Stereoselective Interactions of Phosphoenolpyruvate Analogues with Phosphoenolpyruvate-utilizing Enzymes*

THOMAS NOWAK‡ and ALBERT S. MILDYAN§
From The Institute for Cancer Research, Philadelphia, Pennsylvania 19111

SUMMARY

The optically pure D and L isomers of 2-phospholactate were synthesized by phosphorylation of the appropriate lactic acids. The interactions of these analogues and of phosphoglycolate with several enzymes that catalyze reactions of phosphoenolpyruvate were examined by kinetics and by direct binding studies measuring the longitudinal relaxation rate of water protons.

With pyruvate kinase, the $K_I$ of D-phospholactate (21 $\mu M$) and of phosphoglycolate (52 $\mu M$) agree with the $K_I$ of phosphoenolpyruvate (26 $\mu M$) while the $K_I$ of L-phospholactate (380 $\mu M$) is 18-fold greater. This difference in affinity is confirmed in the direct binding studies. The enhancement of the ternary pyruvate kinase-Mn-inhibitor complex of D-phospholactate ($\epsilon_T = 1.9$) and of phosphoglycolate ($\epsilon_T = 1.7$) agree with that of phosphoenolpyruvate ($\epsilon_T = 2.2$), but differs from that of L-phospholactate ($\epsilon_T = 3.8$), indicating a structural difference in this latter metal bridge complex.

With enolase, the inhibition was not stereospecific since the $K_M$ values of both isomers of phospholactate and of phosphoglycolate were equal (370 $\mu M$) and larger than the $K_M$ of 2-phosphoglycerate (6.7 $\mu M$). Equal affinities for the analogues were confirmed by direct binding studies, but unequal ternary enhancements were observed for the substrate phosphoenolpyruvate ($\epsilon_T = 3.7$), D-phospholactate ($\epsilon_T = 11.0$), phosphoglycolate ($\epsilon_T = 8.4$), and L-phospholactate ($\epsilon_T = 5.8$) because of structural differences in their respective ternary complexes. Phosphoenolpyruvate carboxykinase, which catalyzes a reaction with a mechanism homologous with that of pyruvate kinase, shows the opposite inhibition pattern since it is preferentially inhibited by L-phospholactate ($K_I = 28 \mu M$) as compared with D-phospholactate ($K_I = 292 \mu M$). None of the analogues inhibited the reaction catalyzed by phosphoenolpyruvate synthetase. The patterns of inhibition by the phospholactates of pyruvate kinase ($D > L$), enolase ($D = L$), and carboxykinase ($L > D$) are explained in terms of the known stereocchemistries of the reactions catalyzed by these enzymes and the structures at their respective active sites.

Two enzymes that catalyze reactions of phosphoenolpyruvate, enolase (1) and PEP carboxykinase (2), have been shown to be inhibited by the substrate analogue, 2-phospholactate. The absolute stereocchemistries of the reactions catalyzed by these enzymes (3, 4), as well as pyruvate kinase (5), have recently been established. The present work examines the interaction of the enantiomers of phospholactate with four enzymes that utilize PEP, in order to determine whether the inhibition of these enzymes is also stereospecific and to attempt to rationalize their specificity in terms of the stereospecificity of the respective reactions. Although the literature contains several samples of stereoselective inhibition of enzymes (6), the structural basis for such inhibition has rarely been established. A recent example of stereospecific inhibition has been found with pyruvate carboxylase (7) for which L-malate is a 30-fold more potent inhibitor than D-malate. This difference appears to be due to bidentate coordination of L-malate and monodentate coordination of D-malate by the enzyme-bound Mn$^{2+}$ (7).

EXPERIMENTAL PROCEDURE

Materials—Rabbit muscle pyruvate kinase and rabbit muscle enolase were purchased from Boehringer und Sohne (Mannheim, West Germany), as was lactate dehydrogenase, malate dehydrogenase, and potato acid phosphatase. Yeast enolase was a generous gift from Dr. David H. Hanlon, chicken liver PEP carboxykinase was provided by Drs. Harold Kolenbrander and Merton Utter, and the PEP synthetase from Escherichia coli was a gift from Drs. Kenneth Berman and Mildred Cohn. Optically pure D and L isomers of lactic acid were purchased from Pierce Chemicals, Rockford, Illinois, and 2-phosphoglycolate was purchased as the tricyclohexylamine salt from General Biologicals, Chagrin Falls, Ohio. The NADH, IDP, 2-phosphoglycolate was purchased from Boehringer und Sijhne (Mannheim, West Germany), as was lactate dehydrogenase, malate dehydrogenase, and potato acid phosphatase. Yeast enolase was a generous gift from Dr. David H. Hanlon, chicken liver PEP carboxykinase was provided by Drs. Harold Kolenbrander and Merton Utter, and the PEP synthetase from Escherichia coli was a gift from Drs. Kenneth Berman and Mildred Cohn. Optically pure D and L isomers of lactic acid were purchased from Pierce Chemicals, Rockford, Illinois, and 2-phosphoglycolate was purchased as the tricyclohexylamine salt from General Biologicals, Chagrin Falls, Ohio. The NADH, IDP, 2-phosphoglycolate, and AMP were purchased from Sigma, and PEP was purchased from Calbiochem. All other reagents used were of the highest purity commercially available.

Phospholactate Synthesis—The syntheses of the D and L isomers of 2-phospholactic acid were performed by a modification of

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‡ Postdoctoral Fellow of the National Institutes of Health.
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† The abbreviation used is: PEP, phosphoenolpyruvate.
the phosphorylation procedure described by Yoshikawa, Kato, and Takenishi (8) on the methyl esters of the lactic acids. N-
Nitrosomethylurea was prepared (9), dried overnight in a vacuum desiccator, and stored at 4°C. The optical purity of lactic acid isomer was first converted to its methyl ester with diazomethane by dissolving 1 g of lactic acid in 50 ml of absolute methanol at 0°C and slowly adding an ethereal solution of an excess of diazomethane generated from the nitrosomethylurea (10). Care was taken to add the highly toxic, potentially explosive diazomethane under a hood and behind a shield with the use of protective equipment. After the methylation was complete, the reaction was allowed to stand for approximately 1 hour until the excess yellow color disappeared and bubbling stopped. The solution was taken to dryness in a flash evaporator, and the viscous yellow methyl ester was phosphorylated in 40 ml of dry trimethylphosphate with a 10-fold excess of ice-cold POC13. The mixture was kept at 0°C for 18 hours. The reaction was terminated by pouring the mixture over 100 g of ice and rapidly adjusting the pH to 9 with saturated LiOH, keeping the temperature below 4°C. The milky white suspension was maintained at pH 9 with LiOH at room temperature for 2 hours, and the lithium phosphate precipitate was then removed by centrifugation and rinsed with water. The combined supernatants were washed with a 2-fold volume of chloroform to remove the trimethylphosphate, and the volume of the aqueous layer was then reduced to about 150 ml in a flash evaporator. To this solution, 22 ml of 5 M KOH were then added, and the solution was allowed to saponify overnight at room temperature. The pH was adjusted to 9.5, and 2 volumes of acetone were added. A flocculent precipitate immediately appeared, which was allowed to age at 0°C for 2 hours. The waxy white precipitate was filtered, washed with acetone, and kept in a desiccator under vacuum.

D- and L-P-lactate were purified on a Dowex 1-X8-200 Cl column. To a column (19 × 1.6 cm) of previously treated resin, 0.2 g of product in 100 ml of water was applied to the column and washed with 50 ml of water and 25 ml of 10 mM HCl. The column was then eluted with 50 mM HCl, and the fractions were monitored by their absorbance at 210 nm. The peaks were assayed for total phosphate (11). The unsaponified product, which eluted first, was well separated from the phospholactate. The peak fractions were combined and taken to dryness in a flash evaporator, with redissolution of the viscous product with acetone and the solution was maintained at pH 9 with LiOH at room temperature for 30 minutes. The milky white suspension was then dissolved in 5 ml of water, decolorized with charcoal, and titrated with fresh 1 M KOH. Both products had titratable groups of pK 1.7, 3.6, and 7.0, and their carboxy-methyl esters had titratable groups of pK 1.8 and 6.5. The L isomer of phospholactate required 3.1 mmoles to titrate the first group, 3.4 mmoles for the second, and 3.7 mmoles for the third group, indicating that at least 88% of the product was phospholactate. The three groups for D-P-lactate required 0.36, 0.35, and 0.37 mmoles of KOH, respectively, suggesting 98% purity. Proton nuclear magnetic resonance spectra with a Varian HA-100-15 nuclear magnetic resonance spectrometer gave essentially identical spectra for both isomers. The parameters of the nuclear magnetic resonance spectra were as expected from chemically related compounds. Thus, the methyl doublet ($J = 7$ cps) was at 1.9 ppm downfield from a tetramethylsilane standard, and the $C_2$ proton octet was at 4.85 ppm downfield from tetramethylsilane with coupling constants $J_{M-H} = 7$ cps and $J_{P-H} = 8.5$ cps. In the D-P-lactate sample, an additional singlet was observed at 4.19 ppm downfield, which might be ascribed to a methyl group of some unsaponified methyl ester. This could account for less than 13% of the sample. There is no trace of this singlet with the L-P-lactate sample.

Samples of D- and L-P-lactate were treated with potato acid phosphatase and assayed for L-lactic acid (12) and inorganic phosphate (11). With D-P-lactate, all of the expected P1 was released and there was less than 2% L-lactate detected, whereas with L-P-lactate the release of P1 was accompanied by the release of 86% of the amount of L-lactate expected, as determined with L-lactic dehydrogenase (12). This discrepancy, although not yet understood, is probably not due to racemization of the product. It may be concluded that both preparations are at least 85% pure.

**Enzymic Assays—**Pyruvate kinase was assayed in 0.1 M Tris-Cl and 0.1 M KCl, pH 7.5, measuring the rate of appearance of pyruvate by coupling the pyruvate kinase with lactate dehydrogenase in the presence of 0.16 mM NADH (13). The rate of disappearance of NADH was followed continuously at 340 nm and at 25°C with a recording spectrophotometer. Yeast enolase was assayed at 25°C by a modification of a previously used assay (14), in a 1-ml volume containing 50 mM Tris-Cl, pH 7.5; 0.5 mM KCl; 0.1 mM MnCl2, and varying concentrations of D-glycerate 2-phosphate. After the addition of enzyme to the reaction in a 0.01-ml volume, the rate of appearance of PEP was measured at 220 nm, with an expanded recording scale of 0 to 0.1 absorbance. The muscle enolase was assayed at 30°C in the presence of 1 mM MnCl2.

PEP carboxykinase was assayed by a slight modification of the method described by Berman and Cohn (16). The reverse reaction of PEP synthetase was measured by a modification of the method described by Berman and Cohn (16). The reaction mixture contained 50 mM potassium phosphate, pH 6.5; 0.2 mM KCl; 1 mM AMP; 5 mM MgCl2; 0.1 mM NADH; 25 µg of lactate dehydrogenase; and PEP in 1 ml. The reaction was initiated with the addition of PEP to give a final volume of 1 ml. The rate of disappearance of NADH was followed spectrophotometrically at 340 nm over a 0.1 absorbance range.

The reverse reaction of PEP synthetase was measured by a slight modification of the method described by Berman and Cohn (16). The reaction mixture contained 50 mM potassium phosphate, pH 6.5; 0.2 mM KCl; 1 mM AMP; 5 mM MgCl2; 0.1 mM NADH; 25 µg of lactate dehydrogenase; and PEP in 1 ml. The reaction was initiated with the addition of enzyme, and the rate of NADH disappearance was followed at 25°C and 340 nm at an absorbance range of 0 to 0.1.

**Determination of Binding and Enhancement Parameters of Ternary Complexes—**Binding experiments with pyruvate kinase were carried out and the data were calculated as described previously (17). The enzyme was separated from ammonium sulfate by passage through a Sephadex G-25 (Pharmacia) column, and solutions of 0.38 to 1.1 mg of pyruvate kinase and 50 mM MnCl2 containing 50 mM Tris-Cl, pH 7.5, and 0.1 mM KCl in 0.05 ml were titrated with identical solutions which contained in addition one of the analogues of PEP.

To prepare metal-free enolase, the enzyme (10 to 20 mg) was dissolved in 1 ml of 50 mM Tris-Cl, pH 7.5, containing 1 mM EDTA and dialyzed against one change of 500 ml of 50 mM Tris-Cl containing 1 mM EDTA, pH 7.5, for 12 hours at 4°C and a second change of 500 ml of Tris-Cl buffer alone. The enzyme was then passed through a column of chelex overlying
FIG. 1. Double reciprocal plots of the initial velocity of the pyruvate kinase reaction as a function of PEP concentration in the presence of varying concentrations of D-P-lactate, L-P-lactate, and P-glycolate. A, the reactions contained the following concentrations of D-P-lactate: Curve 1, 0 μM; Curve 2, 22 μM; Curve 3, 55 μM; Curve 4, 110 μM; and Curve 5, 220 μM. B, the reactions contained the following concentrations of L-P-lactate: Curve 1, 0 μM; Curve 2, 0.233 mM; Curve 3, 0.582 mM; Curve 4, 1.17 mM; Curve 6, 2.33 mM. C, the reactions contained the following concentrations of P-glycolate: Curve 1, 0 μM; Curve 2, 88 μM; Curve 3, 220 μM; Curve 4, 440 μM; Curve 5, 880 μM. In addition, the reaction vessels all contained 100 mM Tris-Cl buffer, pH 7.5; 100 mM KCl; 4 mM MnCl₂; 1 mM ADP; 0.16 mM NADH; and 25 μg of lactate dehydrogenase and PEP as indicated. The reactions in A were started with the addition of 0.5 μg of pyruvate kinase, in B with 0.54 μg, and in C with 0.46 μg of pyruvate kinase to give a final volume of 1 ml. The temperature was maintained at 25°C.

TABLE I

| Analogue     | Inhibitor and binding constants of PEP analogues with pyruvate kinase |
|--------------|---------------------------------------------------------------------|
|              | Kinetics | Proton relaxation           |
|              | Kᵢ       | Kᵢ₀   | Eᵢ₀ |
| PEP          | 26 ± 3   | 15 ± 5 | 2.2 ± 0.2 |
| L-P-lactate  | 383 ± 56 | 182 ± 3 | 3.8 ± 0.1 |
| D-P-lactate  | 21 ± 5   | 5.9 ± 3.2 | 1.9 ± 0.1 |
| P-glycolate  | 52 ± 8   | 17.4 ± 3.8 | 1.7 ± 0.1 |

* For this preparation of pyruvate kinase, the enhancement of the binary complex, eᵢ₀ = 25.6 ± 0.7.

RESULTS

Inhibitor Constants of Substrate Analogues for Pyruvate Kinase

The three analogues, D- and L-P-lactate and phosphoglycolate, were tested as inhibitors with respect to PEP in the Mn-pyruvate kinase system. Double reciprocal plots of the PEP concentration, with respect to the initial velocity at varying concentrations of D- and L-P-lactate and phosphoglycolate, are shown in Fig. 1. Secondary plots of slopes versus inhibitor concentration were linear for each analogue, indicating that all are linear competitive...
inhibitors. A summary of the kinetic results for the substrate and the substrate analogues is given in Table I. The $K_M$ for PEP with the Mn-enzyme is of the same order as the limiting Michaelis constant ($K'_M = 48 \mu M$) and dissociation constant ($K = 41 \mu M$) previously measured (17). The $K_I$ values for $\alpha$-P-lactate and phosphoglycolate are of the same order as the $K_M$ for PEP. The $\beta$-P-lactate isomer is approximately one-twentieth as potent an inhibitor as $\alpha$-P-lactate.

**Dissociation Constants and Enhancements of Ternary Complexes of Pyruvate Kinase with Manganese**—The manganese enzyme complex was titrated with PEP and each of the three PEP analogues as described under "Experimental Procedure" and in Reference 17. Typical titration curves of pyruvate kinase with $\alpha$- and $\beta$-P-lactate are shown in Fig. 2, in which the paramagnetic contribution to the longitudinal relaxation rate of water, $1/T_1$, is plotted against the reciprocal of the inhibitor concentration. A mixture of Mn and enzyme was titrated to an end point value with each ligand at various enzyme concentrations but at constant Mn. Curvature was observed at high concentrations of ligand because the enhancement has a finite value in absence of the ligand. The curvature observed at high ligand concentrations was due to an approach to an end point in the titration in which the Mn$^{++}$ is bound in a ternary complex. The $K_S$ and $e_T$ values obtained from such titrations as described under "Experimental Procedure" and in Reference 17 are given in Table I, together with the enhancement of the binary complex, $e_B$, for this enzyme preparation. The $K_S$ values, which are in reasonable agreement with the dissociation constants obtained kinetically, confirm the observation that $\alpha$-P-lactate and phosphoglycolate bind to pyruvate kinase more tightly than $\beta$-P-lactate by more than an order of magnitude, corresponding to a greater free energy of binding of $2.0 \pm 0.2$ kcal per mole. The $e_T$ values suggest that both $\alpha$-P-lactate and phosphoglycolate displace the same number of water ligands from the enzyme-bound Mn and that the conformation about the Mn site is apparently the same as that seen with the substrate PEP, whereas the poorer inhibitor $\beta$-P-lactate does not displace the water protons as well because it does not produce the same conformation at the Mn site on the enzyme.

**Inhibitor Constants of Substrate Analogues for Yeast Enolase**—Following the treatment described under "Experimental Procedure," the yeast enolase was judged to be "metal-free" since $0.4 \mu g$ caused a change of less than 0.001 absorbance unit per min at $25^\circ$ in the absence of any added divalent cation. The $K_M$ of $2$-phosphoglycerate was determined for the Mn enzyme and compared with the $K_I$ values of the three PEP analogues.

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**TABLE III**

| Analogue            | $K_S$  | $e_T^a$ |
|---------------------|--------|---------|
| PEP                 | $<30$  | 3.7     |
| $\alpha$-P-lactate  | 600 ± 150 | 5.8     |
| $\beta$-P-lactate   | 500 ± 100 | 11.6    |
| $\beta$-glycolate   | 600 ± 200 | 8.4     |

*a* The enhancement of the binary complex $e_B = 15 \pm 1.5$. $T = 24.5 \pm 0.5^\circ$. The experimental error in $e_T$ is $<10\%$.

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**TABLE II**

| Analogue           | $K_M$  | $K_I$  |
|--------------------|--------|--------|
| PEP                | $6.7 \pm 0.7$ | $350 \pm 50$ |
| $\alpha$-P-lactate | $395 \pm 45$ | $91 \pm 11$ |
| $\beta$-P-lactate  | $200 \pm 20$ | $54 \pm 3$ |

*a* Commercial yeast enolase (Sigma) assayed with $1 \text{ mm MnCl}_2$. 

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Fig. 2. Titrations of MnCl$_2$ and pyruvate kinase with $\alpha$-P-lactate and $\beta$-P-lactate. A plot of the reciprocal of the paramagnetic contribution to the proton relaxation rate of water ($1/T_{1p}$) versus a reciprocal of the concentrations of ligand added is shown. In Curve A, pyruvate kinase (0.55 mg) was titrated in the presence of 50 $\mu M$ MnCl$_2$ and 0.1 M KCl in 50 $\mu l$ with a solution containing 200 $\mu M$ $\alpha$-P-lactate in addition to the same concentration of enzyme, MnCl$_2$, and KCl. In Curve B, pyruvate kinase (0.64 mg) was titrated in the presence of 50 $\mu M$ MnCl$_2$ and 0.1 M KCl in 50 $\mu l$ with a solution containing 2.82 mM $\beta$-P-lactate in addition to the same concentration of enzyme, MnCl$_2$, and KCl. After a complete titration of the Mn-enzyme by the ligand small additions of concentrated $\alpha$-P-lactate (18.8 mM) or $\beta$-P-lactate (5.5 mM) were made to reach their respective end points and the relaxation time corrected for the small dilution; temperature, 24.5$^\circ$.
The $K_M$ of PEP was not determined since the assay involves measurement of the rate of change of PEP, and the equilibrium of the reaction is in the direction of PEP by approximately 80% (18). Since the $K_M$ values for PEP and phosphoglycerate are similar for the Mg$^{2+}$-activated enzyme (1, 19) and our $K_M$ of 2-phosphoglycerate is smaller by at least an order of magnitude, it was technically impossible to measure the $K_M$ of PEP.

The kinetic data shown in Table II indicate no stereospecific inhibition by the analogues, none of them being judged a “strong” inhibitor since their $K_I$ values are 30 to 60 times larger than the $K_M$ of phosphoglycerate. All three, however, are linear competitive inhibitors of yeast enolase.

### Table IV

| Analogue          | $K_M$ | $K_I$ |
|-------------------|-------|-------|
| PEP               | 11.1 ± 1.3 |       |
| L-P-lactate       | 27.7 ± 3  | 292 ± 5 |
| D-P-lactate       | 729 ± 29* | 640 ± 25* |
| P-glycolate       |        |       |

* Mixed type inhibitor.

### Dissociation Constants and Enhancements of Ternary Complexes of Enolase with Manganese—As described, manganese-activated yeast enolase was titrated with each of the three substrate analogues and with PEP (equilibrium mixture of PEP and d-glycerate 2-phosphate), and the dissociation constants of the ternary complexes and the enhancement values were measured. The results are given in Table III. The dissociation constants, which agree with the kinetically determined constants, substantiate the finding that there is no stereospecific binding of the two phospholactate isomers. The binary enhancement of the Mn-enolase complex (15.0 ± 0.5) is in reasonable agreement with that (13.8 ± 1.1) found by Cohn (20). The addition of PEP de-enhances the relaxation rate of water in a manner consistent with an enzyme-metal-substrate bridge complex and gives a ternary enhancement value of 3.7 in agreement with that observed for the addition of phosphoglycerate (3.2) by Cohn and Leigh (21).

Although the binding constants for the three substrate analogues are the same, their ternary enhancement values vary, indicating different structures at the Mn$^{2+}$ site on the enzyme for each of the inhibitors. These differences may be reflected in a difference in the number of water molecules replaced on the Mn by the different inhibitors, a difference in their effect on the rotation of water on the Mn$^{2+}$, a change in the ligand field experienced by the Mn$^{2+}$, or a combination of these effects. Further investigations are currently underway to examine these effects. On the basis of the ternary enhancement values, L-P-lactate mimics PEP most closely on the enzyme, while D-P-lactate and phosphoglycerate have quite different effects.

Kinetic studies of the muscle enzyme were performed at 30° with 1 mM MnCl$_2$. The results (Table II) show that, with the Mn enzyme under our assay conditions, the $K_M$ of the substrate 2-phosphoglycerate is of the same order of magnitude as for yeast enolase; however, the $K_I$ of the substrate analogues are lower by a factor of 5. As with yeast enolase, no significant stereospecific inhibition (i.e., no difference between D- and L-P-lactate) is observed.

### Inhibitor Constants of Substrate Analogues for PEP Carboxykinase—The three substrate analogues were examined as inhibitors of PEP carboxykinase, and the double reciprocal plots are shown in Fig. 3. As indicated by secondary plots, both isomers of phospholactate are linear competitive inhibitors with respect to PEP, the L isomer being the better inhibitor by an order of magnitude (Table IV). The phosphoglycerate, however, is a mixed type inhibitor. The competitive portion of the inhibition reflected by its effect on the slope of the double reciprocal plot shows it to be an even less effective inhibitor than D-P-lactate.

In experiments not shown, L-P-lactate was found to be an uncompetitive inhibitor against HCO$_3^-$ at subsaturating PEP concentration (12.3 μM) but at saturating IDP (1.25 mM) concentration.

### Inhibition Studies of PEP Analogues with PEP Synthetase—PEP synthetase catalyzes a reversible two-step reaction of pyruvate and ATP to give PEP, AMP, and P$_i$, the reaction going through a phosphorylated enzyme intermediate (22). The reaction can be studied in the reverse direction only with Mg$^{2+}$ in the presence of varying concentrations of D- and L-P-lactate and P-glycolate. A the reactions contained the following concentrations of L-P-lactate: Curve 1, 0 μM; Curve 2, 47 μM; Curve 3, 94 μM; Curve 4, 141 μM; and Curve 5, 235 μM. B the reactions contained the following concentrations of D-P-lactate: Curve 1, 0 μM; Curve 2, 288 μM; Curve 3, 576 μM; Curve 4, 864 μM. In addition the reaction vessels contained 50 mM Tris-Cl, pH 7.5; 50 mM KHCO$_3$; 1.25 mM IDP; 2 mM glutathione; 1 mM MnCl$_2$; 0.16 mM NADH; 20 μg of malate dehydrogenase; and 6.2 μg of enzyme. The temperature was maintained at 25° and the reaction was initiated by the addition of the appropriate amount of PEP to give a final volume of 1 ml.
Fig. 4. Stereochemistry of reactions of PEP catalyzed by pyruvate kinase (5), phosphoenolpyruvate carboxykinase (3), and enolase (4). BH represents the base on the enzyme responsible for the protonation of PEP.

Fig. 5. Stereochemistry of PEP analogues compared with PEP. LPL, L-phospholactate; P-Gly, phosphoglyceraldehyde; DPL, n-phospholactate.

presence of a large excess of inorganic phosphate at a lower pH (6.5) (16). When the enzyme was assayed in the reverse direction, the $K_M$ of PEP was measured as 17.9 ± 2.4 μM. At a PEP concentration of less than the $K_M$ value (14 μM) in the presence of a large excess of PEP analogue (11 mM phosphoglycolate, 10 mM L-P-lactate, or 9 mM n-P-lactate), no inhibition of activity was observed.

DISCUSSION

Several enzymes that utilize PEP as a substrate have been studied to determine the effect of stereospecific PEP analogues on the activity of these enzymes. The stereochemistry of several PEP-utilizing enzymes has already been investigated (3-5), thus serving as a guide for the interpretation of our data. We have divided the enzymes into two classes on the basis of their general mechanism. As exemplified in Fig. 4, three enzymes studied fall into Class I, in which the tautomeric shift of electrons during the reaction is toward C-3.

In pyruvate kinase, the enzyme catalyzes the transfer of a phosphoryl group to ADP, and an electrophile, H⁺, is added to C-3 from the si face according to the nomenclature of Hanson (23), or above the plane of the PEP molecule if written in a "right-handed" manner as shown in Fig. 4 (5). The products of the reaction are pyruvate and ATP.

The enzyme PEP carboxykinase catalyzes a homologous reaction of PEP (2) in which the phosphoryl group is transferred from PEP to either IDP or GDP, and the electrophile carbonium ion of CO₂ adds from above the plane of the PEP to the C-3, resulting in the products oxalacetate and ATP (3).

PEP synthetase catalyzes a reaction similar to pyruvate kinase in which the enzyme functions as the phosphoryl acceptor and a H⁺ is the electrophile. Pyruvate is liberated, and the phospholyated enzyme reacts with AMP and Pi to give the products ATP and free enzyme (16).

Enolase falls into the second mechanistic class since the electron shift of the double bond is in the opposite direction, toward C-2 where the electrophile (proton) is added. A hydroxyl ion is added, trans to C-3 yielding the stereospecific product d-glyceraldehyde 2-phosphate and, of course, no phosphoryl group transfer takes place. It has been shown that the H⁺ is added from above the plane of PEP and the addition of OH⁻ comes from below the plane (4).

The structure of the substrate analogues used is compared with PEP in Fig. 5. Keeping the phosphate and carboxyl groups on C-2 in the same orientation and plane as PEP, n-P-lactate lies with its methyl group below the plane of PEP, and L-P-lactate lies with its methyl group above the plane of PEP. Phosphoglycolate has no C-3 group.

A qualitative summary of the effects of the PEP analogues on the enzymes studied is shown in Table V.

With pyruvate kinase, both n-P-lactate and phosphoglycolate bind as tightly to the enzyme as does PEP, as measured by kinetics and by proton relaxation rate determinations, and the enhancements of these ternary complexes ($e_R$) are in agreement. Hence, it may be inferred that the pyruvate kinase-Mn complex binds PEP, n-P-lactate, and phosphoglycolate in an identical manner. The phosphoryl groups are probably coordinated by the Mn (24), and the carboxyl groups, which provide an additional 2.8 kcal per mole of binding energy, are held by a group 6 A away (25), possibly the monovalent cation (26). The methyl group of n-P-lactate, which would lie below the plane of PEP on the enzyme (Fig. 5), apparently does not influence its binding since phosphoglycolate lacks a methyl group and binds as well.

When L-P-lactate binds to this manner, its methyl group, which points up above the plane of PEP at the C-3 position (Fig. 4), is sterically hindered by the proton donor on pyruvate kinase which must be located above the C-3 of PEP (5). Hence, L-P-lactate would be expected to bind more weakly than PEP, phosphoglycolate, or n-P-lactate, as observed, and produce a complex of altered structure as indicated by its larger $e_T$ value. The greater $e_T$ value with L-P-lactate can be attributed either to a different conformation of the enzyme about the Mn site in the ternary complex or to the displacement of less water from the coordination sphere of the enzyme-bound Mn.

With enolase, the proton donor resides above the C-2 rather than the C-3 of PEP. Hence, the methyl group of L-P-lactate can be accommodated, and no stereoselective binding is observed. The similar dissociation constants and $K_M$ values for L- and n-P-lactate and phosphoglycolate indicate that the methyl
groups do not influence the affinity of these analogues for the enzyme-Mn complex. All of the analogues, however, appear to bind more weakly, by at least an order of magnitude, than do the substrates PEP and D-glycerate 2-phosphate. Apparently sp^3 hybridization at C-2 prevents the tight binding of the analogues at the substrate site unless another properly oriented binding group is present. Thus, the substrate D-glycerate 2-phosphate, which has an OII instead of an H at C 3 of D-P-lactate, appears to bind 50 times tighter than D-P-lactate (Table II). Furthermore, to bind tightly at the substrate site, an analogue with sp^3 hybridization at C-2 must have a β configuration, since L-glycerate 2-phosphate fails to inhibit the enzyme. Although the ternary complexes of each inhibitor have approximately the same stability, their enhancement values are quite different. Thus, the ternary complex with the equilibrium mixture of substrates (~80% PEP) de-enhances the relaxation rate of water by 75%, while the L-P lactate ternary complex de-enhances by 61%, phosphoglycolate by 46%, and D-P-lactate by 23%. These observations can be fit most simply by assuming that, in the binary complex, the enzyme is acting as a bidentate ligand, leaving 4 water molecules coordinated on the metal. The addition of PEP displaces 3 water molecules, L-P-lactate displaces 2, and phosphoglycolate displaces 2, while D-P-lactate displaces only 1 molecule of water. As pointed out elsewhere (17), such differences in e could also be due to differences in the water exchange rates on Mn, or in the correlation time for the Mn-water interactions. Thus, an alternative explanation for the relatively high e value with D-P-lactate is that its methyl group, which is located below the plane of the CO of PEP, is closer to the hydration sphere of Mn^{2+} than is the methyl group from L-P-lactate and the former thus hinders the rotation of the water on the metal. This would result in an increase in the correlation time, τ_e, and a greater e value. Studies are planned to investigate which of these phenomena are occurring.

At present, it may be suggested that the de-enhancements produced by PEP and all of its analogues are consistent with the formation of enolase-Mn-ligand bridge complexes, as previously proposed (20, 21, 27).

Lower K_f values of the analogues were observed for yeast enolase at 30° than at 25° (Table II), suggesting that the free energy of binding of the inhibitors is due solely to a favorable entropy term. The K_M for 2-phosphoglycerate (6.7 µM) is 10 to 20 times lower than those previously reported for the Mg^{2+}-activated enzyme (1, 19) and the Mn^{2+} enzyme (19); however, our lower value agrees with the dissociation constant measured by kinetic protection against inactivation by glycidol phosphate (28).

Muscle enolase, although studied under slightly different conditions of temperature and MnCl_2 concentrations, shows a similarly low K_M for the substrate, and again there is no stereospecific inhibition by the PEP analogues.

PEP carboxykinase, which reacts with the same stereocchemistry as pyruvate kinase (3), is inhibited stereospecifically by the opposite isomer of phospholactate. Thus, L-P-lactate is a better inhibitor by a factor of 10 than D-P-lactate (Table IV). The methyl group of L-P-lactate contributes to its affinity since phosphoglycolate is a significantly weaker and mixed type inhibitor.

An explanation for the stereospecific inhibition of PEP carboxykinase by the L isomer of phospholactate may be found in the kinetic scheme (29) and in the stereochecmy (3) of the reaction. The enzyme from liver mitochondria has been shown to bind PEP prior to CO_2 in the reaction sequence (29) and, judging from the stereochecmy, the CO_2 binding site must be located above the plane of PEP near C-3 (3). Hence, the methyl group of L-P-lactate, which points above the plane of the PEP near the C-3 position, may be accommodated in the CO_2 binding site. This additional mode of binding could make L-P-lactate a better inhibitor than D-P-lactate. It is of interest that the CO_2 binding site of carboxic anhydrase may also provide a hydrophobic environment for this substrate as judged by infrared difference spectrosocpy (30). Apparently the undersurface of the PEP site is rather specific for PEP and does not accommodate D-P lactate or phosphoglycolate as well. The Uncompetitive inhibition with respect to CO_2 by L-P-lactate is consistent with this interpretation of our results, since CO_2 combines only with the enzyme-Mn-PEP complex (29) and L-P-lactate combines with a different form of the enzyme, namely, the binary enzyme-Mn complex. Further experiments are underway to test this hypothesis more directly.

PEP synthetase, which catalyzes a two-step reaction, is quite different from the other enzymes studied in that no inhibition by the PEP analogues was detected. Although the conditions required in the kinetic experiment were far from optimal for the binding of phospholactate (low pH, high phosphate, Mg^{2+} instead of Mn^{2+}), the complete absence of inhibition is surprising. One possible explanation may be the absence of a tight binding site for PEP or its analogues on this enzyme. Thus, the rate of phosphorylation of the enzyme within the ternary complex may be greater than the rate of formation of the ternary complex. Alternatively, only the planar vinyl system of PEP may lie on the active site.

Stereochemical (3-5, 31) and binding studies (17, 20, 24) suggest that enzymes which catalyze diverse reactions of PEP bind this substrate in a similar manner. The present results indicate, however, that such enzymes may bind analogues of

### Table V

| Enzyme                                             | Relative affinity for substrates and PEP analogues |
|----------------------------------------------------|---------------------------------------------------|
| Pyruvate kinase (muscle)                           | PEP = n-PL > P-glycolate >> L-PL                   |
| PEP carboxykinase (chicken liver)                  | PEP > L-PL >> d-PL > P-glycolate                   |
| Enolase (yeast and muscle)                         | P-glycolate >> P-glycolate = L-PL = d-PL           |
| PEP synthetase (E. coli)                           | No binding detected                               |

a > implies a factor of 2 or 3; >> implies an order of magnitude difference.

b PL, phospholactate.

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F. Wold, private communication.
PEP quite differently because of the specific geometries of their active sites. Thus, pyruvate kinase interacts strongly with n-P-lactate, but weakly with L-P-lactate, possibly as a result of steric interaction between the methyl group of L-P-lactate and the proton donor of the enzyme. The opposite binding preference is observed with PEP carboxykinase, in which the methyl group of L-P-lactate may contribute to its binding by being held in the CO₃ site. Enolase shows no preference since its proton donor is near C-2, and it binds all analogues with an sp³ C-2 by an order of magnitude more weakly than it does the substrate. PEP synthetase appears to have a much lower affinity for all of the analogues of PEP.

Regardless of the mechanisms, the relative effectiveness of the D and L isomers of phospholactate in inhibiting pyruvate kinase (a carbohydrate-catabolizing enzyme) and PEP carboxykinase (a carbohydrate-anabolizing enzyme) may be of pharmacological interest. Thus, D-P-lactate, which has a 14-fold lower Kᵢ for pyruvate kinase, would tend to prevent glycolysis and L-P-lactate, which has a 1C-fold lower Kᵢ for PEP carboxykinase, would tend to prevent gluconeogenesis, assuming means could be found to introduce these phosphorylated compounds into the appropriate cells.

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