Hydrolysis of S-Substituted Monoesters of Phosphorothioic Acid by Alkaline Phosphatase from Escherichia coli

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SUMMARY
Alkaline phosphatase from Escherichia coli catalyzes the hydrolysis of S-substituted monoesters of phosphorothioic acid of the type RSPO_{3}Na_{2} (R = -CH_{2}CH_{2}NH_{2}, -CH_{2}CHNHCOCH_{3}, -CH_{2}COOH, or -CH_{2}CH_{2}COOC_{2}H_{5}), at the S-P bond, to yield orthophosphate and the corresponding thialcohols.

The rate of enzymic hydrolysis of cysteamine S-phosphate was measured at different pH values and substrate concentrations. The pH profile of the rate of hydrolysis, as well as the $K_m$ and $V_{max}$ values obtained, are similar to the corresponding values obtained for p-nitrophenyl phosphate. Cysteamine S-phosphate competitively inhibits the hydrolysis of p-nitrophenyl phosphate. Inorganic phosphate and phosphorothioate competitively inhibit the enzymic hydrolysis of both cysteamine S-phosphate and p-nitrophenyl phosphate. Phosphorothioate is hydrolyzed by alkaline phosphatase, in a manner analogous to inorganic orthophosphate, to yield orthophosphate and hydrogen sulfide.

It is suggested that cysteamine S-phosphate and p-nitrophenyl phosphate are bound to the same catalytic site of alkaline phosphatase.

EXPERIMENTAL PROCEDURE
Materials—Chromatographically purified alkaline phosphatase (7) (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (E. coli) was obtained as a suspension containing 14 mg of enzyme per ml of 0.5 M MgSO_{4} from Worthington. The suspension was diluted with conductivity water to a final concentration of 1 mg per ml, and the clear solution obtained was stored at 4°C. p-Nitrophenyl phosphate was obtained from Sigma; aminoethanol and aminoethanol O-phosphate were purchased from British Drug Houses. Cysteamine S-phosphate (1), N-acetylcysteamine S-phosphate (3), trisodium phosphorothioate (8) barium S-(carboxymethyl)phosphorothioate (2), and disodium S-[2-(methoxycarbonyl)ethyl]phosphorothioate (2) were prepared and purified according to the literature. The other compounds used were of analytical grade.

In the preparation of $^{32}$P or $^{35}$S-labeled trisodium thiophosphate and cysteamine S-phosphate (1), phosphorus trichloride labeled with $^{32}$P or $^{35}$S was used. Both were obtained from the Radiochemical Centre, Amersham, England. The $^{35}$S-labeled cysteamine S-phosphate and phosphorothioate had specific activities of 10 μC per μmole, whereas the $^{32}$P-labeled preparations had an activity of 20 μC per μmole.

Stock solutions of cysteamine S-phosphate (0.03 M) and of p-nitrophenyl phosphate (0.03 M) in conductivity water were kept in the deep freeze. A solution of DTNB (2.0 mg per ml) in 0.05 M veronal buffer, pH 8.0, was kept at room temperature and used within several days after preparation. Veronal-HCl buffer was prepared according to Dawson et al. (9).

Methods—The pH measurements were carried out with a pH meter, model 22, Radiometer, with a type G-202B glass electrode and a type K4312 calomel electrode. The electrodes were standardized with the aid of standard buffer solutions (10).

Spectrophotometric measurements were made with a Zeiss model PMQ spectrophotometer. Quartz cuvettes with a light path of 10 mm were used.

High voltage electrophoresis was performed on Whatman No. 1 paper at 36 volts per cm in an apparatus of the type described by Michl (11), with pyridine-acetic acid-water buffer, pH 6.5, according to Ryle et al. (12).

The electrophoretograms were stained with ninhydrin (0.25%, w/v, in acetone) for detection of compounds with free amino groups, with a phosphate reagent (13) for detection of organic or inorganic phosphate.

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inorganic phosphate, and with t-butyl hypochlorite (14) for
detection of compounds containing amide groups. Labeled
compounds were detected by scanning for radioactivity with a
Nuclear-Chicago Actiograph 11, model L-100a.

Cysteamine (or other thiol) concentrations were determined by
the method of Ellman (15) with DTNB. We found that
the reaction of DTNB with cysteamine to yield 2-nitro-5-thiobenzoic
acid is essentially instantaneous under the experimental condi-
tions used (see below). The molar absorptivity of 2-nitro-5-
thiobenzoic acid at 412 m\(\mu\) was found (assuming complete
reaction) to be 13,600, in accord with Ellman (15). The molar
absorptivity did not change over the pH range 6.5 to 9.8.

The rate of hydrolysis of phosphomonothioesters by alkaline
phosphatase was determined with the above assay. The reaction
mixtures consisted of substrate (10\(^{-6}\) to 3 \(\times\) 10\(^{-3}\) M), DTNB
(1 \(\times\) 10\(^{-4}\) M), and alkaline phosphatase (0.5 \(\mu\)g per ml) in the
appropriate buffer. The absorbance at 412 m\(\mu\) was measured
every 10 sec for 3 min, and the velocity of the reaction was
calculated by assuming \(E_{412} = 13,600\). The velocity was
constant with time over the first few minutes in each case.

The rate of enzymatic hydrolysis of \(p\)-nitrophenyl phosphate was
derived from the amounts of nitrophenol liberated with time.
Nitrophenol was assayed spectrophotometrically at 400 m\(\mu\),
taking into consideration the change in molar absorptivity with
pH and ionic strength \(E_{400} = 17,200\) at pH 9.0 in 1.5 M
NaCl. Reaction mixtures contained \(p\)-nitrophenyl phosphate (10\(^{-8}\) to
3 \(\times\) 10\(^{-5}\) M) and alkaline phosphatase (0.5 \(\mu\)g per ml) in the
appropriate buffer. Absorbance measurements were made every
10 sec for 3 min. The changes in absorbance were linear with
time during the first 5 min.

No temperature control was provided, and all measurements
were carried out at room temperature.

RESULTS

Hydrolysis of Cysteamine \(S\)-Phosphate by Alkaline Phosphatase

— In accord with the findings of Akerfeldt (16), it was found that
in aqueous solution cysteamine \(S\)-phosphate is not hydrolyzed to
any detectable extent within 24 hours at 25° in the pH range of
7.0 to 10.0. Fast hydrolysis of cysteamine \(S\)-phosphate in this
pH range could be effected, however, in the presence of alkaline
phosphatase. The enzymic hydrolysis was accompanied by the
liberation of free sulfhydryl groups, which could be detected with
the aid of DTNB as described under “Experimental Procedure.”
The course of hydrolysis of cysteamine \(S\)-phosphate (2 \(\times\) 10\(^{-8}\) M)
by alkaline phosphatase (0.5 \(\mu\)g per ml) at different pH values
(pH 6.8 to 9.0) at 25° is given in Fig. 1. Within the first 5 min a
linear reaction was obtained for each of the pH values tested.
The rate of reaction was found to increase markedly on raising
the pH from 6.8 to 9.0.

To obtain preliminary information as to the products formed
by the enzymic hydrolysis of the organic phosphomonothioester
used, cysteamine \(S\)-phosphate (10\(^{-2}\) M) was incubated with
alkaline phosphatase (10 \(\mu\)g per ml) for 20 hours at pH 7.0, 8.0,
8.5, or 9.0 at 25°, and 25-\(\mu\)l aliquots were withdrawn and sub-
jected to high voltage electrophoresis for 30 min at pH 6.5.
The electrophoretograms were stained with ninhydrin or with
phosphate reagent. The results summarized in Fig. 2 show that
all of the cysteamine \(S\)-phosphate was converted into a new
ninhydrin-positive and phosphate-negative compound moving
toward the cathode and a new phosphate-positive and ninhydrin-
negative compound moving toward the anode. The mobility of
the new ninhydrin-positive spot was identical with that of an
authentic sample of cysteamine, whereas the mobility of the
phosphate-positive spot was identical with that of inorganic
phosphate. It should be noted, however, that the electrophoretic
mobility of ethanolamine is practically identical with that of
cysteamine under the experimental conditions used. Moreover,
the mobilities of inorganic phosphate and phosphorothioate,
although separable, are rather close. The possible enzymic
hydrolysis of cysteamine \(S\)-phosphate at the C—S bond to yield
ethanolamine and thiophosphate is not, therefore, excluded by
the above results.

Final proof as to the hydrolysis of cysteamine \(S\)-phosphate by
alkaline phosphatase at the S—P bond was obtained by using
cysteamine \(S\)-phosphate labeled with \(^{32}\)P or with \(^{35}\)S in two differ-
ent sets of experiments. When the \(^{32}\)P-labeled cysteamine \(S-
phosphate was used as substrate under the experimental condi-
tions given in Fig. 2, all of the original radioactivity was found,
after complete enzymic hydrolysis, in the spot corresponding to
the inorganic phosphate. When \(^{35}\)S-labeled cysteamine \(S-
phosphate was used as substrate under similar conditions, all of
the radioactivity was found, after complete-enzymic hydrolysis, in
the spot corresponding to cysteamine.

Hydrolysis of N-Acetylcysteamine \(S\)-Phosphate, \(S\)-(Carboxymeth-
yl)phosphorothioic Acid, and \(S\)-[d-(Methoxycarbonyl)ethyl]phos-
phorothioic Acid, Catalyzed by Alkaline Phosphatase—Alkaline

![Fig. 1. Hydrolysis of cysteamine \(S\)-phosphate by alkaline phosphatase at different pH values. The amount of cysteamine formed was followed with the aid of DTNB according to the procedure given in "Methods." The reaction mixture was 0.002 M in substrate, 0.02 M in Veronal buffer, and 1.5 M in NaCl and contained 0.5 \(\mu\)g of enzyme per ml. Incubation temperature was 22°.](http://www.jbc.org/)
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Electrophoretogram of an incubation mixture of cysteamine S-phosphate with alkaline phosphatase. The enzymic hydrolysis was carried out at pH 9.0 for 20 hours, and the high voltage electrophoresis was performed at pH 6.5. For further details, see the text. Hatching with solid lines indicates positive reaction with ninhydrin spray; hatching with dashes indicates positive reaction with phosphate spray. The following abbreviations are used: CASP, cysteamine S-phosphate; CA, cysteamine; PSH, phosphorothioate; Pi, inorganic phosphate; E, alkaline phosphatase.

Phosphatase is a nonspecific phosphomonoesterase. It was thus of interest to investigate whether its hydrolysis of phosphothioesters is also nonspecific. Reaction mixtures 0.01 M in one of the above substrates and 0.1 M in Veronal buffer, pH 9.0, were incubated with enzyme (10 μg per ml) for 24 hours. In control experiments the enzyme was omitted. Aliquots (25 μl) were withdrawn and subjected to high voltage electrophoresis, and the paper was stained with phosphate reagent. Each of the substances used yielded only one phosphate-positive spot, possessing a mobility identical with that of inorganic phosphate. The phosphate-positive spots present in the control experiments disappeared in the experiments with enzyme. From these experiments one can conclude that alkaline phosphatase digests the S-substituted monoesters of phosphorothioic acid irrespective of the ester residue.

The rates of hydrolysis of the above phosphothioesters were determined under standard conditions (0.02 M Veronal buffer (pH 9.0), 1.5 M NaCl, 0.5 μg of enzyme per ml, 0.002 M substrate, and 0.0004 M DTNB). The rates found (1.9 to 2.0 x 10^-8 mole of substrate ml^-1 min^-1 per 0.5 μg of enzyme per ml) were very similar to the rate of enzymic hydrolysis of cysteamine S-phosphate (2.1 x 10^-8 mole ml^-1 min^-1 per 0.5 μg of enzyme per ml) under the same conditions.

Kinetics of Hydrolysis of Cysteamine S-Phosphate Catalyzed by Alkaline Phosphatase in Veronal Buffer

Qualitative observations revealed that the hydrolysis of cysteamine S-phosphate catalyzed by alkaline phosphatase is dependent on pH, salt concentration, and the nature of the buffer used. The investigation of the effect of pH on the reaction rate was limited to the pH range 6.8 to 9.6 in 0.02 M Veronal buffer. Experiments at various concentrations of sodium salt at different pH values were carried out. The results of these experiments are given in Fig. 3. The rates were evaluated as described under “Experimental Procedure.” It is

![Fig. 2. Electrophoretogram of an incubation mixture of cysteamine S-phosphate with alkaline phosphatase. The enzymic hydrolysis was carried out at pH 9.0 for 20 hours, and the high voltage electrophoresis was performed at pH 6.5. For further details, see the text. Hatching with solid lines indicates positive reaction with ninhydrin spray; hatching with dashes indicates positive reaction with phosphate spray. The following abbreviations are used: CASP, cysteamine S-phosphate; CA, cysteamine; PSH, phosphorothioate; Pi, inorganic phosphate; E, alkaline phosphatase.](http://www.jbc.org/)

![Fig. 3. The effect of the concentration of NaCl on the rate of hydrolysis, v, of cysteamine S-phosphate (A) and p-nitrophenyl phosphate (B) catalyzed by alkaline phosphatase at different pH values. The reaction mixtures were 0.002 M in substrate and 0.02 M in Veronal buffer and contained 0.5 μg of enzyme per ml.](http://www.jbc.org/)
apparent that the rate of hydrolysis at each of the pH values studied does not vary markedly with salt concentration in the range from 1.2 to 2.0 M NaCl. Therefore, the pH dependence of the hydrolysis of cysteamine S-phosphate catalyzed by alkaline phosphatase was measured in the presence of 1.5 M NaCl.

Fig. 4 shows the results of these experiments. The rate of hydrolysis passes through a maximum value at pH 9.0 (Fig. 4, Curve B). A similar pH optimum was obtained in the absence of salt (Fig. 4, Curve A). The pH dependence of the rate of hydrolysis of cysteamine S-phosphate in the presence of 1.5 M NaCl did not vary when the concentration of substrate was changed from $3 \times 10^{-3}$ M to $5 \times 10^{-4}$ M. From the variation of the rate of enzymic hydrolysis of cysteamine S-phosphate with substrate concentration at pH 9.0 in 0.02 M Veronal buffer in the presence of 1.5 M NaCl (Fig. 5), a Michaelis constant, $K_m$, of $9.4 \times 10^{-5}$ M and a maximum rate of hydrolysis, $V_{max}$, of $2.1 \times 10^{-8}$ mole of substrate ml$^{-1}$ min$^{-1}$ per 0.5 $\mu$g of enzyme per ml were calculated.

Kinetics of Alkaline Phosphatase-catalyzed Hydrolysis of p-Nitrophenyl Phosphate—In order to investigate the relation between the alkaline phosphatase-catalyzed hydrolyses of phosphomonoesters and phosphate O-esters, the reaction of p-nitrophenyl phosphate with alkaline phosphatase was studied under the same conditions of enzyme, substrate, and Veronal buffer concentrations used for the cysteamine S-phosphate reactions. The variation of rate with salt concentration and pH was determined and was found to be similar to the variations found in the rates of cysteamine S-phosphate hydrolysis (see Figs. 3 and 4).

The $V_{max}$ and $K_m$ were determined for p-nitrophenyl phosphate under the same conditions used for cysteamine S-phosphate (pH 9.0, 0.02 M Veronal buffer, 1.5 M NaCl) $V_{max}$ for p-nitrophenyl phosphate was found to be $2.9 \times 10^{-8}$ mole ml$^{-1}$ min$^{-1}$ per 0.5 $\mu$g of enzyme per ml, i.e. about 1.4 times that for cysteamine S-phosphate, while $K_m$ was found to have essentially the same value for both compounds.

Competition between Cysteamine S-Phosphate and p-Nitrophenyl Phosphate in Enzymic Reaction—The similarity of the hydrolysis of cysteamine S-phosphate and p-nitrophenyl phosphate led to the suggestion that the same enzyme site binds both substrates, and that the mechanism of the enzymic hydrolysis of the S–P bond corresponds to that of the O–P bond. Accordingly, compounds containing the S–P bond would be expected to compete with compounds containing the O–P bond for a common site. Thus, competition of cysteamine S-phosphate and p-nitrophenyl phosphate in the enzymic reaction is to be expected.

For simplicity, the change of absorbance at 400 m$m$ due to the hydrolysis of p-nitrophenyl phosphate was measured (see "Methods") in the presence of various amounts of cysteamine S-phosphate. Increasing the amount of cysteamine S-phosphate in the reaction mixture increased the inhibition of the enzymic hydrolysis of p-nitrophenyl phosphate. The experiments were...
The kinetic behavior of cysteamine S-phosphate hydrolysis by alkaline phosphatase has proved to be very similar to that of the enzymatic hydrolysis of p-nitrophenyl phosphate, a typical O-ester substrate. The reactions have the same pH maximum, pH 9.0, in the presence of 1.5 mM NaCl, and the same dependence on salt concentrations. Both of these alkaline phosphatase phosphate hydrolysis by inorganic phosphate and found it to be very close to that of the cysteamine S-phosphate reaction.

**Inhibition by Phosphorothioate of Enzymic Hydrolysis of p-Nitrophenyl Phosphate**—The possibility that phosphorothioate behaves toward alkaline phosphatase in a manner analogous to inorganic phosphate was considered. Because of the experimental difficulties in measuring the hydrolysis of cysteamine S-phosphate in the presence of phosphorothioate, the inhibition of the hydrolysis of p-nitrophenyl phosphate by phosphorothioate was measured. In this set of experiments, the concentration of p-nitrophenyl phosphate was varied from $3 \times 10^{-5}$ M to $2 \times 10^{-2}$ M, and phosphorothioate, from $3 \times 10^{-2}$ M to $1 \times 10^{-3}$ M. The hydrolysis of p-nitrophenyl phosphate was followed spectrophotometrically at 400 nm, and the results were analyzed as in the case in which inorganic phosphate served as inhibitor. The $K_i$ found was equal to $6.0 \times 10^{-3}$ M, which is of the same order as that found for inorganic phosphate.

**Hydrolysis of Phosphorothioate by Alkaline Phosphatase**—The enzymic hydrolysis of phosphorothioate at the S-P bond was demonstrated by using $^{35}$S-labeled phosphorothioate. The experiments were carried out as follows. A 0.02 M solution of $^{35}$S-labeled phosphorothioate in 0.05 M Veronal buffer at pH 9.0 was prepared. To 2.0 ml of this solution, 4.0 ml of 0.1 M Veronal buffer at pH 9.0, 3.5 ml of water, and 0.5 ml of alkaline phosphatase (100 µg per ml) were added. In the control experiments, the enzyme was omitted. Aliquots of 50 µl were taken at 5-min intervals for up to 2 hours at room temperature, and were subjected to high voltage electrophoresis at pH 6.5 for 20 min. The electrophoretogram was scanned for $^{35}$S-containing compounds. Under our experimental conditions, after 2 hours no radioactive spot remains at the position corresponding to $^{35}$S-labeled phosphorothioate, while the same spot gives a positive reaction for inorganic phosphate. On the basis of this experiment one can conclude that alkaline phosphatase hydrolyzes phosphorothioate at the S-P bond.

**DISCUSSION**

Alkaline phosphatase is known to catalyze the hydrolysis of phosphomonoesters as well as that of phosphoramidates. We have found that the action of this enzyme on phosphoromonoesters is similar to its action on phosphate O-esters. Considerable evidence indicates, in fact, that the same enzyme site is involved in both kinds of alkaline phosphatase actions.

When phosphoromonothioesters, $R-SPO_2H_2$ (in our case $R = -CH_2CH_2NH_2, -CH_2CH_2NHCOCH_3, -CH_2COOH, or -CH_2CH_2CO_2H_2$) are incubated with alkaline phosphatase, the release of free sulfhydryl groups is observed. The point of cleavage was determined to be the P-S bond by high voltage electrophoresis analysis of the products of hydrolysis of $^{32}$P- and $^{35}$S-labeled cysteamine S-phosphate. This is analogous to the P-O cleavage observed in the action of the same enzyme on phosphorothioate O-esters. Furthermore, the cleavage of a number of phosphoromonothioesters with different ester substitutents at similar rates indicates that this activity, like phosphate O-ester cleavage, has little specificity for the structure of the ester.

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The kinetic behavior of cysteamine S-phosphate hydrolysis by alkaline phosphatase has proved to be very similar to that of the enzymatic hydrolysis of p-nitrophenyl phosphate, a typical O-ester substrate. The reactions have the same pH maximum, pH 9.0, in the presence of 1.5 mM NaCl, and the same dependence on salt concentrations.
reactions show typical Michaelis-Menten kinetics and, in fact, have surprisingly similar kinetic constants: $V_{max} = 2.9 \times 10^{-3}$ mole ml$^{-1}$ min$^{-1}$ per 0.5 µg of enzyme per ml, and $K_m = 9.5 \times 10^{-7}$ M for the hydrolysis of p-nitrophenyl phosphate; $V_{max} = 2.1 \times 10^{-3}$ mole ml$^{-1}$ min$^{-1}$ per 0.5 µg of enzyme per ml, and $K_m = 9.4 \times 10^{-7}$ M for the hydrolysis of cysteamine S-phosphate, under the same experimental conditions.

The identity of the enzyme sites for the cysteamine S-phosphate hydrolysis and p-nitrophenyl phosphate hydrolysis activities of alkaline phosphate has been most clearly shown in a number of competition experiments. Cysteamine S-phosphate competitively inhibits the alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate. Furthermore, the hydrolyses of both cysteamine S-phosphate and p-nitrophenyl phosphate by alkaline phosphatase are competitively inhibited by inorganic phosphate and phosphorothioate, which we have found to have the same $K_i$ in either reaction. These findings, all taken together, indicate that the replacement of the linking oxygen in a phosphate ester by sulfur has very little effect on its reactivity toward alkaline phosphatase. The mode of binding, reaction mechanism, and functional groups (as witnessed by pH and salt dependence) involved in the hydrolysis of either O- or S-linked substrates appear to be very similar. The only apparent difference between the action of alkaline phosphatase on the two substrates is in rates of hydrolysis.

A further interesting observation is the similarity of phosphate and phosphorothioate with respect to alkaline phosphatase. Phosphorothioate was found, like inorganic orthophosphate, to inhibit competitively the hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase. What is more, phosphorothioate is itself hydrolyzed by alkaline phosphatase at the P—S bond, just as inorganic phosphate has been shown to be hydrolyzed at the P—O bond (17).

The nearly complete analogy between the reactions of phosphorothioate and its derivatives with alkaline phosphatase and those of phosphate and its derivatives tempts one to conclude that alkaline phosphatase does not interact directly with the linking sulfur or oxygen in these compounds. Since sulfur and oxygen have different van der Waals radii, polarizabilities, and basicities in these compounds, any interaction with them, either in binding or in catalysis (i.e. protonation), would be expected to lead to major differences in the behavior of these two types of compounds in the presence of the enzyme. The lack of such differences, whatever its complete explanation, is of significance in considering the mechanism of alkaline phosphatase action.

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