Hydrolysis of a Stable Oxygen Ester of Phosphorothioic Acid by Alkaline Phosphatase*

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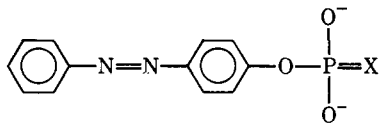
ABSTRACT: The hydrolysis of an O ester of phosphorothioic acid, *p*-phenylazophenyl phosphorothioate (I), by alkaline phosphatase of *Escherichia coli* is reported. Kinetics of hydrolysis of I and its oxygen analog, *p*-phenylazophenyl phosphate (II), show that both substrates have similar K_m values, $\sim 10^{-4}$ M, but I is hydrolyzed ~200 times slower than II. In 1 M Tris pH 8, V_{max} is 7 μmoles/hr per mg of enzyme for I and 3300 μmoles/hr per mg for II. Ester II is hydrolyzed 100-fold more rapidly by the alkaline form of the enzyme than by the acid form, while enzymic hydrolysis of I is almost pH independent from pH 5 to 9. Compound I is a competitive inhibitor of the hydrolysis of II, and hydrolysis of both esters is enhanced by Tris which serves as an acceptor for thiophosphate as well as phosphate in the transphosphorylation reaction catalyzed by alkaline phosphatase.

Hydrolysis of the O esters of phosphorothioic acid by *Escherichia coli* alkaline phosphatase has been the subject of conflicting reports in the literature. Neumann (1968) has reported that *p*-nitrophenyl phosphorothioate is not hydrolyzed, while Breslow and Katz (1968) have reported the hydrolysis of this compound by the *E. coli* enzyme at a rate 100-fold slower than the oxygen analog, *p*-nitrophenyl phosphate. Neumann has also reported the isolation of a 1:1 complex between the enzyme and a ^{35}S derivative of *p*-nitrophenyl phosphorothioate by a gel filtration technique (Neumann, 1968). Eckstein (1966) has reported that the O-substituted derivatives of nucleotides of phosphorothioic acid are not hydrolyzed by alkaline phosphatase. In an attempt to synthesize a chromophoric substrate analog suitable for titration of the active sites of alkaline phosphatase we synthesized an arylazoaryl phosphorothioate (I). This compound cannot



I, X = S
II, X = O

be used as an active-site label, since it is hydrolyzed by the crystalline *E. coli* alkaline phosphatase. We report here a study of the hydrolysis of I by the *E. coli* enzyme as well as a comparative study of the hydrolysis of its oxygen analog (II) by the same enzyme.

Materials and Methods

Reagents. All chemicals were reagent grade and were used without further purification. *p*-Phenylazophenol was obtained from Eastman (Rochester, N. Y.).

Synthesis of p-Phenylazophenyl Phosphate Esters I and II. The preparation of ester I involved modifications of the method of Neumann (1968). A suspension of freshly prepared sodium salt of *p*-phenylazophenol in dry reagent grade tetrahydrofuran was slowly added at room temperature to a solution of freshly redistilled thiophosphoryl chloride in tetrahydrofuran in 50% excess. After stirring at room temperature for *ca.* 8 hr, sodium chloride was filtered off, the filtrate evaporated to dryness, and the *p*-phenylazophenyl phosphorothiodichloridate was recrystallized from ether–hexane as deep-red plates, mp 70–74°. Mass spectral analysis gave the correct molecular ion in the appropriate chlorine isotope ratios, as well as characteristic fragments. A solution of this phosphorothiodichloridate in ethanol was added to an ethanolic solution of 4 equiv of sodium hydroxide and heated for 15–20 min, during which time sodium chloride precipitated. After filtration, the solution was poured in water, the pH adjusted to 8.0, and a solution of barium chloride slowly added with rapid stirring. The bright yellow barium salt was isolated and exhaustively triturated with tetrahydrofuran, ethanol, and water at the boiling point. A sample taken for elemental analysis was dried under vacuum at elevated temperature. Combustion data conforms to the barium salt of I as the dihydrate. *Anal.* Calcd for $C_{12}H_8BaN_2O_3PS \cdot 2H_2O$: C, 30.97; H, 2.79; N, 6.02. Found: C, 30.81; H, 2.30; N, 6.30. The procedure of King and Nicholson (1939) was employed in the preparation of II. To a stoichiometric amount of phosphorus oxychloride cooled in an ice bath under a drying tube was slowly added a solution of *p*-phenylazophenol in rigorously dried pyridine with constant shaking. The resulting mixture was heated to reflux for 10 min, pyridine added, the solution cooled, and 5.0 ml of water cautiously added. The resulting dark red solution was poured into water, the pH adjusted to 8.0, and barium chloride slowly added. The resulting bright yellow precipitate was successively triturated with tetrahydrofuran, ethanol, and water at the boiling point. A sample for analysis was dried under vacuum. Combustion data corresponds to the anhydrous barium salt of II. *Anal.* Calcd for $C_{12}H_9BaN_2O_4P$: C, 35.11; H, 2.18; N, 6.78. Found: C, 34.90; H, 2.73; N, 6.75.

Enzymatic Assay. Crystalline alkaline phosphatase from *E. coli* was prepared as previously described (Applebury and Coleman, 1969; Applebury *et al.*, 1970). Stock solutions of substrate were made up either in 0.01 M Tris–0.01 M $MgCl_2$ or in 1.0 M Tris–0.01 M $MgCl_2$. Because of solubility problems the upper limit of concentration of both substrates I and II

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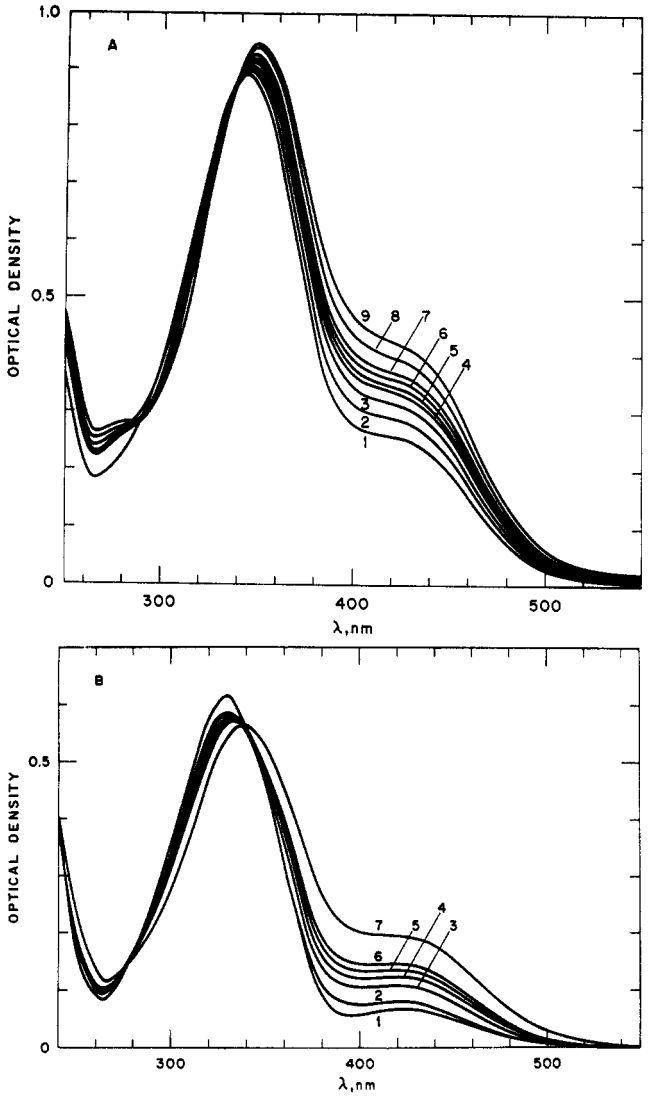

FIGURE 1: Changes in the absorption spectrum. (A) Of the phosphorothioate (I) during hydrolysis by alkaline phosphatase. Conditions: 1×10^{-4} M substrate, 1.68×10^{-6} M enzyme, and 0.01 M Tris–0.01 M $MgCl_2$, pH 8.0, 25°. (1) Initial spectrum without enzyme, (2) 15 min, (3) 25 min, (4) 40 min, (5) 55 min, (6) 70 min, (7) 85 min, (8) 155 min, and (9) 4 hr after adding enzyme. (B) Of the phosphate (II) during hydrolysis by alkaline phosphatase. Conditions: 4×10^{-5} M substrate, 8.4×10^{-8} M enzyme, and 0.01 M Tris–0.01 M $MgCl_2$, pH 8.0, 25°. (1) Initial spectrum without enzyme, (2) 1 min, (3) 4 min, (4) 15 min, (5) 60 min, and (6) 90 min after adding enzyme.

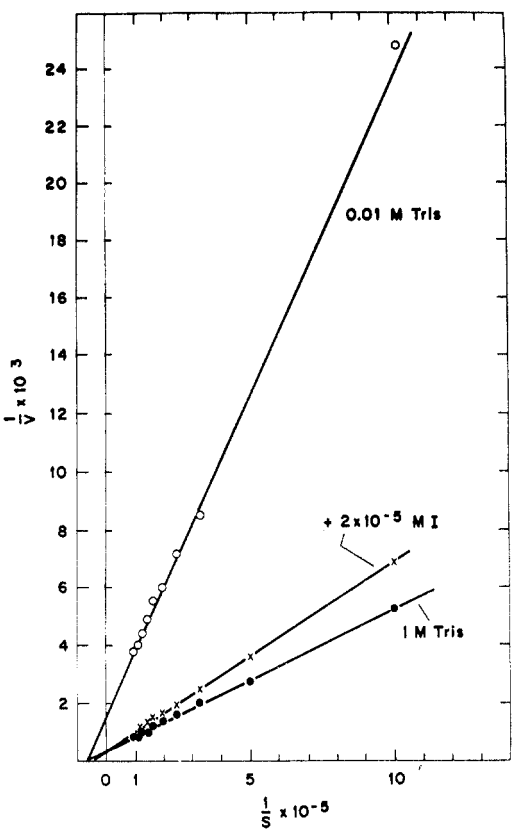

FIGURE 2: Double-reciprocal plot of the velocity of the hydrolysis of II *vs.* substrate concentration. Conditions: 25°, pH 8.0; (○) 0.01 M Tris–0.01 M $MgCl_2$; (●) 1 M Tris–0.01 M $MgCl_2$; (×) 1 M Tris–0.01 M $MgCl_2$, 2×10^{-5} M phosphorothioate.

conveniently obtained in the usual assay buffers is 1×10^{-4} M. Heating to 80° dissolves the crystalline material. Assays were carried out at 25° by adding either 5 or 20 μl of stock enzyme solution to 1 ml of substrate. The former applies to assays in 1 M Tris, the latter to assays in 0.01 M Tris. A stock enzyme concentration of 8.4×10^{-5} M was used for the hydrolysis of I while a stock solution of 8.4×10^{-6} M was used for the hydrolysis of II. Rates were determined from the linear portions of the progression curves and concentrations of substrate and enzyme were adjusted such that hydrolysis over the portion of the curve used did not exceed 10%. Phosphate inhibition causes minimal deviation under these conditions, as determined by adding 10^{-6}–10^{-5} M phosphate to the reaction mixture.

Inorganic phosphate and thiophosphate were determined by the method of Ames (1966) thiophosphate being hydrolyzed to phosphate by the acid molybdate reagent.

Results

The visible and near-ultraviolet absorption spectrum of the phosphorothioate (I) and the changes accompanying hydrolysis of this ester by alkaline phosphatase in 0.01 M Tris–0.01 M $MgCl_2$ are shown in Figure 1A. The product, *p*-phenylazophenol, is available commercially and its spectrum is identical with that of the final product of enzymatic hydrolysis. In 0.01 M Tris, the absorption maximum of the phosphorothioate is at 342 nm, $\epsilon = 1.74 \times 10^4$ M^{-1} cm^{-1}, while the phenol has maximum absorption at 350 nm, $\epsilon = 1.90 \times 10^4$ M^{-1} cm^{-1}. The greatest changes in absorption on hydrolysis of the ester occur between 420 and 460 nm. It was elected to follow hydrolysis at 440 nm where the $\Delta\epsilon$ between the ester and phenol is 5.4×10^3 M^{-1} cm^{-1}. Since the phosphorothioate is hydrolyzed over 100 times slower than its oxygen analogue (see below) the amount of enzyme added to the reaction pictured in Figure 1A had significant absorption at 280 nm and accounts for the initial large deviation of the spectrum in this region. Although one isosbestic point is somewhat disturbed by the enzyme absorption, two are present, one at 290 nm and one at 338 nm (Figure 1A). The rate of hydrolysis of I by alkaline phosphatase is directly proportional to the enzyme concentration. Tris (1 M) has a small effect on the extinction coefficient of the ester (I), but a large effect on the extinction coefficient of the product phenol, especially the relative intensity of absorption at 350 and 440 nm. In 1 M Tris $\Delta\epsilon$ (440) between the ester and the phenol is 7.9×10^3 M^{-1} cm^{-1}.

Spectral changes during the hydrolysis of the analogous phosphate ester (II) in 0.1 M Tris–0.01 M $MgCl_2$ are shown in Figure 1B. The phosphate ester has maximum absorption at

TABLE I: K_m and V_{max} for the Hydrolysis of Phosphoric and *O*-Phosphothioic Acid Esters of *p*-Phenylazophenol by Alkaline Phosphatase at pH 8.0, 25°.

Substrate and Conditions	K_m (M)	V_{max} μmoles/hr per mg of Enzyme	ROH[a] / P_i
I. *p*-Phenylazophenyl phosphorothioate			
0.01 M Tris	Indeterminant	>0.4	2.4
0.10 M Tris			12.4
1 M Tris	0.5×10^{-4}	7.3	58.5
II. *p*-Phenylazophenyl phosphate			
0.01 M Tris	1.6×10^{-4}	670	1.1
1 M Tris	1.6×10^{-4}	3300	3.4

[a] ROH was determined from the optical density of the reaction mixture at 440 nm. Phosphate or phosphate derived from thiophosphate, was determined by the method of Ames (1966). Ratios were determined on reaction mixtures in which 40–60% hydrolysis or transfer had occurred.

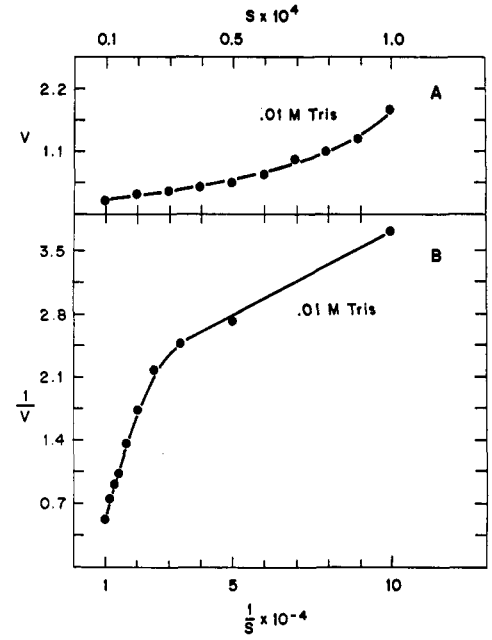

FIGURE 3: (A) Velocity of hydrolysis of I *vs.* substrate concentration (B) Lineweaver–Burk plot of the data in part A. Conditions: 0.01 M Tris–0.01 M $MgCl_2$, pH 8.0, 25°.

330 nm, ϵ 1.60×10^4 M^{-1} cm^{-1}. As in the phosphorothioate case it was elected to follow hydrolysis at 440 nm where $\Delta\epsilon$ between the ester and the phenol is 4.75×10^3 M^{-1} cm^{-1}. Isosbestic points are present at 278 and 338 nm. Due to changes in the extinction coefficients of both the ester (II) and the phenol product induced by 1 M Tris, $\Delta\epsilon$ (440) applying to the hydrolysis of II in 1 M Tris is 4.54×10^3 M^{-1} cm^{-1}.

The kinetics of hydrolysis of II by alkaline phosphatase are plotted in double-reciprocal form in Figure 2. The compound behaves as a typical phosphate ester substrate for alkaline phosphatase, but with a slightly larger K_m, 1.6×10^{-4} M (Table I). The usual marked stimulation of the reaction by Tris is observed, V_{max} rising ~5-fold in 1 M Tris without any change in K_m (Figure 2). The maximum velocity in 1 M Tris is 3300 μmoles/hr per mg of enzyme and is almost identical to the rate of hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase under similar conditions (Malamy and Horecker, 1964; Applebury and Coleman, 1969). The phosphorothioate (I) is a competitive inhibitor of the enzymatic hydrolysis of II (Figure 2). The K_I for I calculated from these data at pH 8 is 3.6×10^{-5} M, while the K_I calculated from similar data at pH 9.5 is 4×10^{-5} M.

The hydrolysis of I by alkaline phosphatase in 0.01 M Tris does not follow Michaelis–Menten kinetics, but shows apparent substrate activation as substrate concentrations approach 10^{-4} M (Figure 3A). A marked break occurs in the Lineweaver–Burk plot at $\sim 4 \times 10^{-5}$ M substrate (Figure 3B). Hydrolysis of the phosphorothioate is also subject to the marked stimulation by Tris (Figure 4). The velocity is increased ~5-fold at 10^{-4} M substrate and the substrate activation is relatively much less since the velocity shows close to a hyperbolic dependence on substrate concentration (Figure 4). The maximum velocity in 1 M Tris, however, is only 0.20% of the rate of the hydrolysis of the phosphate ester by alkaline phosphatase in 1 M Tris (Table I). The kinetic parameters calculated for the hydrolysis of I and II by alkaline phosphatase are given in Table I.

In order to separate the stimulation of hydrolysis caused by the ionic strength effect of Tris from that due to the transphosphorylation of Tris, the alcohol to phosphate ratios were determined for both substrates at various Tris concentrations (Table I). Tris (1 M) causes the usual increase in this ratio in the case of the oxyphosphate, but a much larger increase in the ratio if the phosphorothioate is used.

The pH dependence for the hydrolysis of I and II is given in Table II for pH values between 5.0 and 9.5 in 0.01 M Tris–0.01 M $MgCl_2$, 25°. The P=O compound shows a pH–rate profile typical for the hydrolysis of phosphate monesters by alkaline phosphatase. The rate rises approximately 100-fold between pH 7 and 9 following a sigmoid curve with an apparent pK_a of ~7.5. In marked contrast hydrolysis of the phosphorothioate is almost pH independent over the same pH

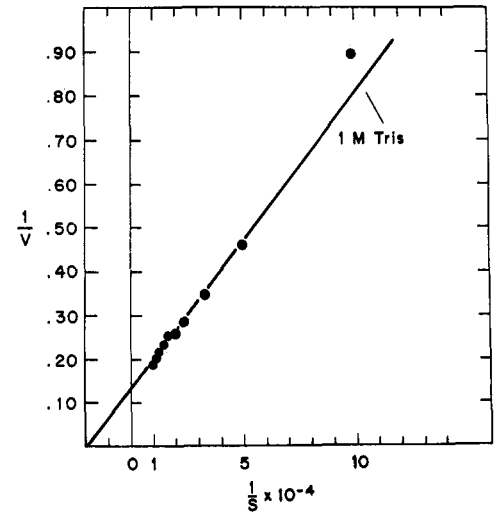

FIGURE 4: Double-reciprocal plot of the velocity of hydrolysis of I *vs.* substrate concentration in 1 M Tris–0.01 M $MgCl_2$, pH 8.0, 25°.

TABLE II: Hydrolysis of *p*-Phenylazophenyl Phosphorothioate and *p*-Phenylazophenyl Phosphate by Alkaline Phosphatase as a Function of pH.[a]

pH	Velocity (μmoles of Substrate Hydrolyzed/hr per mg of Enzyme)[b]	
	P=S	P=O
5.0	0.58 ± 0.05	3.2 ± 0.07
6.0	0.55 ± 0.05	3.1 ± 0.07
7.0	0.50 ± 0.04	3.1 ± 0.24
7.5	0.61 ± 0.07	150 ± 18.0
8.0	0.67 ± 0.07	261 ± 8.5
8.5	0.69 ± 0.13	320 ± 10.0
9.0	0.34 ± 0.06	310 ± 11.0
9.5	0.06 ± 0.01	280 ± 9.0

[a] Conditions: 0.01 M Tris–0.01 M $MgCl_2$, 1×10^{-4} M substrates, 25°. [b] For purposes of determining the pH–rate profile, hydrolysis of the phosphorothioate was followed at 370 nm which is an isosbestic point for the phenol and phenolate product, while hydrolysis of the oxy compound was followed at 440 nm determining $\Delta\epsilon$ as a function of pH.

range. The fall in activity at pH values above 9 has been observed with other substrates of alkaline phosphatase (Neumann *et al.*, 1967).

Discussion

The present data clearly establish that the oxygen esters of phosphorothioic acid are hydrolyzed by alkaline phosphatase. The great advantage of the *p*-phenyl azophenylphosphorothioate is that it can be isolated as a stable crystalline compound. In our experience the isolation of the *p*-nitrophenyl phosphorothioate is difficult and the compound decomposes on attempted isolation as also reported by Breslow and Katz (1968). Contamination by a small amount of the oxyphosphate also complicates the enzymatic data (Breslow and Katz, 1968). The fact that the *p*-phenyl azophenylphosphorothioate is hydrolyzed approximately two orders of magnitude slower than the analogous phosphate ester is further evidence for the conclusion of Breslow and Katz (1968) that the hydrolytic mechanism for alkaline phosphatase involves an addition–elimination mechanism rather than a metaphosphate intermediate. The latter mechanism, believed to operate in the nonenzymatic hydrolysis of I and II, results in much more rapid hydrolysis of the phosphorothioate (Breslow and Katz, 1968).

The possible presence of a phosphoenzyme intermediate (Engström, 1962; Schwartz and Lipmann, 1961; Barrett *et al.*, 1969; Reid *et al.*, 1969; Applebury *et al.*, 1970) or a rate-limiting conformational change in the enzymic mechanism (Halford *et al.*, 1969; Reid and Wilson, 1971), however, complicates any straightforward explanation of why the hydrolysis of the ester is slowed so drastically by the presence of P=S rather than P=O. This complex enzymatic mechanism makes uncertain direct assumptions about the addition–elimination mechanism *vs.* the metaphosphate mechanism based on the reactions of model systems. Ester I is a competitive inhibitor of the enzymatic hydrolysis of II (Figure 2) which suggests that the same active site is involved. The presence of burst kinetics for phosphate ester hydrolysis at low pH by alkaline phosphatase and the catalysis of transphosphorylation from the substrate to a suitable phosphate acceptor at alkaline pH with a constant alcohol to free phosphate ratio regardless of the nature of the R group on the phosphate ester (Dayan *et al.*, 1964; Barrett *et al.*, 1969) has been taken to mean that a phosphoenzyme is a kinetically significant intermediate in the enzyme mechanism. One of the best phosphate acceptors is Tris and the large acceleration of the hydrolysis of the phosphorothioate by Tris (Figure 4) indicates that a transfer of the thiophosphate group can also be catalyzed by alkaline phosphatase. Since stimulation of phosphate ester hydrolysis by Tris includes an ionic strength effect (Trentham and Gutfreund, 1968), the alcohol phosphate ratio must be determined to demonstrate that Tris induces a rise in this product ratio reflecting the phosphorylation of Tris. This rise is seen in the case of the phosphorothioate as well as that of the oxyphosphate (Table I). A striking finding in the transphosphorylation reaction is the large enhancement in the relative efficiency of Tris as an acceptor compared to water when the group being transferred by the enzyme is thiophosphate rather than phosphate (Table I). No chemical reason for this is immediately apparent and it must reflect some feature of the enzymatic mechanism, perhaps the participation of the metal ion. It has previously been reported that the nature of the metal can affect the transphosphorylation reaction (Simpson and Vallee, 1969).

It has been shown that alkaline phosphatase catalyzes the hydrolysis of thiophosphate with the release of H_2S which is analogous to the exchange of ^{18}O from water into inorganic phosphate also catalyzed by the enzyme (Neumann *et al.*, 1967). If the active-site serine residue is involved in the hydrolysis of the thiophosphate, the present data raise some interesting questions in regard to the mechanism of hydrolysis of inorganic thiophosphate *vs.* the hydrolysis of the phosphorothioate. If the serine attacks the phosphorus in both cases, the lack of a good leaving group in the first case may lead to the preferential expulsion of H_2S from the thiophosphate and the formation of a normal phosphoserine intermediate. On the other hand, the presence of a good leaving group in the ester, *e.g.*, *p*-nitrophenyl or *p*-phenylazophenyl, is likely to lead to the formation of a thiophosphorylserine with the expulsion of ROH. The slow hydrolysis of the thiophosphoryl intermediate may account for the slow hydrolysis of the ester. If a slow dethiophosphorylation exists it may be possible to isolate the thiophosphoryl-enzyme throughout the pH range. This would account for the isolation of an ^{35}S- or ^{32}P-labeled enzyme at pH 8.7 reported by Neumann (1968) after incubation of alkaline phosphatase with ^{35}S- or ^{32}P-labeled *p*-nitrophenyl phosphorothioate. The stability of the thiophosphoryl-enzyme, the partition of products into H_2S or thiophosphate and the rapid-flow kinetics for substrate I are currently under study.

The rate-limiting step at alkaline pH for the hydrolysis of the phosphate ester has been the subject of considerable discussion. Some evidence has been presented suggesting the dephosphorylation remains the rate-limiting step at alkaline pH as well as at acid pH where burst kinetics are observed (Barrett *et al.*, 1969; Reid *et al.*, 1969). More recent evidence, however, suggests that a rate-limiting conformational change may be operative at alkaline pH (Halford *et al.*, 1969; Reid and Wilson, 1971). For the oxyphosphates, both phosphorylation and dephosphorylation of the enzyme appear to be pH dependent (Fernley and Walker, 1969).

In contrast to usual phosphate monesters, enzymatic hydrol-

ysis of the phosphorothioate is almost pH independent in the region where ester hydrolysis by alkaline phosphatase usually shows a sigmoid rate profile with a pK_a near pH 7.5. The rates of hydrolysis of the two substrates are the same order of magnitude at pH 5 (Table II). It is the great enhancement of the hydrolysis rate of II by the alkaline form of the enzyme that is not seen in the case of I. This does not appear to be a binding constant effect since the K_i for the phosphorothioate is the same at pH 9.5 as it is at pH 8.0. This finding may suggest that the pH-dependent change in the enzyme that accelerates the step in the reaction responsible for the pH–rate profile does not appreciably enhance the rate of this step if the ester contains the P=S bond. Alternatively, the presence of the P=S bond might have changed the mechanism so that the rate-limiting step is not the same at alkaline pH. If the PO_3^{2-} group of the normal substrate does interact directly with the Zn(II) ion at the active site, the thiophosphate interaction could be considerably altered from that of the oxyphosphate and might interfere with the rapid dissociation of the liganded product.

Another perhaps more likely possibility is that the hydrolysis of a thiophosphoryl-enzyme remains a relatively slow step throughout the pH range. Such an intermediate is likely to be serine–phosphorothioate, another O ester of phosphorothioic acid. The hydrolysis of this intermediate is likely to be much slower than its oxygen analog if an addition–elimination reaction is involved at this step.

The apparent "substrate activation" by the phosphorothioate at low Tris concentrations is not readily explained. Substrate activation at substrate concentrations $>10^{-3}$ M has been reported for the oxyphosphates (Heppel *et al.*, 1962; Simpson and Vallee, 1970). At low substrate concentrations only one active site per enzyme dimer appears to be operating (Schwartz, 1963; Pigretti and Milstein, 1965; Ko and Kezdy, 1967; Fernley and Walker, 1969; Reid *et al.*, 1969) and the substrate activation has been interpreted to reflect negative homotropic interactions between the identical subunits such that a second substrate binding site has much less affinity for substrate than the first. The activation would hence reflect the binding of the second substrate at relatively high concentrations. It is possible that binding of a second substrate molecule is shifted to lower substrate concentrations, $\sim 5 \times 10^{-5}$ M, in the case of the phosphorothioate, but more detailed kinetic studies will be required to prove this. The picture is complex since the substrate activation does not appear to be as great in 1 M Tris. It may be obscured, however, by the large Tris activation if the latter operates primarily on the first site under these conditions. Further study of the reasons for the large drop in enzymatic rate when sulfur is substituted for oxygen should reveal additional features of the alkaline phosphatase mechanism.

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