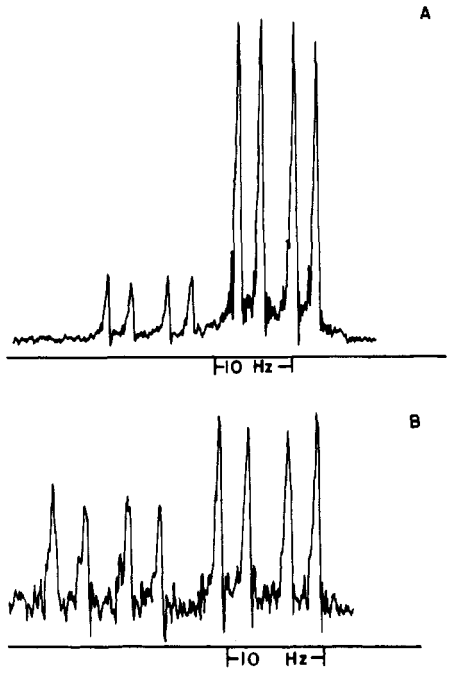

FIGURE 2: Proton nuclear magnetic resonance spectrum (100 MHz) of the upfield methyl protons of (A) an *E,Z* (15:85) mixture of phosphoenol-α-ketobutyrate before UV irradiation and (B) an *E,Z* (40:60) mixture of phosphoenol-α-ketobutyrate after 1.25 h of UV irradiation. The ^{31}P decoupler was not used for these spectra.

Table I: Kinetic Constants for *Z*- and *E*-PEB as Substrates for Pyruvate Kinase

substrate	divalent cation	K_m (μM)	V_{max} (unit/mg)	V_m/K_m (mg^{-1} min^{-1})
PEP[a]	Mn^{2+}	19.0	100	5.3×10^3
Z-PEB	Mn^{2+}	4.6	0.035	7.6
E-PEB	Mn^{2+}	5.4	0.054	10.0
PEP[a]	Mg^{2+}	35	200	5.7×10^3
Z-PEB	Mg^{2+}	5.3	0.040	7.6
E-PEB	Mg^{2+}	500	0.050	0.10

[a] Abbreviation for P-enolpyruvate.

this assay were 0.05% of that measured with P-enolpyruvate as substrate. A direct assay measuring $^{14}CO_2$ incorporation was also performed. Neither isomer showed incorporation of $^{14}CO_2$. The limits of detection for this assay were <2% of that measured with P-enolpyruvate as substrate.

Both the *E* and the *Z* isomers of PEB were shown to act as substrates in the pyruvate kinase catalyzed reaction, by using the LDH-coupled enzyme assay. The Mn^{2+}-activated enzyme gave a Michaelis constant (K_m) of 4.6×10^{-6} M and a V_{max} of 0.035 μmol min^{-1} mL^{-1} mg^{-1} (unit/mg) for the *Z* isomer and a K_m of 5.4×10^{-6} M and V_{max} of 0.054 unit/mg for the *E* isomer. The Mg^{2+}-activated enzyme gave a K_m of 5.26×10^{-6} M and a V_{max} of 0.040 unit/mg for the *Z* isomer and a K_m of 5.0×10^{-4} M and a V_{max} of 0.050 unit/mg for the *E* isomer. The V_{max} values for the Mn^{2+}- and the Mg^{2+}-activated enzymes are comparable for these two substrate analogues. These results are in contrast to the differences in V_{max} measured with Mg^{2+} (100%) and with Mn^{2+} (50%) as activators and with P-enolpyruvate as substrate. The K_m of the *E* isomer with the Mg^{2+}-activated enzyme is 2 orders of magnitude greater than that measured for *E*-PEB with the Mn^{2+}-activated enzyme. These kinetic parameters have been summarized in Table I. α-Ketobutyrate has been previously shown to be a good substrate for lactate dehydrogenase (Stubbe & Kenyon, 1971).

Neither the *E* nor the *Z* isomer of PEB showed substrate activity in the enolase reaction. These experiments were performed with both the Mg^{2+}- and the Mn^{2+}-activated enzymes with identical results. Detectable limits for activity of enolase were 0.009% of the activity measured with 2-PGA as substrate.

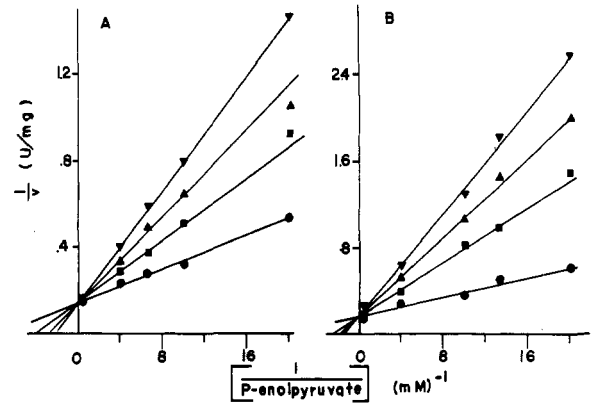

FIGURE 3: Double-reciprocal plots of $1/v$ vs. 1/[P-enolpyruvate] for chicken liver PEPCK with fixed varying concentrations of *E*-PEB and *Z*-PEB. The experimental conditions utilized were as follows: Tris-HCl, pH 7.4 (65 μmol), KCl (100 μmol), $MnCl_2$ (4 μmol), IDP (2 μmol), mercaptoethanol (143 μmol), $KHCO_3$ (200 μmol), NADH (0.14 μmol), and 22 eu of malate dehydrogenase in a final volume of 1 mL. The reaction was initiated by the addition of PEPCK. The concentrations of P-enolpyruvate were as indicated. In (A), the concentrations of *E*-PEB used were (●) 0, (■) 1.45, (▲) 2.32, and (▼) 3.48 μM. In (B), the concentrations of *Z*-PEB were (●) 0, (■) 60, (▲) 100, and (▼) 140 μM.

E- and Z-PEB as Enzyme Inhibitors. Chicken liver PEPCK is stereoselectively inhibited by the two diastereomers of PEB. The results of a kinetic inhibition study are shown in Figure 3. Both isomers showed linear competitive inhibition with respect to P-enolpyruvate; secondary plots of the data (not shown) are linear. The inhibition constant, K_I, for *Z*-PEB was 32 μM while the K_I value for *E*-PEB was 1.5 μM. A K_m/K_I ratio of 5.2 for the *Z* isomer and 110.7 for the *E* isomer were calculated; the *E* isomer is 21.3 times as effective an inhibitor as the *Z* isomer. Kinetic experiments were also attempted by using Mg^{2+} as the activating cation. The results of such experiments were less sensitive since PEPCK, an enzyme which has a low turnover number, shows only 2% V_{max} with Mg^{2+} when compared to the V_{max} with Mn^{2+} as the divalent activator. When Mg^{2+} is used as the activator (at 5 mM), the kinetic results yield a K_m for P-enolpyruvate of 4.2 mM. Inhibition kinetics show a K_I value for *Z*-PEB of 4.0 mM and a K_I value for *E*-PEB of 4.2 mM under these conditions. These results are summarized in Table II.

PEPCK from *Ascaris* is also stereoselectively inhibited by the two isomers of PEB. Both isomers showed linear competitive inhibition with respect to P-enolpyruvate. The inhibition constant, K_I, for *Z*-PEB was 108 μM while the K_I value for *E*-PEB was 3.5 μM. As observed with avian liver PEPCK, the *E* isomer is a more potent inhibitor than the *Z* isomer. The results of the inhibition studies are summarized in Table II.

The Mn^{2+}-activated pyruvate kinase is also stereoselectively inhibited by the two isomers of PEB. Both isomers show linear competitive inhibition with respect to P-enolpyruvate and yield a K_I for *Z*-PEB of 0.45 μM while the K_I value for *E*-PEB was 15 μM. A K_m/K_I ratio of 42.2 for the *Z* isomer and 1.26 for

Table II: Inhibition Constants for *Z*- and *E*-PEB

isomer	enzyme	K_I	K_m/K_I [f]	$K_{I,Z}/K_{I,E}$
Z [a]	PEPCK [c]	32 ± 1 μM	5.2	21.0
E [a]		1.5 ± 0.3 μM	111.0	
Z [b]	PEPCK [c]	4.0 ± 0.6 mM	1.0	0.95
E [b]		4.2 ± 0.5 mM	1.0	
Z [a]	PEPCK [d]	108 ± 3 μM	6.6	31
E [a]		3.5 ± 0.4 μM	204	
Z [a]	pyruvate kinase	0.45 ± 0.04 μM	42.2	0.030
E [a]		15.0 ± 1.0 μM	1.26	
Z [b]	pyruvate kinase	7.1 ± 1.2 μM	4.9	0.014
E [b]		49.5 ± 10 μM	0.071	
Z [a]	enolase	11 ± 2 μM	7.54	<0.0037
E [a]		>3 mM	<0.012	
Z [b]		7.6 ± 1.5 μM	6.58	0.095
E [b]		80 ± 2 μM	1.03	
Z [b]	pyruvate,-phosphate dikinase	ND [e]		
E [b]		ND [e]		

[a] Measured with the Mn^{2+}-activated enzyme. [b] Measured with the Mg^{2+}-activated enzyme. [c] Chicken liver P-enolpyruvate carboxykinase. [d] *Ascaris* P-enolpyruvate carboxykinase. [e] None detectable. [f] The K_m values are those for P-enolpyruvate measured in the same experiment for PEPCK and for pyruvate kinase and for 2-PGA measured with enolase.

the *E* isomer were calculated. With pyruvate kinase, the *Z* isomer is 33 times as effective an inhibitor as is the *E* isomer. The Mg^{2+}-activated enzyme demonstrates the same stereoselectivity of inhibition. The *Z* isomer yields a K_I of 7.0 μM while the *E* isomer yields a K_I of 450 μM.

Enolase showed the greatest degree of stereoselectivity with respect to the two isomers of PEB. With the Mn^{2+}-activated enolase, preliminary studies with the *E* isomer showed weak noncompetitive inhibition (K_I = 1.1 mM). This experiment was repeated under conditions where the concentration of free Mn^{2+} remained constant at 10 μM. For these experiments, the K_d value for Mn–PEB was assumed to be identical with the value of Mn–PEP (1.9 mM) (Nowak & Lee, 1977), and the total [Mn^{2+}] was varied with the concentration of substrate and *E*-PEB. In this experiment, little (K_I > 3 mM) or no inhibition was obtained. Kinetic studies performed with Mg^{2+}-activated enolase showed a K_I value for *E*-PEB of 80 μM. The K_I values for *Z*-PEB of 7.6 μM and 11 μM were obtained for the Mg^{2+}- and Mn^{2+}-activated enolase respectively (Table II).

Pyruvate,phosphate dikinase shows no apparent inhibition by either the *E* or *Z* isomers (K_I > 28 mM). The K_m for PEP measured by using Mg^{2+} as the activating cation is 82 μM. The results of these inhibition studies are summarized in Table II.

Interactions of P-enolpyruvate and the E and Z Isomers of PEB to Enzyme–Mn^{2+} Complexes by PRR Titrations. The binding of P-enolpyruvate and the *E* and *Z* isomers of PEB to the chicken liver PEPCK–Mn complex was investigated by PRR titrations (Figure 4). The results of these titrations were evaluated by a computer fit to the data which takes into account all possible equilibria. The quality of the fits are expressed by the percent standard deviation of the calculated value of ϵ_T. The results of these titrations demonstrate that P-enolpyruvate and *E*-PEB interact with the enzyme–Mn complex with the same K_3 value (0.25×10^{-6} M) but with different values for ϵ_T (8.1 ± 0.2 and 3.5 ± 0.5, respectively). The *Z*-PEB interacts 2 orders of magnitude weaker than the other two ligands ($K_3 = 30 \times 10^{-6}$ M) and yields an ϵ_T of 5.40 ± 0.26. The quality of these fits were relatively insensitive

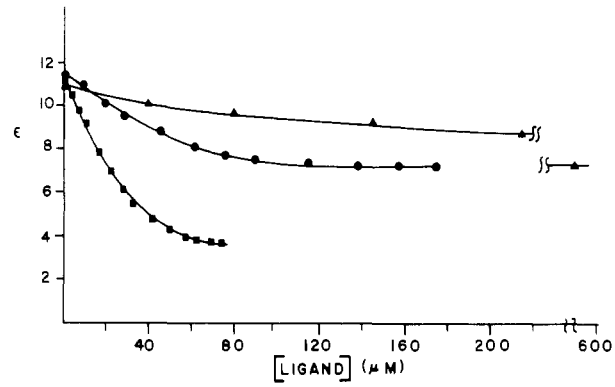

FIGURE 4: Titration of chicken liver PEPCK–Mn with P-enolpyruvate, *E*-PEB, and *Z*-PEB. A plot of observed enhancement (ϵ^*) vs. ligand concentration is made. A solution containing 1.09×10^{-4} M enzyme in 65 mM Tris-HCl, pH 7.4, containing 0.1 M KCl and 4×10^{-5} M Mn^{2+} in a final volume of 0.05 mL is titrated with a solution containing an identical solution and including either P-enolpyruvate (●), *E*-PEB (■) or *Z*-PEB (▲). The titrations shown are best fit with K_3 values of 0.25 μM, 0.25 μM, and 25 μM, respectively.

Table III: Summary of PRR Titration Parameters

enzyme	ligand	K_3 (μM)	ϵ_T	K_D (μM)
PEPCK [a]	PEP [b]	0.25 ± 0.10	8.10 ± 0.20	
	Z-PEB	30 ± 5	5.40 ± 0.26	50
	E-PEB	0.25 ± 0.10	3.50 ± 0.50	
PK [c]	PEP [b]	2.5 ± 1.0	2.03 ± 0.10	
	Z-PEB	0.45 ± 0.13	2.08 ± 0.30	70
	E-PEB	13 ± 2	7.08 ± 0.18	
enolase	PEP [b]	1.0 ± 0.5	6.75 ± 0.50	
	Z-PEB	4.0 ± 1.0	5.50 ± 0.50	1.3
	E-PEB	7.5 ± 2.5	6.50 ± 0.10	

[a] Chicken liver P-enolpyruvate carboxykinase. [b] Abbreviation for phosphoenolpyruvate. [c] Abbreviation for pyruvate kinase.

to the value of K_s. Equally good fits could be obtained by assuming a value of K_s from 1.0×10^{-5} to 1.0×10^{-1} M. As pointed out by one referee, this insensitivity to K_s suggests that experimental conditions are such that significant formation of metal–ligand or enzyme–ligand complexes does not occur. The results of the titrations are summarized in Table III.

The binding of P-enolpyruvate and of *Z*-PEB to the pyruvate kinase–Mn complex was very similar (P-enolpyruvate: $K_3 = 2.5 \times 10^{-6}$ M, ϵ_T = 2.03; *Z*-PEB: $K_3 = 0.5 \times 10^{-6}$ M, ϵ_T = 2.08). The *E* isomer of PEB appears to bind differently than either P-enolpyruvate or *Z*-PEB. The titration data with *E*-PEB yield $K_3 = 15 \times 10^{-6}$ M and ϵ_T = 7.0 ± 0.28. In contrast to PEPCK, the computer fits expressed by the percent standard deviation were sensitive to values of K_s. The optimal K_s value for P-enolpyruvate was 1.5×10^{-4} M, the same as the value for *Z*-PEB. An optimal K_s value of 3×10^{-4} M was obtained for *E*-PEB.

The interactions of P-enolpyruvate and *Z*-PEB with the enolase–Mn complex also showed similarities (Figure 5) (P-enolpyruvate: $K_3 = 1 \times 10^{-6}$ M, ϵ_T = 6.75; *Z*-PEB: $K_3 = 4 \times 10^{-6}$ M, ϵ_T = 5.5 ± 0.5). An anomaly is observed with *E*-PEB. Although no interaction of this ligand with enolase–Mn is detected kinetically, *E*-PEB binds tightly to the enolase–Mn^{2+} complex ($K_3 = 7.5 \times 10^{-6}$ M) (Figure 5). The ϵ_T value obtained (6.5 ± 0.1) is the same as that obtained for P-enolpyruvate and for *Z*-PEB. As with PEPCK, the quality of the fits to the enolase–Mn data were relatively insensitive to the values for K_s. The results of the titrations are summarized in Table III.

Although good fits to all of the data were obtained, the percent standard deviation in the fits increased dramatically

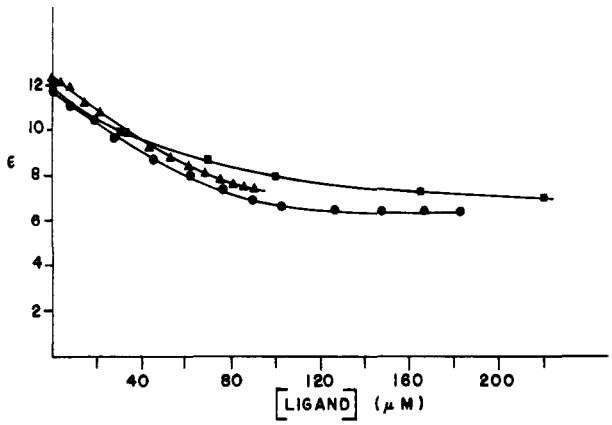

FIGURE 5: Titration of yeast enolase–Mn with P-enolpyruvate, *E*-PEB, and *Z*-PEB. A plot of observed enhancement (ϵ^*) vs. ligand concentration is made. A solution containing 1.21×10^{-4} M enzyme in 65 mM Tris-HCl, pH 7.4, containing 0.1 M KCl and 4×10^{-5} Mn^{2+} in a final volume of 0.05 mL is titrated with an identical solution which also contains either P-enolpyruvate (●), *E*-PEB (■), or *Z*-PEB (▲). The solution containing P-enolpyruvate is an equilibrium mixture of P-enolpyruvate and 2-PGA. The titrations are best fit by using K_3 values of 1 μM, 5 μM, and 3 μM, respectively.

for tight binding ligands ($K_3 < 2$ μM). This is a common observation and reflects extreme sensitivity in the first several titration points.

Discussion

The *E* and the *Z* isomers of phosphoenol-α-ketobutyrate were synthesized and obtained in pure form. The purity of the two isomers was easily determined by high-resolution ^1H NMR. The *Z* isomer was identified on the basis of the larger H–P coupling constants normally observed for trans coupling in contrast to cis coupling. The chemical shifts differ significantly between the two diastereomers, enabling a clear distinction between the diastereomers to be made.

Kinetic results indicate neither of the isomers was a substrate for avian liver PEPCK or *Ascaris* PEPCK. The two diastereomers of PEB also showed no substrate activity with either enolase or pyruvate,phosphate dikinase. Activity with enolase was tested by using either Mn^{2+} or Mg^{2+} with the same results.

Both isomers were shown to be substrates for pyruvate kinase. The stereoselectivity of the two isomers appears to be metal ion dependent. The Mn^{2+}-activated enzyme shows very little stereoselectivity with the V_{max}/K_m ratio approximately the same for the two isomers. The K_m values are essentially the same, and the V_{max} is only slightly larger for the *E* isomer as compared to the *Z* isomer (Table I). Although the V_{max}/K_m ratio was approximately the same for the two isomers, a large difference in their K_I values was measured ($K_{I,Z} = 0.45$ μM, $K_{I,E} = 15$ μM, $K_{m,PEP} = 19$ μM). The K_I values, measured with Mn^{2+} as the divalent cation, are dissociation constants as determined by PRR titration studies (Table III). The data demonstrate stereospecificity in the binding of these analogues to the pyruvate kinase–Mn complex. The bulky methyl group trans to the phosphoryl group to be transferred appears to be detrimental to binding. The inequality of the K_I (K_3) values and the K_m values for the analogues suggests that analogous to the kinetic mechanism with P-enolpyruvate, the reaction is not rapid equilibrium. The reaction is apparently random ordered. The large decrease in V_m/K_m for the analogues (Table I) appears to be an effect on catalysis (k_{cat}) and not on binding (K_m). Differences in the rate-determining step are thus suggested with these analogues. The chemical reaction with P-enolpyruvate as substrate occurs with a minimum of two steps, phosphoryl transfer followed by protonation of the resulting enolate. The rate-determining step appears to be product departure (Robinson & Rose, 1972). The protonation of the enolate of α-ketobutyrate may become rate limiting with the PEB analogues.

The Mg^{2+}-activated enzyme showed stereoselectivity between the two analogues as substrates. This cation appears to affect the V_{max}/K_m ratio by affecting both catalysis and binding. The V_{max} values appear nearly identical for these analogues regardless of whether Mg^{2+} or Mn^{2+} is the activating cation. The rate-limiting step(s) for pyruvate kinase with these analogues may therefore be identical with either Mg^{2+} or Mn^{2+}. The K_m for *E*-PEB is 2 orders of magnitude greater than the K_m of *E*-PEB with Mn^{2+} as the cation and also 2 orders of magnitude greater than *Z*-PEB with Mg^{2+} as the cation. These results suggest that the cation can effect the structure of the catalytic site of pyruvate kinase such that the interaction of structural analogues of P-enolpyruvate are perturbed. These data suggest that the cations affect the symmetry of the substrate binding site. Furthermore, the protonation of the enolate of pyruvate or α-ketobutyrate by a specific base at the catalytic site is also affected. This was previously demonstrated by a measurement of the effect of cations on tritium partitioning between substrate and solvent by pyruvate kinase (Robinson & Rose, 1972). Steric factors, possibly caused by the cation, the substrate analogues, or both may be sufficient to cause distortion or misorientation of the base required for protonation within the catalytic site. Stereochemical studies of the protonation of the butyrates have demonstrated that this reaction does not give absolute stereospecificity (Adlersberg et al., 1977) in contrast to the protonation of the enolate of pyruvate (Rose, 1970). These results suggest that a significant population of the enzyme-bound enolate of α-ketobutyrate is released into the medium prior to protonation. These results were obtained with the Mg^{2+}-activated enzyme, but such experiments have not been investigated with the Mn^{2+} enzyme.

The large difference in K_m values between the two isomers with the Mg^{2+}-activated enzyme allows for the preferential degradation of the *Z* isomer utilized in preparation of pure *E* isomer. This purification procedure can only be successful if Mg^{2+} is the activating divalent cation. The Mg^{2+}, which has a larger atomic radius by 0.25 Å than Mn^{2+}, specifically decreases the "stickiness" of *E*-PEB. The cations appear to play an important role in the interaction of the phosphoryl donor substrate with the enzyme. The details of such effects are still under investigation.

The structures of the E·Mn·PEB complexes are also different. The final enhancement (ϵ_T) for the *Z* isomer ($\epsilon_T = 2.08 \pm 0.30$) is very similar to that of P-enolpyruvate ($\epsilon_T = 2.08 \pm 0.10$). The final enhancement for the *E* isomer is quite different ($\epsilon_T = 7.08 \pm 0.18$), however.

Reuben & Cohn (1970) have obtained data from magnetic resonance studies which they interpret as the existence of two conformational forms of pyruvate kinase: an "active" form, which is stabilized by monovalent cations which activate the pyruvate kinase reaction, and an "inactive" form. Reed & Cohn (1973) came to similar conclusions on the basis of electron paramagnetic resonance (EPR) studies. From PRR and EPR data, they interpreted results to suggest that the "active" enzyme–Mn–P-enolpyruvate complex gave an anisotropic Mn^{2+} EPR spectrum for the ternary complex which had an ϵ_T of 2. The inactive ternary complex has an isotropic Mn^{2+} EPR spectrum which corresponds to a complex with a much larger ϵ_T value (Reed & Cohn, 1973). These postulates are interesting in light of the fact that both *E*- and *Z*-PEB are

equally "good" substrates for pyruvate kinase–Mn yet *Z*-PEB yields an ϵ_T similar to the active complex where *E*-PEB yields an ϵ_T similar to the inactive complex. A PRR titration with *Z*-PEB in the absence of an activating monovalent cation is identical with the titration in the presence of K^+. These results suggest no effect of K^+ on *Z*-PEB binding to pyruvate kinase–Mn. An analogous experiment performed with *E*-PEB yields no change in enhancement, suggesting no interaction with this substrate to the enzyme in the absence of an activating monovalent cation. EPR spectra of these complexes have not yet been obtained.

Avian PEPCK and *Ascaris* PEPCK were both stereoselectively inhibited by the two isomers of PEB. The K_I values were different for the two enzymes, although both showed a preference for the *E* isomer. The $K_{I,Z}/K_{I,E}$ ratio was very similar for the two enzymes (Table II). PEPCK isolated from both the cytosol and the mitochondria of guinea pig, monkey, and rat also showed a preference for the *E* isomer with $K_{I,Z}/K_{I,E}$ values very similar to those found with the above enzymes (Duffy et al., 1981). PRR titrations were performed by using avian liver PEPCK–Mn and the ligands *E*-PEB, *Z*-PEB, and P-enolpyruvate. The value of $K_3 = 30 \pm 5$ μM was calculated for *Z*-PEB, which is in excellent agreement with the kinetically determined inhibitor constant, $K_I = 32$ μM. The value of K_3 for *E*-PEB (0.25 ± 0.10 μM) is also in agreement with its kinetically determined constant, $K_I = 1.5$ μM. Structural differences were also noted in the binding of these ligands to the PEPCK–Mn complex. From a perusal of the data in Figure 4, the titration curve of P-enolpyruvate is nearly parallel to that of *Z*-PEB, with an analogous value for ϵ_T suggesting similar environmental changes occurring about the bound Mn^{2+} in the formation of the respective ternary complexes. The *E* isomer, however, binds tighter than the *Z* isomer and gives a lower ϵ_T. The simplest interpretation of these observations is that the *E* isomer displaces additional H_2O from the enzyme-bound Mn^{2+} or hinders the motion of the water upon binding to the enzyme–Mn complex more than the *Z* isomer or the substrate P-enolpyruvate. These effects are probably steric in nature.

Enolase showed stereoselectivity with respect to the two isomers of PEB. The extreme stereoselectivity appears to be metal ion dependent. The *Z*-PEB shows the same inhibition constants for the Mn^{2+}- ($K_I = 11$ μM) or the Mg^{2+}- ($K_I = 7.6$ μM) activated enzyme. The *E*-PEB demonstrates inhibition with the Mg^{2+}-activated enzyme ($K_I = 80$ μM) but not with the Mn^{2+}-activated enzyme.

Furthermore, an inhibition study with enolase–Mn performed at 10 μM *Z*-PEB and at 10 μM *Z*-PEB containing 10 μM *E*-PEB yielded identical results. These kinetic experiments also substantiate the observation that *E*-PEB elicits no kinetic effects upon enolase–Mn^{2+} activity. Enolase showed the same stereoselectivity of inhibition as pyruvate kinase and the opposite of that of PEPCK.

Upon performance of the PRR titrations, an anomaly was observed. The *E* isomer bound tightly ($K_3 = 7.5 \pm 2.5$ μM) to the E–Mn complex, although little or no inhibition was seen kinetically. It is noted that the kinetic experiments were performed under conditions in which metal was in excess of enzyme while the PRR titrations were performed under conditions in which enzyme was in excess of metal. It was initially considered that different forms of the enzyme were present in the kinetic and in the binding experiments. It was probable that the *E* isomer could only bind to the enzyme which was not saturated with metal. Upon saturation with metal, a conformational change perhaps takes place which no longer allowed the binding of the analogue to occur. Therefore PRR titrations were repeated under conditions of excess enzyme, excess metal, and equimolar enzyme and metal. Under all conditions, tight binding of the *E* isomer to the enolase–Mn complex was seen, suggesting that the metal occupancy of enolase was not the reason for the anomaly observed.

Both *Z*- and *E*-PEB bind in a structurally similar fashion to the enolase–Mn complex as does P-enolpyruvate ($\epsilon_{T,\text{P-enolpyruvate}} = 6.75 \pm 0.50$, $\epsilon_{T,Z\text{-PEB}} = 5.50 \pm 0.50$, and $\epsilon_{T,E\text{-PEB}} = 6.50 \pm 0.10$). The tight, apparently selective binding of *E*-PEB to enolase–Mn with no kinetic effect is still unexplained.

Pyruvate,phosphate dikinase showed a strict stereoselectivity toward the two isomers of PEB. No inhibition was seen with either isomer, indicating that a substantial difference in binding sites exists between pyruvate,phosphate dikinase and PEPCK, pyruvate kinase, and enolase. These results suggest a strict steric requirement for P-enolpyruvate interaction with this enzyme.

The results presented demonstrate that although there is homology in the reaction and in the stereochemistry of the reactions between pyruvate kinase (Rose, 1970) and PEPCK (Rose et al., 1969) (presumably pyruvate,phosphate dikinase has the same stereospecific addition as other P-enolpyruvate-utilizing enzymes), these enzymes exhibit stereospecific differences in binding of the diastereomers of PEB. This has also been previously observed with diastereomers of 2-phospholactate (Nowak & Mildvan, 1970). Thus, there must be substantial steric differences at the catalytic sites of these related enzymes. These differences occur at the site of addition at C-3 of P-enolpyruvate. The differences in the catalytic sites at this location are related to the nature of the group (H^+, CO_2, and OH^-) added to P-enolpyruvate. The physical nature of these differences awaits more refined studies into their catalytic sites. The activating cations appear to influence the structures of these catalytic sites.

These analogues, in principle, can be used to specifically inhibit either the glycolytic or the gluconeogenic pathway. By understanding the stereospecific requirements of related enzymes, it should be feasible to rationally design chemotherapeutic reagents.

One possible utilization of such stereospecific inhibitors is the inhibition of the anaerobic metabolism in parasitic worms. The metabolic pathway in these parasites differs from that of mammalian hosts. In mammalian systems, the terminal steps of glycolysis are catalyzed by pyruvate kinase and lactate dehydrogenase. Parasitic worms lack significant pyruvate kinase and utilize PEPCK to obtain oxalacetate from P-enolpyruvate (Bueding & Saz, 1968). Preliminary experiments with the flatworm *Hymenalipus* and the roundworm *Ascaris* showed no change in motility upon addition of *E*-PEB to its medium. There was also no significant change in the number of parasites found in *Hymenolipus*-infected rats fed or injected with solutions of *E*-PEB. There is no evidence, however, that these compounds were actually ingested by the parasite. Additional studies are still required.

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Rapid Analysis of Estrogen and Progesterone Receptors Using Gel-Exclusion High-Performance Liquid Chromatography†

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ABSTRACT: Estrogen and progesterone receptors prepared from mouse, rat, and human uteri, as well as from human breast cancers, have been characterized by gel-exclusion high-performance liquid chromatography. The qualitative relationships previously established by sedimentation analysis between the cytoplasmic [aggregated (~8S), deaggregated (~4S), and trypsinized (~3.6S)] and nuclear (~5S) forms of the rat uterine estrogen receptor were maintained by this technique. Differences in the partition of estrogen and progesterone receptors from the same species as well as interspecies differences in these receptors were reproducibly observed. Multiple forms of human estrogen and progesterone receptors could clearly be resolved in a single analysis and were distinct from serum steroid binding tissue contaminants. Separation analyses, performed at flow rates up to 2 mL min^{-1}, were capable of resolving all receptor forms in 10-12 min with the column returning to base line in 25 min. With this exclusion gel column (TSK-G3000SW) as a background upon which to reference different receptor forms, eight distinct partitions or elution positions have been enumerated. This approach has considerable promise for the rapid characterization of different forms of steroid-receptor proteins. Moreover, it should provide a critical advantage in minimizing the opportunities for receptor modification during separation analysis and in maximizing the opportunity to study short-lived interactions between receptors and physiologic or pharmacologic ligands.

Estrogenic and progestational steroid hormones exert their effects upon target tissues by passing through the cell membrane, interacting with a cytoplasmic-binding protein, and, as an activated binding complex, translocating to the nucleus where interaction with chromatin presumably initiates certain events responsible for alterations in the rates of transcription, translation, and even replication (Jensen & DeSombre, 1972, 1973; Katzenellenbogen & Gorski, 1975; O'Malley & Means, 1974; Yamamoto & Alberts, 1976). These intracellular steroid-binding proteins are referred to as steroid "receptors", and their presence or expression provides a method for identifying the potential for endocrinologic response in various tissues. Since endocrine therapy can be of considerable benefit in the management of sex hormone responsive cancers, there is an obvious value to performing receptor determinations in order to distinguish those patients who, because of their receptor-positive status, are likely to respond to endocrine therapy (Block et al., 1975; Horwitz et al., 1975; Osborne et al., 1980). Steroid-receptor determinations can be based upon principally quantitative analysis by employing assays involving dextran-coated charcoal, hydroxylapatite, or protamine sulfate (Garola & McGuire, 1977a,b, 1978; Rosner et al., 1980; Zava et al., 1976). Moreover, receptor assays involving sucrose- or glycerol-gradient analysis, gel filtration, column chromatography,

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