Mechanisms of Hydrolysis of O-Phosphorothioates and Inorganic Thiophosphate by Escherichia coli Alkaline Phosphatase*

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SUMMARY

Hydrolyses of phosphate and O-phosphorothioate monooesters by Zn(II) and Co(II) alkaline phosphatases (Escherichia coli) are compared. Although the rate of hydrolysis of the O-phosphorothioate by the Zn(II) enzyme is ~100-fold slower than the hydrolysis of phosphate esters, the kinetic data of the transient and steady state phases of the reaction show that the stepwise mechanisms are apparently identical. Reaction of the Zn(II) enzyme with O-phosphorothioate at acid pH results in the incorporation of covalently linked S into the enzyme during the transient phase of the reaction, and H₂S is not released. Thus a thioenzyme forms analogous to the thioenzyme formed by phosphate esters. In contrast, H₂S and Pi are the products of hydrolysis of inorganic thioenzyme by the enzyme. The variation in the absolute and relative magnitudes of the rate constants describing (thio)phosphorylation and de-(thio)phosphorylation of the enzyme can be explained on the basis of the steric and electronic effects of sulfur substitution in retarding nucleophilic displacement processes involved in the generation and decomposition of reaction intermediates.

Sulfur substitution reduces the negative homotropic interactions between the two active sites such that at concentrations of O-phosphorothioate >10⁻⁴ M a 2-ntm burst of phenol is observed in the pre-steady state phase of the reaction. Enzyme-catalyzed hydrolysis of thioenzyme and solvent-phosphate -OH exchange appear to proceed via a rate-limiting, pH-independent phosphorylation. The catalytic rate of H₂S release from thioenzyme is ~10-fold greater than the rate of hydrolysis of O-phosphorothioate, but ~50-fold less than the rate of hydrolysis of phosphate esters. Substitution of Co(II) for the Zn(II) of alkaline phosphatase increases the rate of O-phosphorothioate hydrolysis by 4-fold, but results in a 5-fold reduction in the rate of phosphate ester hydrolysis and a 10-fold drop in the rate of thioenzyme hydrolysis. While these variations are not fully explicable at present, they reflect differences in the rates of the same mechanistic steps, emphasizing the involvement of the metal ion in all processes required for catalytic hydrolysis.

The O-phosphorothioate analogs of phosphate esters have been useful tools in the study of the mechanisms of several enzymes catalyzing phosphate ester hydrolysis. Although more slowly hydrolyzed than its oxygen analog, uridine 2':3'-O-0,cyclophosphorothioate is a substrate for pancreatic ribonuclease and ³²P incorporation into the two stereoisomers possible with the sulfur analog has assisted differentiation of an in-line from an adjacent mechanism of hydrolysis (1). In marked contrast 3':5'-dithymidine phosphorothioate is a competitive inhibitor of snake venom and spleen phosphodiesterase and is not hydrolyzed (2).

The differences in enzymatic reactivity observed can be attributed to alterations in the physical properties and structural features of the ester on substitution of the P=S function for P=O. For example, the presence of the P=S group reduces the susceptibility of the central phosphorous atom to nucleophilic attack; the rate of hydrolysis of the phosphorothioate triesters is markedly reduced from that of the oxygen analog, $k_a/k_o = 0.1$ to 0.005 (3, 4). This rate effect is attributed to the reduction in polarization of the phosphorus by the less electronegative sulfur atom (5). On the other hand, the rate of hydrolysis of phosphate monooesters by the metaphosphate mechanism displays the opposite effect, increasing in the presence of P=S (6). In addition to the important differences in reactivity, the interatomic distance of the P=S bond is 1.94 Å compared to 1.57 Å for the P=O bond. The van der Waals radius of sulfur is also considerably larger than oxygen (5). These structural differences may have important consequences in the formation of the enzyme-substrate complexes as well as effecting perturbations of certain steps in the enzymatic pathway of hydrolysis.

The O-phosphorothioate monooesters were initially believed to be competitive inhibitors of alkaline phosphatase (7), but have been shown to be substrates, although hydrolyzed much more slowly than their phosphate analogs ($k_a(PO_4) \approx 2 \times 10^4 \ k_a(RO_4)$) (5, 8–10). This result suggested that a detailed
study of the enzymatic reactivity of this class of substrates could provide insight into the mechanism of action of alkaline phosphatase. A preliminary report has been published (9).

MATERIALS AND METHODS

Enzymes and Chemicals—Crystalline alkaline phosphatase was prepared from Escherichia coli (either strain C-90 or CW-3747) as described by Applebury et al. (11). The CW-3747 strain was kindly supplied by Prof. Milton Schlesinger of Washington University, St. Louis, Mo. Co(II) alkaline phosphatase was prepared as previously described (11, 12). Buffer solutions, HCl, and NaOH were prepared metal-free (13). All chemicals were reagent grade. Protein concentrations were determined spectrophotometrically at 278 nm with E₁cm = 0.72 (14). For molar calculations a molecular weight of 80,000 was used (15). Elemental ¹¹⁸S was obtained from New England Nuclear Corp.

Substrate Synthesis—Aryl phosphorodichloridates and phosphorothiodichloridates, the immediate precursors of the desired substrates, were prepared by pyridine-catalyzed reaction of the appropriate alcohol and POCl₃ or PSCl₃ as described by Tolkmith (16). Yields of 70% were obtained in all cases.

p-Phenylazophenylphosphorodichloridate (m.p. 99–100°C)

\[ \text{C}_9\text{H}_8\text{N}_2\text{O}_3\text{PCl}_3 \]

Calculated: C 45.74, H 2.88, N 8.80, P 10.16
Found: C 45.89, H 2.94, N 8.82, P 10.10

p-Phenylazophenylphosphorothiodichloridate (m.p. 74–76°C)

\[ \text{C}_9\text{H}_8\text{N}_2\text{O}_3\text{PSCl}_3 \]

Calculated: C 43.52, H 2.74, N 8.40, S 8.98, P 9.55
Found: C 43.07, H 2.88, N 8.40, S 9.49, P 9.10

p-Nitrophenylphosphorothiodichloridate (m.p. 32.5°C)

\[ \text{C}_9\text{H}_5\text{NO}_3\text{PSCl}_3 \]

Calculated: C 26.64, H 1.36, N 5.39
Found: C 26.48, H 1.48, N 5.14

p-Phenylnazophenyl-0-phosphorothioate: 1 g (3 mmoles) of p-phenylazophenylphosphorodichloridate was dissolved in 10 ml of freshly distilled 1,4-dioxane, 6 ml of 2 N NaOH (32 mmoles) were added dropwise. As reaction proceeded, a fine precipitate of NaCl appeared. The solution was stirred at room temperature for 1 hour, filtered, and diluted to ~80 ml with H₂O. The pH was adjusted to ~8, and 6 ml of 1 M BaCl₂ (6 mmoles) were added dropwise to the rapidly stirred solution. The barium salt of the phosphorothioate immediately precipitated as a flocculent orange-yellow solid. The solution was allowed to stand at 4°C for 1 hour to promote aggregation and the solid was filtered and dried; yield 90%.

p-Phenylnazophenyl-0-phosphorothioate, barium salt, yellow-orange powder

\[ \text{C}_9\text{H}_8\text{N}_2\text{O}_3\text{PSBa} \]

Calculated: C 33.54, H 2.11, N 6.52, P 7.21, S 7.46
Found: C 33.54, H 2.30, N 6.53, P 5.49, S 7.19

p-Phenylnazophenyl phosphate and p-nitrophenyl-0-phosphorothioate were prepared by hydrolysis of the respective dichloridates as described above.

p-Phenylnazophenyl phosphate, barium salt, pale yellow powder

\[ \text{C}_9\text{H}_8\text{N}_2\text{O}_3\text{PBa} \]

Calculated: C 34.85, H 2.19, N 6.77, P 7.48
Found: C 33.24, H 2.14, N 6.54, P 8.08

p-Nitrophenyl-0-phosphorothioate, barium salt, pale yellow powder

\[ \text{C}_9\text{H}_5\text{NO}_3\text{SPBa} \]

Calculated: C 16.79, H 1.72, N 3.94, P 15.55
Found: C 15.79, H 1.72, N 3.94, P 15.55

Trisodium thio phosphate was synthesized by aqueous hydrolysis of thiophosphoryl chloride according to the procedure described by Åkerfeldt (17).

Isolation of the O-phosphorothioate esters by barium precipitation results in isolation of product contaminated by the oxophosphate analog (cf. analytical data for the p-nitrophenyl ester). While the degree of contamination does not interfere with the generation of clean spectral curves during total hydrolysis (see Fig. 1), the presence of even small amounts (1 to 2%) of the oxophosphate analog may seriously interfere with analysis of the pre-steady state hydrolysis observed in stopped flow studies.

Isolation of highly purified O-phosphorothioate esters was accomplished by preparative thin layer chromatography of the hydrolysate (18). The solution is applied to Silica G plates (2000-μm thickness, obtained from Analtech Inc.) which have been activated by heating at 100°C overnight. The plates are then developed with acetonitrile-water (88:12) and dried, and the desired bands are scraped off, extracted with the appropriate buffer solution, and filtered and centrifuged to remove residual silica particles. For the most sensitive experiments (particularly the pre-steady state kinetics) it is desirable to use freshly chromatographed material, since partial conversion of O-phosphorothioate to phosphate occurs in stored solutions with time, due to apparent oxidation of sulfur.

H₂S Assay—Release of H₂S on reaction of O-phosphorothioate and thio phosphate was monitored by acid quenching of reaction mixtures at appropriate time intervals and determination of the amount of H₂S using a modification of the methylene blue method as described by Roy and Trudinger (19).

Synthesis of ³²P-Labeled Compounds—[³²P]Thiophosphoryl chloride was obtained on reaction of elemental ¹¹⁸S with phosphorothioic acid in the presence of aluminum chloride catalyst according to the procedure described by Kottwitz (20). [³²P]Thiophosphate was obtained on hydrolysis of ³²PSCl₃ as described above.

The procedure utilized for formation of p-phenylazophenyl-0-phosphorothioate was essentially that described by Mushak and Coleman (8), wherein the sodium salt of the alcohol is reacted with a slight excess of ³²PSCl₃. This alternate procedure was used to minimize consumption of labeled compound, although it is less efficient than the method described above.

Enzyme Labeling with ³²P-Labeled Compounds—Equal volumes (100 to 200 μl) of substrate and enzyme solution were mixed in a small test tube and allowed to react for the desired time interval (10 to 30 s). At that point the reaction was quenched by rapid addition of a 10% solution of trichloroacetic acid equivalent to 100 to 200 μl. The suspension of denatured enzyme was then filtered through a 0.45-μm, 25-mm Millipore filter; the filter was washed with ~8 ml of 5% trichloroacetic acid (100 to 200 μl) of substrate and enzyme solution were mixed in a small test tube and allowed to react for the desired time interval (10 to 30 s). At that point the reaction was quenched by rapid addition of a 10% solution of trichloroacetic acid equivalent to ~80 ml of H₂O. The pH was adjusted to ~8, and 6 ml of 1 M BaCl₂ (6 mmoles) were added dropwise to the rapidly stirred solution. The barium salt of the phosphorothioate immediately precipitated as a flocculent orange-yellow solid. The solution was allowed to stand at 4°C for 1 hour to promote aggregation and the solid was filtered and dried; yield 90%.

**RESULTS**

Steady State Hydrolysis of O-Phosphorothioates: Michaelis-Menten Parameters—The spectral data in Fig. 1 show that the enzyme catalyzes the complete hydrolysis of the purified p-phenylazophenyl-0-phosphorothioate. Spectrum B, which represents complete hydrolysis in ~24 hours is identical to the spectrum of authentic p-phenylazophenol. Only two species are involved in this hydrolysis as shown by the isobestic points. Similar data were obtained for the total hydrolysis of p-nitrophenyl-0-phosphorothioate.

Lineweaver-Burk plots for the hydrolysis of p-phenylazophenyl-0-phosphorothioate at several pH values are given in
TABLE I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>Tris</th>
<th>$V_{max}$</th>
<th>$K_m$ x 10^4</th>
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<tr>
<td>$ROPO_2^-$</td>
<td>8.0</td>
<td>0.01</td>
<td>3.2</td>
<td>7.8, 2.7*</td>
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<td>1.0</td>
<td>8.2</td>
<td>27.4</td>
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<tr>
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<td>0.01</td>
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<td>6.6, 2.7*</td>
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<tr>
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<td>0.01</td>
<td>2.4</td>
<td>17.3</td>
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<td>$ROPO_2^-$</td>
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<td>0.10</td>
<td>0.5</td>
<td>0.69</td>
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<tr>
<td>$ROPO_2^-$</td>
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<td>0.01</td>
<td>1020</td>
<td>0.76</td>
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<td>1652</td>
<td>0.84</td>
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<td>1.96</td>
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<tr>
<td>$ROPO_2^-$</td>
<td>5.5</td>
<td>0.10</td>
<td>65</td>
<td>&lt;0.10</td>
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</table>

* R, p-phenylazophenyl.

Two $K_m$ values obtained from biphasic Lineweaver-Burk plots.

a NaOAc.

d 0.01 M NaOAc and 0.1 M NaCl also present.

TABLE II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme metal ion</th>
<th>$V_{max}$, PH 5.9</th>
<th>$K_m$, PH 8</th>
<th>$V_{max}$, PH 5.9</th>
<th>$K_m$, PH 8</th>
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</thead>
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<td>3.60</td>
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<td>Co(II)</td>
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<td>3.60</td>
<td>92.0</td>
<td>3.60</td>
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<td>Zn(II)</td>
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<td>7.80</td>
<td>11.8d</td>
<td>7.80</td>
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<td>Co(II)</td>
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<td>1.80</td>
<td>13.3d</td>
<td>1.80</td>
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<tr>
<td>$HOPO_2^-$</td>
<td>Zn(II)</td>
<td>20.1</td>
<td>1.80</td>
<td>20.1</td>
<td>1.80</td>
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<td></td>
<td>Co(II)</td>
<td>2.2</td>
<td>1.80</td>
<td>2.2</td>
<td>1.80</td>
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</table>

* R, p-phenylazophenyl.

* Conditions: 0.01 M Tris.

c Conditions: 0.1 M sodium acetate; for $ROPO_2^-$, 0.1 M NaCl, 0.01 M Tris, 0.01 M sodium acetate.

d $V_{max}$ values in micromoles per hour per mg of enzyme were calculated from the $^18O$ exchange data in Ref. 11.

Fig. 1. Absorption spectra during complete hydrolysis of $ROPSO_2^-$ catalyzed by alkaline phosphatase, $R = p-C_6H_5N\&H_2C_H_4$. Conditions: $3.94 \times 10^{-6}$ M $ROPSO_2^-$, $3.00 \times 10^{-1}$ M enzyme at 1, 0.25; 5, 50; 4, 100; 5, 500 min; 6, 24 hours after adding enzyme.

Fig. 2A. $V_{max}$ and $K_m$ values for hydrolysis of $p$-phenylazophenyl-O-phosphorothioate and $p$-phenylazophenyl phosphate by alkaline phosphatase.

Fig. 2B. Lineweaver-Burk plots of the velocity of alkaline phosphatase-catalyzed hydrolysis of $p$-phenylazophenyl-O-phosphorothioate versus substrate concentration. A, conditions: $\square$, 0.1 M NaOAc, pH 5.5, $1.0 \times 10^{-8}$ M enzyme; $\bigcirc$, 0.01 M Tris, pH 7.0, $1.0 \times 10^{-8}$ M enzyme; $\triangle$, 0.01 M Tris, pH 8.0, $6.00 \times 10^{-1}$ M enzyme; $\blacktriangle$, 0.01 M Tris, pH 10.0, $2.40 \times 10^{-8}$ M enzyme. B, biphasic nature of Lineweaver-Burk plots at high pH. Conditions: 0.01 M Tris, pH 10, $2.40 \times 10^{-8}$ M enzyme. $\blacktriangle$, see scale on upper abscissa; $\bigcirc$, see scale on lower abscissa. $\cdots \cdots$, extrapolation of right arm of $\bigcirc$ plot on the scale of the $\blacktriangle$ plot.

Hydrolysis of $O$-Phosphorothioate and Inorganic Thio phosphate by Zn(II) and Co(II) Alkaline Phosphatase: pH Dependence—$V_{max}$ values for the hydrolysis of $p$-phenylazophenyl phosphate, the corresponding $O$-phosphorothioate monoester, thio phosphate, and $^18O$ exchange from solvent into inorganic phosphate by Zn(II) and Co(II) alkaline phosphatases are given in Table II. For both metallocnzymes, the phosphorothioate ester is hydro-

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lyzed more slowly than the phosphate analog, although the differential reactivity of the Co(II) enzyme for the two substrates is much reduced relative to the native enzyme, k_{cat}/k_{cat} = 0.1 for Co(II); 0.005 for Zn(II). While the activity of the Co(II) enzyme toward the phosphate esters is 10 to 20% that of the Zn(II) enzyme, Co(II) shows 400% the activity of the Zn(II) enzyme toward the phosphorothioate ester.

Alkaline phosphatase catalyzes the exchange of $^1$H from solvent into phosphate (11, 22). The turnover number for this reaction, calculated from the data of Ref. 11, is ~12 µmoles per hour per mg of enzyme and is pH-independent between pH 5.5 and 8. In an analogous reaction inorganic thiophosphate is a substrate for alkaline phosphatase, the products being H$_2$S and inorganic phosphate (Fig. 3). The catalyzed hydrolysis of thiophosphate, assayed by following the release of H$_2$S, is twice as rapid as $^1$H exchange into inorganic phosphate, but like the latter is also pH-independent (Fig. 4 and Table II). In contrast to O-phosphorothioate hydrolysis, catalysis of thiophosphate hydrolysis by the Co(II) enzyme is only ~10% that of the native Zn(II) enzyme (Table II). Experiments in which the extent of alcohol (ROH) and H$_2$S formation were determined demonstrate that the initial products of enzyme turnover of the phosphorothioate ester are alcohol and thiophosphate. H$_2$S is not released until much later when the thiophosphate product begins to be hydrolyzed (Fig. 3).

$V_{\text{max}}$ for the O-phosphorothioate shows a relatively small but significant pH dependence, sigmoid to high pH (Fig. 4A). Hydrolysis rates rise only ~7-fold for the phosphorothioate, while a 33-fold rise is observed for the phosphate following an apparent pK$_a$ value near 7.5. $V_{\text{max}}$ for O-phosphorothioate hydrolysis appears to follow a significantly lower apparent pK$_a$. In contrast, the pH profiles of $V_{\text{max}}$ for hydrolysis of both substrates by the Co(II) enzyme show a markedly attenuated rise from acid to alkaline pH; only a ~2 fold increase is observed (Fig. 4B). The attenuated rise in rate and larger variability in the rate of the Co(II) enzyme make precise determination of the apparent pK$_a$ of $V_{\text{max}}$ difficult. The lines drawn in Fig. 4B can be considered only as the best approximation of the pH function for the activity of the Co(II) enzyme.

**FIG. 3.** Release of H$_2$S on hydrolysis of thiophosphate by Zn(II) and Co(II) alkaline phosphatase and on hydrolysis of ROPS$_0^-$ by Zn(II) alkaline phosphatase as a function of time; R = p-GpN&g1&N&e1& Conditions: 0, 0.01 M Tris, pH 8, 1.08 x 10$^{-4}$ M Na$_3$PO$_3$, 1.90 x 10$^{-7}$ M Zn(II) enzyme, 25°C, 0.1 M NaOAc, pH 5.5, 1.16 x 10$^{-4}$ M Na$_3$PO$_3$, 1.90 x 10$^{-7}$ M Zn(II) enzyme; 0, 0.01 M Tris, pH 8, 1.08 x 10$^{-4}$ M Na$_3$PO$_3$, 1.90 x 10$^{-7}$ M Zn(II) enzyme; 0, 0.01 M Tris, pH 8, 1.07 x 10$^{-4}$ M Na$_3$PO$_3$, 2.66 x 10$^{-7}$ M Zn(II) enzyme; 0, 0.01 M Tris, pH 8, 4.63 x 10$^{-4}$ M ROPS$_0^-$, 9.22 x 10$^{-6}$ M Zn(II) enzyme; O, 0.1 M NaOAc, pH 5.5, 2.34 x 10$^{-3}$ M ROPS$_0^-$, 5.21 x 10$^{-7}$ M Zn(II) enzyme. No significant H$_2$S is released from the ester even though ROH release shows 80% (O) and 30% (A) hydrolysis to have occurred.

**FIG. 4.** pH dependence of $V_{\text{max}}$ for hydrolysis of ROPO$_3^-$, ROPS$_0^-$, HOPO$_3^-$, and HOPSO$_3^-$ by (A) Zn(II) and (B) Co(II) alkaline phosphatase. O, p-C$_6$H$_5$N$_2$C$_6$H$_4$PO$_3$; p-C$_6$H$_5$N$_2$H$_2$OPO$_3$; O, p-C$_6$H$_5$N$_2$H$_2$OPO$_3$; O, HOPO$_3$; O, HOPSO$_3$. Conditions: for ester hydrolyses, 0.1 M NaCl, 0.01 M Tris, 0.01 M NaOAc; for HOP0$_3^-$ cf. Ref 11; for HOPSO$_3^-$ cf. Fig. 3. Values of $V_{\text{max}}$ are given in micromoles per hour per mg of enzyme (right ordinate). The left ordinate displays the fractional increase in $V_{\text{max}}$ for a given substrate (S) at a given pH (X) from the minimum value of $V_{\text{max}}$ at pH 5.5, arbitrarily setting as 1.66 the difference in $V_{\text{max}}$ observed between pH 5.5 and 10 for p-phenylazophenyl phosphate.
employed at pH 5.5 exceeded the $K_m$ by 1- to 5-fold. The large optical density of the substrate and the small $\Delta$O between substrate and protonated phenol precluded experiments at higher substrate concentrations. Thus it has not been possible to determine if the burst amplitude would rise to 2 moles per mole of dimer at very high substrate concentrations as it does at alkaline pH. The rate constants in Table III at pH 5.5 have been calculated assuming one active site per dimer. In marked contrast, on reaction of the phosphate ester with the Zn(II) enzyme, an alcohol burst is observed only at acid pH (Fig. 6A), in agreement with previous data on other phosphate esters (23, 24). A maximum of 1 mole of alcohol is released in the transient phase at pH 5.5.

Stopped flow reaction of both substrates with the Co(II) enzyme (Fig. 6, B and C) shows marked alterations from the results obtained with the Zn(II) enzyme (Figs. 5 and 6A). A transient phase is observed at both acid and alkaline pH for hydrolysis of the phosphate ester (Fig. 6B), while a much reduced burst is seen on reaction of the phosphorothioate ester with the Co(II) enzyme at alkaline pH (Fig. 6C).

$^{35}$S-labeling Experiments—Quenching of the enzymatic reaction 10 s after mixing solutions of enzyme and p-phenylazophenylphosphorothioate results in significant incorporation of $^{35}$S into the enzyme at acid pH (Fig. 7). Incorporation of $^{35}$S decreases at alkaline pH and follows a pH function similar to that for the stability of the phosphoryl enzyme (Fig. 7) (11). Unless rapid (millisecond) quenching of the labeled enzyme is carried out, less than maximal concentrations of phosphoryl enzyme are isolated at the low pH values (11, 22). This may relate to some denaturation of the enzyme or loss of Zn(II) at low pH or even to a decrease in the level of phosphoryl enzyme once the steady state is achieved. The latter is a possibility if phosphorylation, dephosphorylation, and product dissociation rate constants do not differ greatly. The same qualifications apply to the isolation of the thiphosphoryl enzyme. Since the quenching time was 10 s, the observed maximum value of 0.5 mole of thiphosphoryl enzyme isolated per mole of dimer must be considered a minimum value. In contrast to O-phosphorothioate, reaction of the enzyme with phosphate results in only a small degree of labeling at acid and alkaline pH.

Inhibition of O-Phosphorothioate Hydrolysis by Inorganic Phosphate, Thiophosphate, and p-Nitrophosphophosphate—Inorganic phosphate and thiophosphate are potent competitive inhibitors of the O-phosphorothioate hydrolysis catalyzed by the enzyme. Complete kinetic analyses show competitive inhibition with similar $K_i$ values for phosphate and thiphosphate (Table IV). The $K_i$ values are of the same magnitude as that determined for phosphate inhibition of phosphate ester hydrolysis under the same conditions (Table IV). In contrast, O-phosphorothioate is a very poor inhibitor of phosphate ester hydrolysis. Little inhibition is noted at usual phosphate ester concentrations. By lowering the phosphate ester concentration to $10^{-8}$ M, satisfactory double reciprocal plots can be obtained which give a value for $K_i$ of $10^{-4}$ M which agrees remarkably well with the $K_m$ determined for this substrate under the same conditions, $1 \times 10^{-5}$ M (Table II). The data are, however, inadequate, because of the low concentration of phosphate ester required to unambiguously determine if strictly competitive inhibition is indicated. Phosphate ester is a competitive inhibitor of O-phosphorothioate hydrolysis with a $K_i$ of $3 \times 10^{-3}$ M (Table IV). This value is very similar to the $K_m$ reported for the hydrolysis of the phosphate ester under the same conditions (Table II) (23). Since a single Zn-dependent phosphate-binding site has been identified method of plotting the logarithm of the difference between the pre-steady state curve and the steady state velocity extrapolated to zero time (21). The phosphorylation rate constants, $k_2$, and dephosphorylation rate constants, $k_3$, are calculated according to the expressions given in Table III. $B_{obs}$ is the expected burst amplitude calculated from the values of $k_2$ and $k_3$. Disagreement between $B_{obs}$ and $B_{ex}$ is observed when significant enzyme desaturation occurs at the substrate concentration employed (e.g. the Cu(I1) enzyme reacting with phosphate ester), thus reducing the value of $B_{obs}$ (see Table III). Attempts to correct these values by using $K_m$ values determined from the steady state kinetics do not always produce agreement because of the sensitivity of the expression for $B_{ex}$ to errors in the determination of $K_m$ (see Table III). The transient phase of the reaction of O-phosphorothioate ester with native enzyme (Fig. 5) displays an alcohol burst throughout the pH range from 5.5 to 8.5. The dependence of burst amplitude, but not burst rate, on enzyme concentration (Table III and Fig. 5) confirms that contamination by phosphate ester is negligible. The correspondence of the steady state velocities to those obtained from Lineweaver-Burk plots (Table II) supports the reliability of these data.

At pH 7.0 and 8.5 the observed transient corresponds to release of 2 moles of product. Concentrations of substrate estimated.
on the enzyme (11), these inhibition data strongly imply that the same site is involved in the hydrolysis of both substrates.

**DISCUSSION**

The mechanism of hydrolysis of phosphate monoesters catalyzed by E. coli alkaline phosphatase can be most simply described by the series of steps depicted in Scheme 1. The evidence requiring this minimum description including

\[
E + S \xrightleftharpoons{\kappa_1^{-1}} E \cdot S \xrightarrow{\kappa_1} E - P, \quad \frac{k_1}{k_2} = E \cdot P, \quad \frac{k_1}{k_4} = E + P
\]

**SCHEME 1**

the formation and hydrolysis of a phosphoryl enzyme intermediate has been presented previously (25-27).

**Mechanism of O-Phosphorothioate Hydrolysis**—Comparisons of the chemical features of phosphate monoester (I), O-phosphorothioate monoester (II), phosphate (III), and thiophosphate (IV) and the corresponding covalent enzyme intermediates are summarized in Table V. The leaving groups generated on phosphorylation of the enzyme are placed at the bottom and the remaining substituents lie in a plane perpendicular to the bond broken on attack by serine. The placement of the various groups on the four substrates in analogous geometrical arrangements in Table V implies that they occupy similar positions in the enzyme-substrate complex. The validity of this arrangement of groups for the phosphorothioate ester (II) in comparison to phosphate ester (I) and thiophosphate (IV) is supported by the following evidence.

(a) Phosphorothioate ester hydrolysis involves formation of a thiophosphoryl enzyme and does not include additional solvolysis processes (e.g. conversion of the thiophosphoryl enzyme to phosphoryl enzyme) as shown by the labeling of enzyme with "S at low pH and the release of alcohol product but not H2S (Figs. 3 and 7). H2S is ultimately produced by the subsequent hydrolysis of the thiophosphate product (Fig. 3). (b) \( V_{\text{max}} \) for the hydrolysis of (II) is pH-dependent in a manner similar to \( V_{\text{max}} \) for the hydrolysis of (I) (Fig. 4A). (c) At alkaline pH a relatively greater stability for the enzyme-substrate Michaelis complex compared to the phosphoryl enzyme has been demon-
## Table III

Kinetic parameters determined from stopped flow reactions of enzymatic hydrolysis of ROPSOna and ROPOSnb

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>Enzyme Concentration</th>
<th>Substrate Concentration</th>
<th>Substrate Concentration</th>
<th>pH</th>
<th>Burst Stoichiometry</th>
<th>Transient Rate</th>
<th>Phosphorylation Rate</th>
<th>Dephosphorylation Rate</th>
<th>Velocity</th>
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<tr>
<td></td>
<td></td>
<td>[E] (\times 10^6)</td>
<td>[S] (\times 10^5)</td>
<td>(b_{obs}/[E]_o)</td>
<td>(s_{calc}/[E]_o)</td>
<td>(b)</td>
<td>(k_2)</td>
<td>(k_3)</td>
<td>(k_{cat})</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>0.35</td>
<td>ROPSOnb</td>
<td>3.33</td>
<td>5.5</td>
<td>0.04</td>
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<td>0.26</td>
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<tr>
<td>Co(II)</td>
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<td>ROPSo</td>
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<td>0.16</td>
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<td>Zn(II)</td>
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<td>Zn(II)</td>
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<td>8.0</td>
<td>0.34</td>
<td>8.45</td>
<td>8.14</td>
<td>0.31</td>
<td>0.30</td>
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</table>

- for details of reaction conditions cf. Materials and Methods
- R = p-phenylazophenyl
- molar transient amplitude \(B\)/moles enzyme \([E]_o\)
- calculated values based on observed burst amplitude: transient rate and \(k_{cat}\)
- in most cases \(B_{obs}\) is taken from traces depicted in Figs. 5 and 6; for low values of \(B\), reference was made to traces at greater sensitivity
- \(B_{calc}\) determined from values of \(k_3\) and \(k_{cat}\)
- values include correction for saturation effects
- \(b = \frac{k_3 + (k_2/([S]_o))}{1 + (k_2/([S]_o))}\)
- \(R = \frac{c(\frac{k_2}{k_3} + k_3)^2 + k_2 k_3}{(1 + (k_2/([S]_o))\)}\)
- \(c\) is the active site concentration (21)

Thus, the values of all reaction parameters can be determined using these equations provided that any two of the constants can be obtained independently. The data in this table were obtained in this manner.

strated (27), and a similar relative stability appears to hold for the sulfur-substituted analog (Fig. 7).

While the above data suggest that the general features of the minimal mechanism (Scheme 1) must be similar for phosphate and O-phosphorothioate monoesters, there are significant differences in the rates of the various steps. For the phosphate esters the observation of a transient "burst" of product alcohol at acid pH suggests that dephosphorylation of the phosphoryl enzyme is the rate-limiting step. A similar burst is seen at acid pH during the enzyme-catalyzed hydrolysis of substrate (II) (Fig. 5).
rate-limiting at both acid and alkaline pH. The great stability of the intermediate thiophosphoryl enzyme at acid pH (Fig. 7) by the enzyme, a transient phase is observed during the hydrolysis of the 0-phosphorothioate ester at alkaline pH (Fig. 4). This reduction in rate on sulfur substitution is qualitative reported for the hydrolysis of phosphate monoesters at pH 5.5 (23). This range is too narrow to unambiguously state that dephosphorylation is not at least partially rate-limiting. In fact, the above ratio of $k_2$ to $k_4$ of 1:2 predicts that 10% residual burst should remain (Table III). However, with these rate constants, the burst should be over in 10 ms. Rapid bursts of $E$-$P$ up to 0.5 mole within the first 3 ms of reaction have been observed at high substrate and enzyme concentration using a rapid quenching technique (32). Later decline in the concentration of this intermediate may reflect the relatively increased stability of $E$-$P$ at alkaline pH.

For the O-phosphoorthioate, where a step following phosphorylation is clearly rate-limiting at alkaline pH, the difference between $k_2$ and $k_4$ is only 10-fold (Table III). However, in view of the similarity of the rate constants for phosphorylation, $k_2$, dephosphorylation, $k_3$, and probably also for the dissociation of the non-covalent enzyme-product complex, $k_4$, it is not surprising that rather small changes in the enzyme or the substrate structure may alter the relative values of these constants and change the relative contributions of phosphorylation and dephosphorylation to the steady state rate. It is doubtful that a condition for the hydrolisis of any substrate by alkaline phosphatase exists where the phosphorylation step is completely rate-limiting, i.e. 10 $k_2 < k_4$.

The phosphorylation rate constant, $k_2$, is, however, 0.30 s$^{-1}$ (Table III) compared to phosphorylation rate constants of 14 s$^{-1}$ reported for the hydrolysis of phosphate monoesters at pH 5.5 (23). This reduction in rate on sulfur substitution is qualitatively similar to that observed for the uncatalyzed hydrolysis of phosphate and O,O,O-triester triesters, reactions known to occur via a nucleophilic displacement mechanism (3, 4, 28, 29). The 50-fold drop in $k_2$ for the O-phosphoorthioate in addition to the 200-fold drop in $k_{cat}$ suggests that both phosphorylation (the transient) and dephosphorylation (the rate-limiting step) at pH 5.5 are nucleophilic displacement processes involving serine and water as the respective nucleophiles.

In contrast to the kinetics of hydrolysis of phosphate esters by the enzyme, a transient phase is observed during the hydrolysis of the O-phosphoorthioate ester at alkaline pH (Fig. 4). Thus, a step following thiophosphorylation of the enzyme is rate-limiting at both acid and alkaline pH. The great stability of the intermediate thiophosphoryl enzyme at acid pH (Fig. 7) suggests that at acid pH, dethiophosphorylation is rate-limiting (i.e. $k_4 > k_3 < k_2$), while at alkaline pH breakdown of the enzyme-thiophosphate Michaelis complex must be rate-limiting (i.e. $k_3 > k_2 > k_4$). Inclusion of supplementary, rate-limiting steps involving conformational changes of the enzyme or enzyme complexes are thus not required to explain the enzymatic hydrolysis of the O-phosphorothioates by Zn(II) alkaline phosphatase.

Rate-limiting Phosphorylation and Dephosphorylation—The loss of the burst during the hydrolysis of phosphate esters by the enzyme at alkaline pH (Fig. 6A) has been interpreted to mean that the rate-limiting step for the enzymatic phosphate ester hydrolysis shifts from dephosphorylation (Step 3, Scheme 1) at acid pH to phosphorylation (Step 2, Scheme 1) at alkaline pH (30). Disappearance of a pre-steady state burst does not mean, however, that phosphorylation has become completely rate-limiting.

<table>
<thead>
<tr>
<th>Table IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ values for the inhibition of hydrolysis of p-phenylazophenyl phosphate and O-phosphoorthioate monoesters by inorganic phosphate and thiophosphate and for the inhibition of the phosphoroorthioate by the phosphate and vice versa</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate$^a$</th>
<th>Inhibitor</th>
<th>$K_i \times 10^4$</th>
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<td>ROPO$_3$-</td>
<td>Na$_2$PO$_4$</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Na$_2$P$_2$O$_7$</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>ROPSO$_3$-</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>ROPS$_3$-</td>
<td>Na$_2$PO$_4$</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Na$_2$P$_2$O$_7$</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>R'PO$_3$-</td>
<td>~30.0</td>
</tr>
</tbody>
</table>

$^a$ Conditions: 1 M Tris, pH 8.0.
$^b$ R, p-phenylazophenyl.
$^c$ R', p-nitrophenyl, assayed at 400 nm where absorption spectra of R' and R do not overlap.

Substitution of various first transition and IIB metal ions at the active site has already been shown to profoundly alter the rates of both phosphorylation and dephosphorylation, as well as the relative rates of these steps. For example Cd(II) slows both steps, but dephosphorylation is so radically slowed that 1 mole of phosphoryl enzyme is isolated at neutral pH, and negligible turnover is observed (11). Substitution of sulfur for oxygen in the O-phosphoorthioate substrates also clearly slows both steps, but in the case of the Zn(II) enzyme at alkaline pH, the dissociation of the thiophosphate product appears to be slowed enough to become rate-limiting and a burst remains at alkaline pH (Fig. 5). Substitution of Co(II), however, abolishes most of this burst and increases $k_{cat}$ from 0.08 s$^{-1}$ to 0.35 s$^{-1}$ (Fig. 6C). The rate constants for phosphorylation and dephosphorylation or dissociation of product have moved much closer together (calculated to be 0.63 s$^{-1}$ for $k_2$ and 0.79 s$^{-1}$ for $k_4$, Table III). In contrast, a burst remains when the Co(II) enzyme reacts with phosphate esters (Fig. 6B) (33). Of possible significance here is the differential in binding affinity of

![Graphical representation of pH dependence of enzyme activity](http://www.jbc.org/content/172/4/7199/F7)
At alkaline pH where the phosphorylation rate may make the major contribution to the steady state rate of hydrolysis of the phosphate esters, it has been proposed that a rate-limiting change in conformation precedes chemical phosphorylation in order to account for the lack of influence of the pKₐ of the leaving group on the hydrolytic rate. Mechanisms proposed on this basis are compatible with most of the kinetic data (30, 33, 35-37). On the other hand, the great fall in phosphorylation rate from phosphate ester to O-phosphorothioate is adequately explained on chemical grounds due to the inhibitory effect of sulfur on a nucleophilic mechanism of phosphorylation.

It is not clear that the large R group dependence expected from the model nucleophilic hydrolysis of phosphate triesters applies to the enzyme case. The model system measures the facility with which the leaving group, RO-, can be ejected from the five-coordinate intermediate (or transition state) which is roughly proportional to the pKₐ of ROH (3, 4). In the enzyme case, however, phosphorylation is from an E·S complex, and there is likely a great enhancement of the susceptibility of the phosphorus to nucleophilic attack induced by electronic structure at the active site. Alteration of the relative contributions of bond-making and bond-breaking to the rate may reduce the expected effect of the pKₐ of the leaving group. It is observed that phosphonates with substituents withdrawing electrons from the phosphorus bind more tightly to the enzyme (33, 35).

**Contrasting Mechanisms of O-Phosphorothioate and Thio-Phosphate Hydrolysis**—Sulfur substitution has little effect on the slow rate of enzyme-catalyzed hydroxide (H₂O) reaction with inorganic phosphate or thiophosphate (38, 39) (Fig. 3 and Table 11). This may not be too surprising, since sulfur has become the leaving group rather than being incorporated into the intermediate (Figs. 3 and 7). In addition, enzyme-catalyzed ³²O exchange into phosphate and the analogous hydrolysis of thiophosphate show no pH dependence. Since (I), (III), and (IV)
give rise to the same phosphoryl enzyme intermediate (Table V), it is not possible that the rate-limiting step for $^{18}O$ exchange into inorganic phosphate and thiophosphate hydrolysis is dephosphorylation of the enzyme. A slow, pH-independent, rate-limiting phosphorylation of the enzyme could account for the sluggish reactivity of these species.

The $K_i$ for competitive inhibition of the enzyme reaction by thiophosphate in 1 M Tris is almost the same as that for phosphate inhibition (Table IV), implying that the sulfur occupies a position which does not significantly interfere with binding. On the other hand, $K_i$ for O-phosphorothioate as an inhibitor of oxyphosphate hydrolysis in 1 M Tris is 20-fold larger (Table IV), suggesting that there is significant interference with binding by sulfur in this position relative to the leaving group, not incompatible with the stereocchemistry pictured in Table V.

Supporting the above picture is the fact that hydrolysis of thiophosphate and $^{18}O$ exchange into phosphate (postulated to be controlled by rate-limiting phosphorylation) is 4- to 6-fold faster than hydrolysis of O-phosphorothioate ester where a step much later than phosphorylation is clearly rate-limiting (Table II and Fig. 4). This again reflects the difference in rate of hydrolysis of the oxygen- and sulfur-containing intermediates. That a different rate-limiting step is involved in thiophosphate hydrolysis than in O-phosphorothioate hydrolysis is supported by the opposite effects of substituting Co(II) at the active site, decreasing the rate of the former but significantly increasing the rate of the latter.

Negative Cooperativity—One of the most striking features characteristic of phosphate ester hydrolysis by alkaline phosphatase is the observation that only one active site per dimer appears to function at any one instant under most conditions (11, 40–42). Recently, the validity of these observations has been called into question by Bloch and Schlesinger (43), who have suggested that these results may be ambiguous owing to the presence of endogenous phosphate on the enzyme as isolated. However, under a number of experimental conditions where negative cooperativity has been observed these qualifications do not apply. (a) Phosphate-binding studies have been performed which show the metal ion-dependent binding of only one phosphate per dimer with a low dissociation constant (11). These studies utilized metalloenzymes prepared from metal-free apoenzyme by readorption of metal ions, a process known to remove phosphate (11). The techniques employed are similar to those cited by the above authors in their “purging” process and have been described previously (11). One of the most striking examples is induction of phosphate binding to a metal-free, phosphate-free apoenzyme by the addition of Zn(II). The tight metal ion-dependent binding of only one phosphate ion per dimer is induced and two Zn(II) ions per dimer are required (11). (b) Addition of 1 mole of phosphate or arsenate ion to cobalt enzyme prepared as described above induces dramatic spectral changes which are not enhanced by further phosphate addition above 1 mole per mole of enzyme dimer (44). (c) The transient phase of the hydrolysis of phosphorothioate esters reported here shows a burst amplitude of 2 moles of alcohol released per mole of native Zn(II) enzyme at alkaline pH (Fig. 5 and Table III) demonstrating that native enzyme can, under certain conditions, react at both active sites.

The interpretation of the negative cooperativity or “half-the-sites” reactivity (45) must include a consideration of the evidence which indicates that the enzyme has 2-fold symmetry in the unliganded state (11, 46, 47). Conformational changes induced by substrate binding and propagated between monomer subunits must certainly be related to the binding energy and the topography of the resultant enzyme-substrate complex. That O-phosphorothioates might alter negative cooperativity is not surprising, since the binding energy is different (Tables I and II), and the physical dimensions of the phosphate group are altered.

The negative cooperativity accompanying the binding of O-phosphorothioate to the enzyme is reduced but apparently not completely abolished (Fig. 2). The region of the shift in slopes observed in the Lineweaver-Burk plots also corresponds to the concentration range where a shift from a 1-mole burst to a 2-mole burst and the transient phase has been observed (9). Thus substrate binding takes place over a somewhat wider range of concentration than the 2 log units of concentration expected for two independent sites. For the phosphate esters, binding of 1 and 2 substrate molecules must be separated by 4 or 5 log units of concentration, since there is no evidence for binding of a second phosphate ester until substrate concentrations of $10^{-3}$ to $10^{-2}$ M are attained (41), while the binding constant for the first phosphate ester is near $10^{-4}$ M. Thus substrate- or product-induced conformational changes appear to control the number of active sites reacting with substrate. That these conformational equilibria enter as rate-limiting steps in the mechanism of phosphate ester hydrolysis catalyzed by alkaline phosphatase is less certain.

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Mechanisms of Hydrolysis of O-Phosphorothioates and Inorganic Thiophosphate by *Escherichia coli* Alkaline Phosphatase
Jan F. Chlebowski and Joseph E. Coleman


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