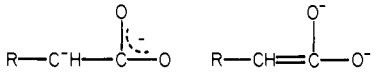
Reaction Intermediate Analogues for Enolase†

Vernon E. Anderson,‡ Paul M. Weiss, and W. W. Cleland*

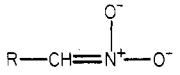
ABSTRACT: A number of compounds that appear to be analogues of the aci form of the normal carbanion intermediate are good inhibitors of yeast enolase. These include (3-hydroxy-2-nitropropyl)phosphonate (I), the ionized (pK = 8.1) nitronate form of which in the presence of 5 mM Mg^{2+} has a K_i of 6 nM, (nitroethyl)phosphonate (III) (pK = 8.5; K_i of the nitronate in the presence of 5 mM Mg^{2+} = 1 μM), phosphonoacetohydroxamate (IV) (pK = 10.2; K_i with saturating Mg^{2+} for the ionized form = 15 pM), and (phosphonoethyl)nitrolate (VII) (K_i at 1 mM Mg^{2+} = 14 nM). The oxime of phosphonopyruvate (VI) has a pH-independent K_i of 75 μM. I, IV, VI, and VII are slow binding inhibitors. All of these compounds are trigonal at the position analogous to C-2 of 2-phosphonoglycerate and contain a phosphono group, but a negatively charged metal ligand at the position isosteric with the hydroxyl attached to C-3 of 2-phosphoglycerate (as in IV) appears to contribute more to binding than a nitro group isosteric with the carboxyl of 2-phosphoglycerate (I and III). These data support the carbanion mechanism for enolase and suggest that the 3-hydroxyl of 2-phosphoglycerate is directly coordinated to Mg^{2+} prior to being eliminated to give phosphoenolpyruvate.

Enolase has been concluded to catalyze the reversible dehydration of 2-P-glycerate to P-enolpyruvate via a carbanion intermediate (Dinovo & Boyer, 1971; Stubbe & Abeles, 1980). The formation of a carbanion α to a carboxyl group has proven to be a general mechanism, not only for the similar elimination reactions of aspartase and fumarase (Porter & Bright, 1980) and aconitase (Schloss et al., 1980) but for the condensation reaction of isocitrate lyase (Schloss & Cleland, 1982) as well. In these reactions, it is thought that the enzyme induces carbanion formation by stabilizing the *aci*-carboxylate, a form of the carbanion with the negative charge delocalized to the carboxyl oxygens:



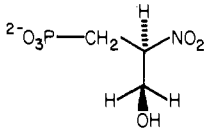
carbanion *aci*-carboxylate

The α protons of nitroalkanes are more acidic than the α protons of carboxylic acids partly because formation of the *aci*-carboxylate is a more favorable process. A nitroalkane in the aci or nitronate form is isoelectronic and isosteric with the aci form of the carbanion intermediate in enzymatic reactions, the only major difference being an extra positive charge introduced by the nitrogen substitution:



According to the theory of Wolfenden (1977), these nitro analogues should be potent inhibitors of enzymes that catalyze reactions with *aci*-carboxylate carbanion intermediates.[1]

Since the nitro analogue of 2-P-glycerate would be an unstable α-nitrophosphate, we have synthesized (3-hydroxy-2-nitropropyl)phosphonate (I), the nitro analogue of methylene-P-glycerate (II),[2] an alternate substrate for enolase (Stubbe & Kenyon, 1972). We also report the syntheses of (nitroethyl)phosphonate (III) and of phosphonoacetohydroxamate (IV), which shows the tightest binding of any known inhibitor of enolase. The inhibition of yeast enolase by these compounds as a function of pH and [Mg^{2+}] is analyzed in detail.



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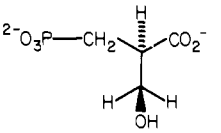

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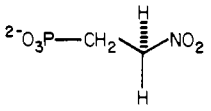

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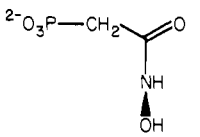

IV

Materials and Methods

Materials. Yeast enolase and (2-aminoethyl)phosphonic acid were from Sigma. Phosphonoacetic acid was from ICN. Diethyl (2-bromoethyl)phosphonate was from Aldrich. Phosphonoalanine was from Calbiochem. [^{14}C]Formaldehyde was from Bio-Rad. 3-Phosphonopropionic acid was from Richmond Organics.

Phosphonoacetohydroxamate (IV). Phosphonoacetic acid was converted to the monoethyl ester by refluxing 1.0 g (7.1 mmol) in 50 mL of absolute ethanol with 0.1 mL of H_2SO_4 for 4 h. After being cooled, the ester was converted to the hydroxamate by adding 20 mL of a freshly prepared solution of 2.0 M NH_2OH–HCl in 3.0 M NaOH. Additional 40% NaOH was added to raise the pH to 13. Any precipitate formed was redissolved by adding water. After 30 min, the reaction was diluted to 1.0 L, titrated to pH 8.0 with HCl, and applied to a 2.5 × 25 cm column of Dowex 1-Cl. The column was washed with 100 mL of water and eluted with a 0–0.1 M LiCl gradient in 20 mM HCl. Phosphonoacetohydroxamate eluted in three 20-mL fractions around 70 mM LiCl, the pooled fractions were titrated to pH 8.0 with LiOH, and the Li^+ salt was recovered by lyophilization and removal

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[1] Such compounds are commonly called transition-state analogues but are really analogues of labile intermediates in most cases (as here) and not of transition states.

[2] Abbreviations: methylene-P-glycerate, 2-(hydroxymethyl)-3-phosphonopropionate; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonate; TLC, thin-layer chromatography.

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of the LiCl by washing twice with dry methanol–acetone (1:4), yield 825 mg (69%). The salt was stable over a 6-month period stored at room temperature over P_2O_5. It was characterized by the UV spectrum in 0.1 N KOH, λ_{max} = 219 nm and ϵ = 9000 cm^{-1} M^{-1}, and the visible spectrum of the ferric complex in 1% $FeCl_3$ in 1.2 N HCl, λ_{max} = 505 nm and ϵ = 275 cm^{-1} M^{-1}. Titration gave p*K*'s of 6.6 and 10.2, and the p*K* of 10.2 was confirmed spectrophotometrically by the increase in absorbance at 240 nm. The proton NMR (in D_2O) showed δ 3.0 (d, J_{PCH} = 19.7 Hz). The 53.6-MHz ^{31}P NMR in D_2O, with an external 85% H_3PO_4 standard, showed δ 12.9 (t, J_{PCH} = 19.4 Hz). The 67.4-MHz ^{13}C NMR (in D_2O) showed δ 183 (d, J_{PCC} = 5 Hz) and δ 49 (dt, J_{PC} = 117 Hz, J_{CH} = 128 Hz). In the proton-decoupled ^{13}C NMR spectrum the δ 49 peaks collapsed to a doublet, J_{PC} = 117 Hz, while the δ 183 peaks were not significantly changed.

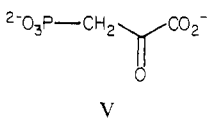
Alternatively, 1 g (8 mmol) of (2-aminoethyl)phosphonic acid was dissolved in a solution containing 160 mL of tetrahydrofuran and 40 mL of 0.4 M *tert*-butylammonium hydroxide (16 mmol). A total of 7.5 g (44 mmol) of *m*-chloroperbenzoic acid in 30 mL of tetrahydrofuran was added and the reaction mixture maintained at 40–45 °C. The reaction was stopped after 2.5 h when 77% (34 mmol) of the *m*-chloroperbenzoic acid had been consumed. Phosphonoacetohydroxamate (IV) was isolated in 40% overall yield by anion-exchange chromatography.

(Nitroethyl)phosphonate (III). Diethyl (2-bromoethyl)-phosphonate was converted to diethyl (2-nitroethyl)-phosphonate by the method of Kornblum & Ungnade (1963). Five grams (20.7 mmol) of diethyl (2-bromoethyl)phosphonate was dissolved in 200 mL of anhydrous ether and slurried with 3.5 g (23 mmol) of $AgNO_2$. The reaction was protected from light and stirred at 4 °C for 1 day and at room temperature for 2 days. The silver salts were removed by filtration through a glass wool plug, and the ether was evaporated under a stream of dry nitrogen. The resulting 1.6 mL of faintly yellow liquid was 5 M in diethyl (2-nitroethyl)phosphonate (39% yield) as judged by the difference in extinction at 240 nm between pH 5.0 and 10.0. The p*K* appeared to be about 7.5, and an extinction coefficient of 10000 cm^{-1} M^{-1} was assumed (Nielsen & Cordes, 1964).

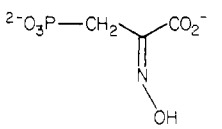
Diethyl (2-nitroethyl)phosphonate was hydrolyzed by the method of McKenna et al. (1977). One milliliter (5 mmol) of the unpurified ester was mixed with 2.1 mL (16 mmol) of bromotrimethylsilane and allowed to react at room temperature for 4 h. The bis(trimethylsilyl) (2-nitroethyl)phosphonate was hydrolyzed by adding the reaction mixture to 100 mL of 0.5 M *N*-ethylmorpholine titrated to pH 8.0 with 1 N HCl. The difference in extinction between pH 5 and 10 at 240 nm indicated that over 85% of the nitro group withstood hydrolysis. The (nitroethyl)phosphonate (III) was purified by titrating the hydrolysate to pH 7.0 with 1 N HCl, applying the solution to a 2.5 × 25 cm column of Dowex 1-Cl, and eluting with 1.6 L of a linear 0–0.3 M LiCl gradient. The fractions containing IV were located by following the slow increase ($t_{1/2}$ = 5 min) in absorbance at 240 nm when an aliquot was added to 0.05 M TAPS, pH 9.0. These fractions were pooled and titrated to pH 10 with LiOH (the titration must be carried out over at least 1 h because of the slow ionization of the compound). This solution was diluted with an equal volume of water and applied to the regenerated Dowex 1-Cl column that had been equilibrated with 5 mM NH_3. The product was eluted with 1.6 L of a 0–0.4 M LiCl gradient in 5 mM NH_3 and was located directly by absorbance at 240 nm. Only those fractions with a λ_{max} of 232 nm and decreasing absorbance to 225 nm were pooled. The pooled fractions were titrated slowly to pH 7.0 with 20 mM HCl and rotary evaporated to a minimal volume. The Li^+ salt was precipitated by addition of 10 volumes of absolute ethanol, collected by centrifugation, and stored over P_2O_5. (Nitroethyl)phosphonate (III) was characterized by the UV spectrum, λ_{max} = 232 and ϵ = 9500 cm^{-1} M^{-1}. At the spectral p*K* of 8.5, the approach to ionic equilibrium was slow ($t_{1/2}$ = 20 min). The 270-MHz proton NMR (in D_2O) showed δ 2.34 (2 H, m) and δ 4.78 (2 H, m). Decoupling the δ 4.78 multiplet collapsed the δ 2.34 peak to a doublet, J_{PCH} = 18.3 Hz. Decoupling the δ 2.34 peak collapsed the δ 4.78 multiplet to a broad singlet. After 3 weeks in D_2O at room temperature, pD 7.0, the δ 4.78 multiplet disappeared and the δ 2.34 peak appeared as a doublet, J_{PCH} = 18.3 Hz, showing that the protons on C-2 had exchanged with the solvent.

(3-Hydroxy-2-nitropropyl)phosphonate (I). I was formed by condensing III with formaldehyde. One hundred milligrams (0.6 mmol) of the Li^+ salt of III was dissolved in 6 mL of water. After titration to pH 10 with KOH, 1.2 mL of 1.0 M [^{14}C]formaldehyde was added, and the reaction was allowed to proceed for 30 min. The reaction mixture was then placed on a 2.5 × 15 cm column of Dowex 1-Cl equilibrated with 10 mM trimethylamine and eluted with 1 L of a linear 0.1–0.2 M LiCl gradient in 10 mM triethylamine. The unreacted III eluted slightly before the ^{14}C-labeled product. The fractions with the highest cpm to A_{240} ratio were pooled. I was characterized by the UV spectrum, λ_{max} = 234.5 nm, the spectral p*K* of 8.1, and the extremely slow approach to the ionic equilibrium at pH 8.1 ($t_{1/2}$ = 250 min).

Phosphonopyruvate (V). Phosphonoalanine (500 mg) was



V



VI

dissolved in 50 mL of methanol containing 0.1 N dry HCl, and the solution was held at 40 °C for 36 h. The reaction was followed by TLC on cellulose plates eluted with butanol–acetic acid–water (7:2:5) and visualized with ninhydrin spray. (This methyl ester could be precipitated as the Li^+ salt by neutralizing with triethylamine and adding an equal volume of methanol saturated with LiCl, but hydrolyzed rapidly in D_2O.) The solution containing the methyl ester was neutralized with 1.5 equiv of *N*-ethylmorpholine and mixed with 100 mL of methanol containing 1 equiv (650 mg) of 3,5-di-*tert*-butyl-*o*-benzoquinone. The mixture was initially red but became light yellow as the reaction proceeded (it was followed spectrophotometrically by the decrease in absorbance at 530 nm). After the reaction was complete (4 h), the Schiff base was hydrolyzed by adding 15 mL of water and adjusting the pH to 4 with acetic acid. After 12 h, the solution was extracted 3 times with an equal volume of $CHCl_3$, and the pooled $CHCl_3$ extracts were then extracted with an equal volume of 10 mM $KHCO_3$. The aqueous phases were combined, titrated to pH 7.5, and loaded on a 2.5 × 25 cm column of Dowex 1-Cl. The solution was eluted with a 1.2 L gradient from 0.15–0.3 M LiCl in 5 mM *N*-ethylmorpholine, pH 7.5. V eluted at 600–660 mL and was identified by (1) its UV absorbance at 255 nm, (2) the change in absorbance at 253 nm when 0.01 mL was added to 2 mL of 1% semicarbazide–HCl and 2% sodium acetate, and (3) total phosphate analysis. The peak fractions were titrated to pH 9 with LiOH, rotary evaporated to a syrup, and diluted with 15 volumes of absolute ethanol.

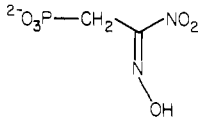
The precipitate was isolated by centrifugation, redissolved in water, and reprecipitated by the addition of ethanol to remove *N*-ethylmorpholine. The final precipitate was redissolved in D_2O, and the solvent was removed under reduced pressure. This process was repeated 3 times to remove ethanol and permit NMR analysis.

The proton NMR showed two closely spaced (0.03 ppm) doublets at δ 3.05 with 20-Hz splitting caused by ^{31}P. Presumably, the upfield, slightly broader doublet corresponds to –CHD– and the other to $-CH_2-$. The ^{31}P NMR in 50% D_2O gave a broad singlet at 7.5 ppm, which became a pentuplet after 24 h (a triplet for $-CH_2-PO_3^{2-}$, a doublet for –CHD–PO_3^{2-}, and a singlet for $-CD_2-PO_3^{2-}$). The J_{HCP} splitting constant of 20 Hz was the same seen in the proton NMR spectrum. The ^{13}C NMR after being allowed to stand in 100% D_2O for several days showed carbons 1 and 2 as a sharp singlet at 170 ppm and a closely split doublet at 202 ppm (split by 6 Hz from long-range ^{31}P coupling), but carbon 3 was not visible. In 30% D_2O with proton decoupling, however, this carbon was seen at 43 ppm as a doublet with 100-Hz ^{31}P coupling (from $-CH_2-$) and a doublet of triplets (J_{CD} = 20 Hz, from –CHD–). These data confirm the structure of V as predominately the keto form but with sufficient enolization to exchange the protons at C-3 on standing for several days.

Phosphonopyruvate (V) was found to have pK values of 6.60 and 2.35 by titration. Its reacts at pH 4.5 with semicarbazide to give a product with λ_{max} = 253 nm and ϵ = 10 000 (based on total phosphate content) and with hydroxylamine to give increased UV absorbance but no discrete peak (at 240 nm, ϵ rises from 700 to 1400). At pH 13, the extinction coefficient is doubled again at 240 nm, but no discrete peak of absorbance is seen.

When phosphonopyruvate (V) was incubated with hydroxylamine at high pH, small amounts of what appeared to be phosphonoacetohydroxamate (IV) were formed. This product (formed at pH 12) chromatographed as a dianion at pH 7.5 (vs. a trianion for the major oxime product VI) but made up less than 5% of the reaction product. Its K_i vs. enolase was pH dependent and was around 20 nM at pH 9. Since high pH with or without hydroxylamine did not convert the major oxime product into this inhibitor, it seems most likely that it is formed by oxidative decarboxyltion of the carbinolamine intermediate (which is formed rapidly at high pH but dehydrated slowly), possibly with hydroxylamine as the oxidant (Scheme I).

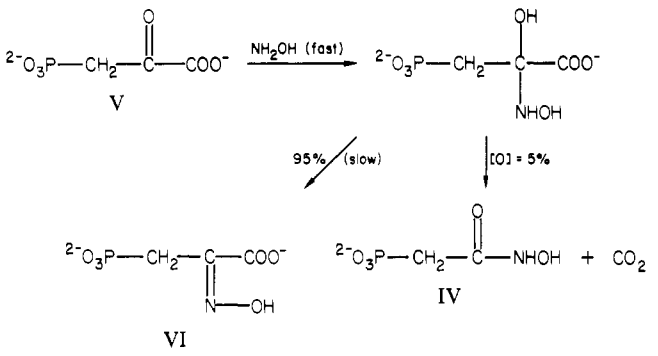
(2-Phosphonoethyl)nitrolic Acid (VII). VII was prepared



VII

by the method of Meyer (1875). One milliliter of 0.5 M sodium nitrite (0.5 mmol) was added to 1 mL of 0.1 M III that had been titrated to pH 10 with LiOH and kept on ice. The mixture was slowly acidified by the addition of five 20-μL aliquots of 1 N HCl over 20 min. During the course of the acidification the solution turned light yellow. Five minutes after the final acid addition, five 20-μL aliquots of N LiOH were added over 20 min to the solution, which turned very deep yellow. This cycle of slow acidification and treatment with base was repeated 3 times. The presence of VII was inferred from the characteristic broad absorption band of the ionized nitrolic acid in the near-UV (Morgan, 1959), λ_{max} = 337 nm. The reaction mixture was titrated with 1 N HCl to pH 6.5

Scheme I



(faint yellow), and the lithium phosphonate salts were precipitated by the addition of 20 mL of absolute ethanol and redissolved in water.

Experimental Methods. The concentrations of inhibitors were determined primarily by ashing a small aliquot with $MgNO_3$ and determining the total phosphate concentration (Ames, 1966). These concentrations were used to determine the extinction coefficients in 0.1 M KOH at the absorption maxima. To be certain the nitro compounds were at ionic equilibrium in the enzyme inhibition assays, stock solutions of (nitroethyl)phosphonate and I were maintained at pH 5.5 and diluted into different pH buffers the day preceding an experiment. 2-P-glycerate and P-enolpyruvate concentrations were determined by converting them to lactate in the presence of MgADP, NADH, pyruvate kinase, lactate dehydrogenase, and (for 2-P-glycerate) enolase and following the concomitant decrease in the NADH concentration at 340 nm.

For measurement of inhibition, various quantities of an inhibitor were added to a 2-mL reaction mixture containing 50 mM TAPS, pH 9, 1 mM magnesium acetate, and variable 2-P-glycerate (0.25 mM for testing column fractions for inhibition). Reactions were begun by addition of enolase (at a level always at least 4-fold lower than the apparent K_i of the inhibitor and as low as 0.1 nM with the slow binding inhibitors) and followed by the increase in absorbance of P-enolpyruvate at 240 nm. All assays were at 25 °C. Bursts were seen when reactions were initiated with enolase and included either phosphonoacetohydroxamate or I. Lags in reaching the steady-state velocity were seen by preincubating enolase and either inhibitor long enough for binding of it to reach equilibrium and starting the assay by addition of 2-P-glycerate. These two inhibitors thus display slow binding inhibitory behavior (Williams & Morrison, 1979).[3]

Enolase concentrations were determined by incubating various concentrations of enzyme with a fixed concentration of phosphonoacetohydroxamate for 10 min at pH 9.0 and 1.0 mM Mg^{2+}, initiating the reaction with a saturating concentration of 2-P-glycerate, and following the initial velocity as shown in Figure 1. At the intersection of the linear assymptote with the horizontal axis, the concentrations of enolase active sites and of phosphonoacetohydroxamate are equal (Williams & Morrison, 1979), if we assume that one molecule of inhibitor binds per active site.

Data Analysis. Kinetic data were fitted to the appropriate rate equations by FORTRAN programs of Cleland (1979)

[3] "Slow binding" is misleading since the second-order rate constant could be diffusion controlled and slow binding behavior is still observed at low enough inhibitor concentrations. It is a slow off-rate ($<0.15\ s^{-1}$) that is required for slow binding inhibition to be seen. A more accurate description would be a "slow equilibrating" or "slow dissociating" inhibitor.

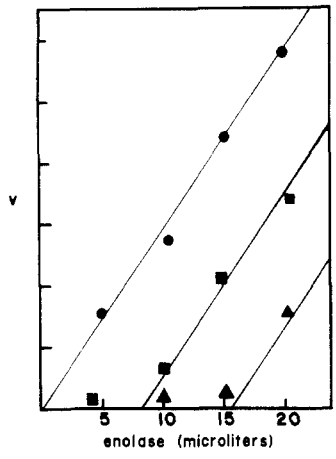


FIGURE 1: Initial velocities of assays (pH 9.0, 1.0 mM Mg^{2+}) initiated with 0.5 mM 2-P-glycerate after a 10-min preincubation of enolase with the following concentrations of phosphonoacetohydroxamate (IV): (●) 0; (■) 15 nM; (▲) 30 nM. The lines are drawn to be parallel and have equal spacing.

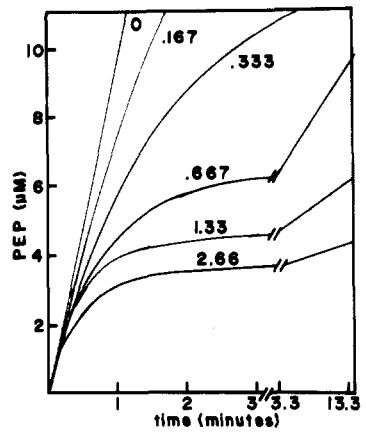


FIGURE 2: Slow binding behavior of phosphonoacetohydroxamate (IV) as an inhibitor. Assay conditions were pH 8.5, 0.5 mM 2-P-glycerate, 0.5 mM Mg^{2+}, and 1.8 nM enolase active sites. The micromolar concentrations of the inhibitor are shown in the figure.

that assume equal variance for the fitted parameter or its logarithm. Data from competitive inhibition experiments were fitted to eq 1. The K_i values from eq 1 as a function of pH

$$v = VA/[K(1 + I/K_i) + A] \quad (1)$$

were fitted to eq 2 when the K_i value increased a factor of 10

$$pK_i = \log [(1/K_{i\,high\,pH})/(1 + H/K)] \quad (2)$$

per pH unit below pK and to eq 3 when it varied from one

$$pK_i = \log \left(\frac{1/K_{i\,low\,pH} + (1/K_{i\,high\,pH})(K/H)}{1 + K/H} \right) \quad (3)$$

plateau value at low pH to another at high pH. The variation of pK_i values as a function of Mg^{2+} concentration was fitted to equations similar to eq 2 and 3 with [Mg^{2+}] replacing H or to eq 4.

$$pK_i = \log [(1/K_{i\,I})(1 + [Mg^{2+}]/K_{I\,Mg})] \quad (4)$$

The time courses of bursts and lags in the presence of the slow binding inhibitors were fitted to eq 5 (where P is net

$$P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \quad (5)$$

product formed), the integrated form of the rate equation that assumes a first-order change in the reaction velocity from the initial velocity, v_0, to the final steady-state velocity, v_s, with a rate constant k (Williams & Morrison, 1979). In burst experiments, the rate of dissociation of a slow binding inhibitor may be determined from the constants of eq 5 as $k_{off} = k(v_s/v_0)$. Since the plot of k vs. inhibitor concentration at a fixed substrate concentration was linear, k_{on} could be calculated from the slope of the line multiplied by $K_i(1 + [2\text{-P-glycerate}]/K_{2PGA})$, where K_i is k_{off}/k_{on} (Williams & Morrison, 1979).

Results

Inhibition by Phosphonoacetohydroxamate (IV). When an assay containing this inhibitor was started with enolase, the curved time courses in Figure 2 were observed. The rate constant for the change in velocity, obtained by fitting each time couuse to eq 3, was a linear function of inhibitor concentration. In experiments where the binding of enolase and phosphonoacetohydroxamate was allowed to come to equilibrium and the assay was started by the addition of 2-P-glycerate, lags were observed. The rate constant for the change

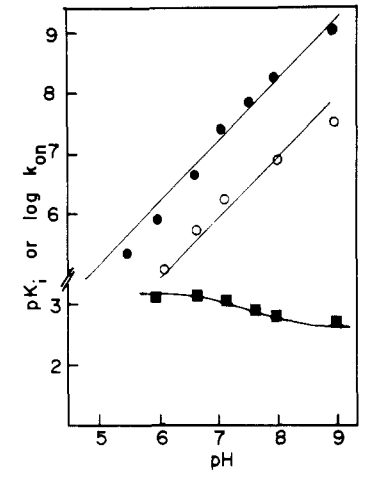


FIGURE 3: Variation of pK_i (●) and log k_{on} [(○) the units are min^{-1} M^{-1}] with pH for phosphonoacetohydroxamate (IV). The lines assume a change of a factor of 10 per pH unit. K_i values were obtained from the final steady-state velocities. [Mg^{2+}] was $5K_{Mg}$. For comparison, pK_i values for P-glycolate (■) fitted to eq 3 are also shown (K_i was 0.61 ± 0.07 mM at low pH and 2.4 ± 0.2 mM at high pH, with a pK value of 7.8 ± 0.2 for the transition).

in velocity was still determined by fitting the time courses to eq 5 (v_s is now larger than v_0). The rate constants for the change in velocity obtained from burst or lag experiments under identical conditions were always equal within experimental error. The k_{on} for phosphonoacetohydroxamate increased approximately a factor of 10 per pH unit as shown in Figure 3. The value of k_{off} decreased as [Mg^{2+}] increased as shown in Figure 4. The inhibition constants for phosphonoacetohydroxamate obtained from the final steady-state velocities as a function of pH and [Mg^{2+}] are shown in Figures 3–5.

Phosphoglycolate Inhibition. The variation of the K_i for P-glycolate as a function of pH and [Mg^{2+}] is presented in Figures 3–5 for comparison with the K_i values for phosphonoacetohydroxamate. The K_i for P-glycolate is relatively pH independent, being 4-fold smaller at low pH than at high pH. Increasing [Mg^{2+}] decreases the K_i for P-glycolate, but the slope of the line in the plot of pK_i vs. log [Mg^{2+}] was less than 1.

Inhibition by the Nitro Compounds. (Nitroethyl)-phosphonate (III) was a competitive inhibitor of enolase, but there was no curvature to the assay time courses beyond that expected from depletion of the substrate (that is, it did not act as a slow binding inhibitor). The pH variation of the K_i

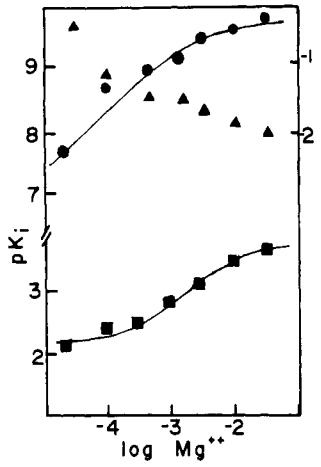


FIGURE 4: Variation of pK_i and log k_{off} with $[Mg^{2+}]$ at pH 9.0: (●) pK_i for phosphonoacetohydroxamate (IV) (data fitted to an equation analogous to eq 2, giving K_{II} = 230 pM and $K_{i\,Mg}$ = 2.0 ± 0.5 mM; see mechanism 6 and eq 7); (▲) k_{off} for phosphonoacetohydroxamate; (■) pK_i for P-glycolate (data fitted to eq 7, giving $K_{i\,Mg}$ = 11.5 ± 2.1 mM, $K_{I\,Mg}$ = 0.29 ± 0.08 mM, K_{II} = 0.16 ± 0.05 mM, and $K_{i\,I}$ = 6.5 ± 1.2 mM; see mechanism 6 and eq 7).

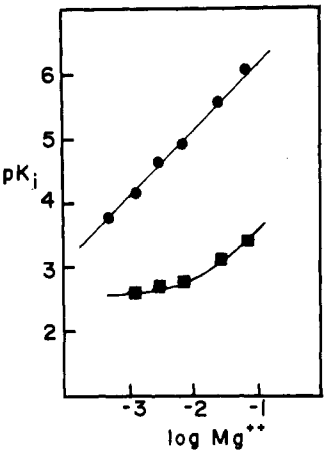


FIGURE 5: Variation of pK_i with $[Mg^{2+}]$ at pH 5.5. The inhibitor was phosphonoacetohydroxamate (IV) [(●) the line assumes that K_i is inversely proportional to $[Mg^{2+}]$] or P-glycolate [(■) the data were fitted to eq 4, giving $K_{i\,I}$ = 2.7 ± 0.2 mM and $K_{I\,Mg}$ = 12.3 ± 0.5 mM; see mechanism 6 and eq 7].

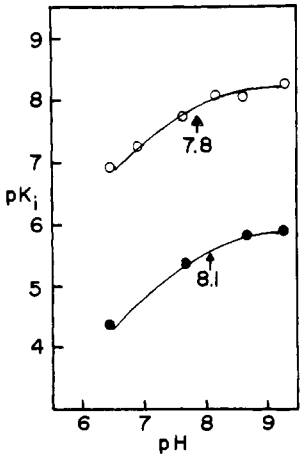
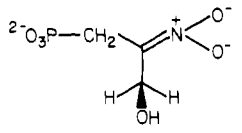


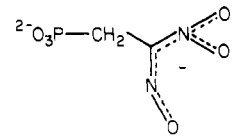
FIGURE 6: Variation of pK_i with pH for (nitroethyl)phosphonate (●) and I (○). The data were fitted to eq 2, giving pK = 8.1 ± 0.1 and a limiting K_i value of 1.2 ± 0.2 μM for (nitroethyl)phosphonate and pK = 7.8 ± 0.1 and a limiting K_i value of 6.4 ± 1.0 nM for I. $[Mg^{2+}]$ was $5K_{Mg}$.

at saturating $[Mg^{2+}]$ is shown in Figure 6. The limiting K_i at high pH is 1.2 ± 0.2 μM, but this value increases by a factor of 10 per pH unit below a pK of 8.1. The inhibition of enolase by I, on the other hand, was time dependent. The half-time for the development of inhibition varied from 5 to 20 min, corresponding to rate constants of 0.14–0.034 min^{-1} and k_{off} value of less than 0.01 min^{-1}. The extremely slow rate constant for the change in velocities required that either high I concentrations or low enolase levels (~0.1 nM) be used to prevent over 15% of the 2-P-glycerate from being consumed before the final steady-state velocity was reached. The K_i for I as a function of pH is shown in Figure 6. The K_i at high pH is 6.4 ± 1.0 nM and increases a factor of 10 per pH unit below a pK of 7.8.

Enolase does not dehydrate I at a significant rate. When I was incubated with 1 mg/mL enolase at pH 8.0, there was no increase in absorption at 230 nm, as would be expected if a vinyl nitro group were formed. Apparently, the nitronate form of I (VIII) is so stable that OH^- cannot be lost from C-3.



VIII



IX

Additionally, when I equilibrated at pH 5.5 was added to a solution of 1 mg/mL enolase at pH 8.0, the rate of ionization of I was not significantly higher than when it was added to the assay mix without enolase. A similar experiment with I equilibrated at pH 10 showed that enolase does not significantly catalyze the protonation of VIII. These data suggest that only the trianion of I binds to and dissociates from enolase.

Inhibition by the Oxime of Phosphonopyruvate (VI). As noted above in describing the synthesis of phosphonopyruvate, treatment of this keto acid with hydroxylamine at high pH leads to formation of small amounts of what appears to be phosphonoacetohydroxamate (IV). The true oxime of phosphonopyruvate (VI), when isolated chromatographically however, showed a pH-independent (over the range 8–9.3) K_i vs. enolase of 75 μM and showed slow binding behavior (that is, the inhibition became stronger with time; the K_i is for the final steady-state phase of the inhibition). The pK of this oxime is about 12, and thus, the K_i of the ionized oxime is less than 3 orders of magnitude lower than that for the trianionic oxime.

Inhibition by (2-Phosphonoethyl)nitrolate (IX). The crude preparation of this compound was a slow binding inhibitor of enolase at pH 9 (the pK of VII should be well below this pH), 1 mM Mg^{2+}, with a K_i based on final steady-state velocities and on the total phosphorus content of the inhibitor solution of 13 ± 4 nM. While some residual (nitroethyl)phosphonate (III) may have been present, this compound inhibits much too weakly to have contributed to the observed inhibition. The only inhibitor that binds tightly enough to enolase to have contributed to this inhibition is phosphonoacetohydroxamate (IV), but it would have had to be 10% of the preparation to account for the observed inhibition. Solutions of nitrolic acid salts decompose on heating to nitrous oxide, nitrite, and a carboxylic acid, but hydroxamates have not been reported as products (Morgan, 1959). While the conclusion must remain tentative until a more thoroughly characterized preparation is obtained, we believe that this inhibition is caused by the nitrolate.

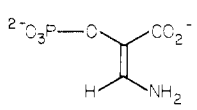
Discussion

Phosphonoacetohydroxamate Inhibition. Oxidation of aliphatic primary amines to nitro compounds by *m*-chloro-

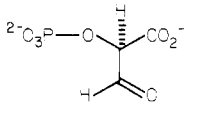
perbenzoic acid has been reported by Robinson et al. (1966). Our attempt to synthesize (nitroethyl)phosphonate (III) by this procedure yielded a potent inhibitor of enolase that analysis proved to be phosphonoacetohydroxamate (IV). Oxidation of primary amines to hydroxamates by peracids has been observed (Bamberger, 1903).

The time-dependent inhibition of enolase observed in Figure 2 implies that the equilibration of the inhibitor binding is slow. The decrease in reaction velocity cannot be attributed to inactivation of enolase, since a final steady-state velocity is reached and since enolase incubated with phosphonoacetohydroxamate in the absence of substrate regains activity with the same rate constant when 2-P-glycerate is added to the incubation. Slow binding behavior suggest a slow unimolecular step after an initial complex has been formed (Williams & Morrison, 1979). If a slow unimolecular step is responsible for the slow binding behavior, then the rate constant for the change in velocity will reach a limiting value when the inhibitor is saturating for formation of the initial complex. However, under all experimental conditions tested, this rate constant was a linear function of the phosphonoacetohydroxamate level. If there is initial reversible binding of the inhibitor prior to a slow unimolecular step, the initial complex of enolase and phosphonoacetohydroxamate must have a dissociation constant larger than 1.0 μM. Such initial binding is likely, but is apparently too weak to be detected at the levels of inhibitor that it is practical to employ.

Slow binding inhibition of enolase has been observed before by Spring & Wold (1971b) with 3-aminoenolpyruvate-P (X).

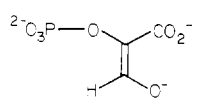


X

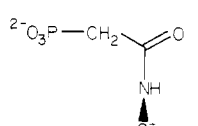


XI

In a stopped-flow study, Lane & Hurst (1974) analyzed the transitory kinetics of the inhibition of enolase by both aminoenolpyruvate-P and tartronate semialdehyde-P (XI). They concluded that X bound in a second-order fashion but that the rate of binding of XI was limited by a unimolecular step. They found that the rate of dissociation of XI was 0.6 min^{-1}, 10 times faster than that for phosphonoacetohydroxamate (IV) under similar conditions. The forward rate constant for the unimolecular step, 12 min^{-1}, was too fast to be detected without a rapid-mixing device. The k_{on} value for X with enolase was 1.5×10^6 min^{-1} M^{-1}, 20-fold slower than the k_{on} of 3.2×10^7 min^{-1} M^{-1} for phosphonoacetohydroxamate at pH 9.0 and, consequently, over 200-fold slower than k_{on} for the trianion of the latter. Unlike the situation with phosphonoacetohydroxamate, however, the inhibition by tartronate semialdehyde-P is pH independent (P. A. Weiss and W. W. Cleland, unpublished experiments), and the removal of the proton from C-2 to give an enolate (XII) on the enzyme is apparently a catalytic process.



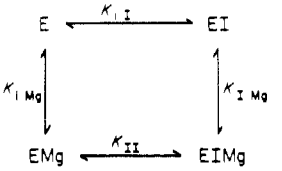
XII



XIII

The slope of 1.0 observed in the plot of pK_i vs. pH, Figure 3, indicates that some group that is normally protonated in the accessible pH range is unprotonated in the enolase–phosphonoacetohydroxamate complex. The nearly pH-independent nature of the P-glycolate K_i suggests that this is not an enzymic group but that the hydroxamate group of phosphonoacetohydroxamate is ionized in its complex with enolase. From the spectral pK of 10.2 and the observed K_i of 230 pM at pH 9 in the presence of infinite Mg^{2+}, the K_i of the trianion of phosphonoacetohydroxamate (XIII) would be about 15 pM. The increase in k_{on} with pH suggests that it is the trianion that initially binds to enolase. If both the doubly and triply ionized forms bound, but the dianion dissociated faster, the decrease in K_i would show up as a decrease in k_{off}, rather than as an increase in k_{on} with increasing pH.

The combination of Mg^{2+} with enolase and an enolase–inhibitor complex is represented by mechanism 6. The apparent



(6)

K_i for the inhibitor as a function of $[Mg^{2+}]$ can be calculated from eq 7, where $K_{i\,Mg}$ and $K_{I\,Mg}$ are dissociation constants

$$\text{app } K_i = K_{i\,I}(1 + [Mg^{2+}]/K_{i\,Mg})/(1 + [Mg^{2+}]/K_{I\,Mg}) \quad (7)$$

of Mg^{2+} from E and EI and $K_{i\,I}$ is the dissociation constant of I from EI. The dissociation constant of I from EIMg is given by

$$K_{II} = K_{i\,I}K_{I\,Mg}/K_{i\,Mg} \quad (8)$$

Figure 7 shows the shape of a plot of eq 7 and illustrates the graphical determination of the four dissociation constants.

At pH 5.5, the linearity of the plot of app pK_i for phosphonoacetohydroxamate vs. $[Mg^{2+}]$ shows that $K_{I\,Mg}$ is less than 0.1 mM and $K_{i\,Mg}$ is greater than 100 mM. The high value for the latter is consistent with the fact that Mg^{2+} normally adds only after 2-P-glycerate or P-enolpyruvate in the reaction (Anderson, 1981), and the low value for $K_{I\,Mg}$ shows that there is great synergism in the binding of phosphonoacetohydroxamate and Mg^{2+}. This is expected because the ionized hydroxamate group is such a good metal ligand. The app pK_i for P-glycolate, however, clearly determines $K_{I\,Mg}$ in this case as 12 mM and $K_{i\,\text{P-glycolate}}$ as 2.7 mM. The synergism in binding of P-glycolate and Mg^{2+} is thus not great, as expected because P-glycolate does not have any group in the position analogous to the hydroxymethyl of P-glycerate, which is thought to coordinate to Mg^{2+} during the reaction (Nowak et al., 1973).

At pH 9, however, it is possible to detect the binding of Mg^2 to the enzyme in the absence of inhibitor [this is too weak to see at pH 5.5 because metal ligands on the protein become protonated at low pH (Anderson, 1981)]. The pK_i profiles for both phosphonoacetohydroxamate and P-glycolate level out at high pH with $K_{i\,Mg}$ values of 2 and 12 mM, respectively. (These values should be the same, but the quality of the data is such that these values are probably not significantly different.) While $K_{I\,Mg}$ for phosphonoacetohydroxamate is again too low to detect, the value is 0.3 mM for P-glycolate. The synergism in the binding of Mg^{2+} and P-glycolate is thus a factor of 50. Since P-glycolate does not contain a ligand for the metal, the effect must come from a conformation change induced by the binding of P-glycolate, which is a pseudosubstrate from C-2 of which a proton is reversibly removed, as shown by the slow exchange with solvent seen by Stubbe & Abeles (1980).

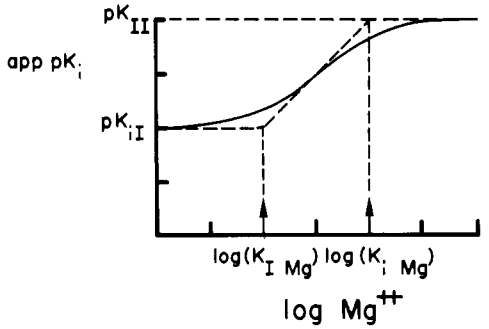
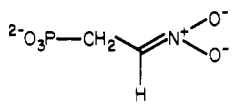


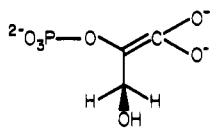
FIGURE 7: A plot of eq 7, showing the graphical analysis for the four kinetic constants in mechanism 6.

The Mg^{2+} concentration in the above studies was always 10-fold greater than the dissociation constant for the first, or "conformational", Mg^{2+} that binds to enolase (Faller et al. (1977). Consequently, the Mg^{2+} dissociation constants detected by the variation in K_i values for phosphonoacetohydroxamate and P-glycolate correspond to the second or catalytic Mg^{2+}, and "free enzyme" is the enolase–[conformational metal] complex. The binding of more than one Mg^{2+} per active site in the absence of substrate was not observed by Brewer (1981), but it is hard to detect dissociation constants over 1 mM, and in the absence of substrate or inhibitor the binding of the second Mg^{2+} may not affect the absorption of enolase at 295 nm used by Brewer & Collins (1980) to detect metal binding. Faller et al. (1977) determined the dissociation constant of the second Mg^{2+} from the enolase–(2-P-glycerate, P-enolpyruvate)–Mg^{2+} complex to be 50 μM. Our data suggest that the K_i value for this second Mg^{2+} from an enolase–phosphonoacetohydroxamate–Mg^{2+} complex is less than 10 μM and from an enolase–P-glycolate–Mg^{2+} complex is around 0.1 mM.

Inhibition by I and (Nitroethyl)phosphonate (III). The spectral pK's of the nitro compounds are within the pH region of enolase activity. Figure 6 demonstrates that the inhibition of enolase by I and (nitroethyl)phosphonate is pH dependent but, unlike the inhibition with phosphonoacetohydroxamate, reaches a maximum at high pH. This is consistent with trianions of these two inhibitors (VIII and XIV) being the inhibitory forms. The apparent pK's of 7.8 and 8.1 observed in the pK_i profiles for I and (nitroethyl)phosphonate are within experimental error of the spectrally determined pK's of 8.1 and 8.5. The replacement of one of the C-2 hydrogens of (nitroethyl)phosphonate by a CH_2OH group reduced the K_i by 150-fold, contributing an additional 3 kcal/mol to the binding energy.



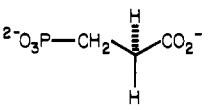
XIV



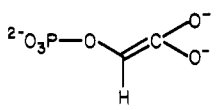
XV

Structure of the Carbanion Intermediate in the Enolase Reaction. The fact that nitronate forms of nitro analogues of aspartate, malate, and isocitrate and citrate were potent inhibitors of aspartase, fumarase, and aconitase, respectively, has been interpreted by Porter & Bright (1980) and by Schloss et al. (1980) to mean that carbanion intermediates were involved in these elimination reactions and that the carbanion had an *aci*-carboxylate structure, with a trigonal rather than tetrahedral ionized carbon. The potent inhibition of enolase by I and (nitroethyl)phosphonate suggests that the same situation applies here. The K_i (6 nM) for the trianion of I (VIII), which is isosteric with the putative carbanion (XV), is 3 orders of magnitude lower than the Michaelis constants of the normal substrates or their methylene-bridged analogues (the major effect of the substitution of CH_2 for oxygen is on V_{max} not on K_m). Thus, the enzyme clearly displays much tighter binding of the reaction intermediate which is trigonal at C-2 and tetrahedral at C-3 than of either substrate, one of which is trigonal at both carbons and the other of which is tetrahedral at both.

Which portions of the intermediate structure are most important for binding? All of the tightly bound inhibitors have a trigonal carbon at C-2 and a phosph(on)ate group. The contribution a trigonal carbon at C-2 makes can be estimated from the ratio of the K_i's for (nitroethyl)phosphonate (1 μM for XIV) and 3-phosphonopropionate (>10 mM, XVI). The



XVI

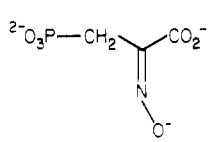


XVII

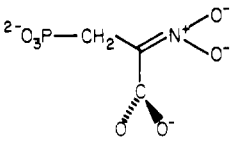
ratio of over 10^4 suggests that enolase stabilizes the tetrahedral to trigonal transition at C-2 by at least 5.5 kcal/mol. The hydroxymethyl group at C-3 contributes 3 kcal/mol to binding, as noted above from comparison of (nitroethyl)phosphonate and I or 2-P-glycerate and P-glycolate. In fact, the nitronate of (nitroethyl)phosphonate (XIV) is really an anlogue of the carbanion form of P-glycolate (XVII), formation of which must be catalyzed by the enzyme during the solvent exchange reaction discovered by Stubbe & Abeles (1980), and XIV binds 3 orders of magnitude tighter than P-glycolate. It is clear, however, that a negatively charged group at the position of C-3 can contribute even more to binding strength than a hydroxymethyl group. The most efficient group is the ionized hydroxamate of phosphonoacetohydroxamate (XIII), and the reason for this is presumably that the negatively charged oxygen can assume an out-of-plane position completely isosteric with the 3-hydroxyl of the carbanion intermediate (since the elimination is trans, the geometry must be as shown in II and XV). The nearly 3 orders of magnitude difference in the binding of I and phosphonoacetohydroxamate suggests that Mg^{2+}, which binds very synergistically with substrates and inhibitors, is directly coordinated to the 3-hydroxyl groups of 2-P-glycerate and of the carbanion intermediate during the reaction, as originally postulated by Nowak et al. (1973).

Negative charges on oxygen attached to C-3 or the equivalent position which cannot assume an out-of-plane position contribute to binding, but not as strongly as the ionized hydroxamate of phosphonoacetohydroxamate. These include the enolate formed catalytically by proton removal from tartronate semialdehyde-P (XII) and the nitrolate IX. The double bonds prevent rotation to the prefered out-of-plane position, and there are two possible in-plane positions; we have no way to tell which occurs on the enzyme, or whether both do. Tartronate semialdehyde-P (XI) binds about as well as the normal substrates, but because only the free aldehyde form should bind and this form is less than 1% of the total (the rest is the *gem*-diol), the true K_i value for the aldehyde will be less than 150 nM. In aminoenolpyruvate-P (X), the neutral amino group (which will be a better metal ligand than a hydroxyl, but not as good as a negatively charged oxygen) is also constrained to be in the in-plane position, and thus, X shows a K_i at pH 8 of 500 nM.[4] The oxime of phosphonopyruvate

(VI) binds more weakly still with a K_i of 75 μM. One might expect the ionized oxime (XVIII) to be a better inhibitor, but



XVIII



XIX

in view of its pK of 12 and the pH-independent K_i (up to pH 9.3), the K_i of XVIII must be above 200 nM. Thus, it is quite critical whether the metal-binding atom attached to C-3 or the atom isosteric with it can assume the out-of-plane position required for the normal carbanion intermediate.

By contrast with the importance with the rest of the molecule, the presence of the carboxyl group, or its nitro analogue, seems to be less important. This group is totally absent in phosphonoacetohydroxamate (IV), the best inhibitor we have. There does not seem to be any way, however, to design an inhibitor with a freely rotating negatively charged oxygen attached to the C-3 position and still maintain a trigonal C-2 with an attached nitronate group unless the enzyme could accommoate a carboxyl group at C-3 as in the tetraanion of (2-nitro-3-phosphono)propionate (XIX). This would be an interesting molecule to test as an enolase inhibitor!

Registry No. I, 89873-28-9; III, 89873-29-0; IV, 89873-30-3; V, 5824-58-8; VI, 89873-31-4; VII, 89873-32-5; P-glycolate, 13147-57-4; phosphonoacetic acid, 4408-78-0; phosphonoacetic acid monoethyl ester, 35752-46-6; (2-aminoethyl)phosphonic acid, 2041-14-7; diethyl (2-bromoethyl)phosphonate, 5324-30-1; diethyl (2-nitroethyl)-phosphonate, 73084-26-1; bis(trimethylsilyl) (2-nitroethyl)phosphonate, 89873-33-6; formaldehyde, 50-00-0; phosphonoalanine, 5652-28-8; enolase, 9014-08-8.

[4] The protonation state of the amino groups should affect this value. Aminoenolpyruvate-P shows a change in its UV spectrum with a pK of 7.5 (Spring & Wold, 1971a), which is probably that for the amino group, and thus, it will be largely in the neutral amino form at pH 8. The pH profile of the decomposition rate of aminoenolpyruvate-P (Spring & Wold, 1971a) shows that the rate increases (presumably a factor of 10 per pH unit) as the pH is lowered until it plateaus at the pK of 7.5. It then shows a further increase to a plateau 3 times faster with a pK for the change of about 6.5 (presumably the phosphate pK). Below pH 3, the rate increases drastically again, presumably as the result of protonation of the carboxyl group. This profile can be explained by assuming that the rate-limiting step for decomposition is protonation of C-2 of the enamine by H^+, followed by addition of water to C-3 and subsequent rapid breakdown of the carbinolamine. The rate plateaus below the pK of the amino group because the level of the free amino form is decreasing a factor of 10 per pH unit as $[H^+]$ is increasing by the same factor. The transition to higher plateau values when the phosphate and carboxyl groups are protonated presumably reflects the increased proportion or reactivity of the carbanion form of the enamine as the number of negative charges in the near vicinity of C-2 is decreased.

References

Ames, B. W. (1966) *Methods Enzymol. 8*, 115.

Anderson. V. E. (1981) Ph.D. Dissertation, University of Wisconsin, Madison.

Bamberger, E. (1903) *Ber. Dtsch. Chem. Ges. 36*, 710.

Brewer, J. M. (1981) *C.R.C. Crit. Rev. Biochem. 11*, 209.

Brewer, J. M., & Collins, K. M. (1980) *J. Inorg. Biochem. 13*, 151.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Dinovo, E. C., & Boyer, P. D. (1971) *J. Biol. Chem. 246*, 4586.

Faller, L. D., Baroundy, B. M., Johnson, A. M., & Euall, R. X. (1977) *Biochemistry 16*, 3864.

Kornblum, N., & Ungnade, H. E. (1963) *Organic Syntheses*, Collect. Vol. IV, p 724, Wiley, New York.

Lane, R. H., & Hurst, J. K. (1974) *Biochemistry 13*, 3292.

McKenna, C. E., Higa, M. T., Cheung, N. H., & McKenna, M.-C. (1977) *Tetrahedron Lett.*, 155.

Meyer, V. (1875) *Justus Liebigs Ann. Chem. 175*, 88.

Morgan, D. J. (1959) *J. Appl. Chem. 9*, 201.

Nielsen, A. T., & Cordes, H. F. (1964) *Tetrahedron, Suppl. 20*, 235.

Nowak, T., Mildvan, A. S., & Kenyon, G. L. (1973) *Biochemistry 12*, 1690.

Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772.

Robinson, C. H., Milewich, L., & Holter, P. (1966) *J. Org. Chem. 31*, 524.

Schloss, J. V., & Cleland, W. W. (1982) *Biochemistry 21*, 4420.

Schloss, J. V., Porter, D. J. T., Bright, H. J., & Cleland, W. W. (1980) *Biochemistry 19*, 2358.

Spring, T. G., & Wold, F. (1971a) *Biochemistry 10*, 4649.

Spring, T. G., & Wold, F. (1971b) *Biochemistry 10*, 4655.

Stubbe, J. A., & Kenyon, G. L. (1972) *Biochemistry 11*, 338.

Stubbe, J. A., & Abeles, R. H. (1980) *Biochemistry 19*, 5505.

Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.

Wolfenden, R. (1977) *Methods Enzymol. 46*, 15.