Purification and Properties of a Phosphohydrolase from Enterobacter aerogenes*

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JOHN A. GERLT† AND GLENN J. R. WHITMAN

From the James Bryant Conant Laboratories, Harvard University, Cambridge, Massachusetts 02138

SUMMARY

A phosphohydrolase from Enterobacter aerogenes which hydrolyzes phosphate mono- and diesters has been purified approximately 50-fold to apparent homogeneity and crystallized. The enzyme is produced when the bacteria utilize phosphate diesters as sole phosphorus source. From sedimentation equilibrium experiments the molecular weight of the native enzyme is 173,000; from sodium dodecyl sulfate polyacrylamide gel electrophoresis the subunit molecular weight is 29,000, indicating that the enzyme is hexameric.

The hydrolytic activity of the enzyme using both mono- and diesters is maximal at pH 5; the $k_m$ of the enzyme for bis-$\beta$-nitrophenyl phosphate is constant from pH 5 to 8.5 whereas that for $\beta$-nitrophenyl phosphate increases about 40-fold as the pH increases over the same range. The phosphodiesterase activity is not inhibited by chelating agents but is inhibited by several divalent metal ions. $^{31}P$ NMR spectroscopy was used to identify the hydrolysis products of glycoside cyclic phosphates. The enzyme-catalyzed hydrolysis of methyl $\beta$-ribofuranoside cyclic 3'-5' phosphate yields exclusively the 5-phosphate whereas that of adenosine 3',5'-monophosphate yields a 4:1 mixture of 3'- and 5'-AMP.

The enthalpies of hydrolysis of cyclic 3'-5' and 2'-3'-nucleotides were reported recently (1, 2). Whereas the enthalpies of hydrolysis of cyclic 2'-3'-nucleotides were in accord with their structure and reactivity, the values reported for the cyclic 3'-5'-nucleotides were unexpectedly exothermic, suggesting that their hydrolysis is accompanied by the relief of significant strain. In order to evaluate the effect of phosphodiester structure on enthalpy of hydrolysis, measurements of the enthalpiies of hydrolysis of an acyclic diester, monocyclic diesters, and other glycoside cyclic phosphates were necessary. Diesters hydrolyze too slowly under conditions compatible with calorimetric techniques to make accurate measurements possible. No catalyst, either non-enzymatic or enzymatic, was known which would hydrolyze the desired phosphodiester. However, Enterobacter aerogenes can utilize dimethyl phosphate as sole phosphorus source (3). In this article we describe the purification and properties of a phosphohydrolase present in Enterobacter aerogenes which hydrolyzes both mono- and diesters of phosphoric acid. A preliminary account of this research has been published (4).

EXPERIMENTAL PROCEDURES

Materials

Barium dimethyl phosphate was prepared by the hydrolysis of trimethyl phosphate (Aldrich) with a small excess of barium hydroxide, followed by recrystallization from aqueous ethanol. The barium salt was converted to the sodium salt by passage through an Amberlite IR-120 (Na+) column.

The preparation of cyclic alkyl and glycoside phosphodiesterase and their enzymatic hydrolysis products is described in the following article (5).

Disodium $\beta$-nitrophenyl phosphate was purchased from Aldrich, sodium bis-$\beta$-nitrophenyl phosphate from Sigma, and nucleotides from P-L Biochemicals and Calbiochem.

Tris base and Tris-HCl were obtained from Sigma. Pipes was from Calbiochem. $^2$ DEAE-cellulose (Whatman DE52) was obtained from Reeve-Angel, Sephadex G-200 from Pharmacia, and Bio Gel HT hydroxylapatite from Bio Rad Laboratories.

Sodium sulfate (salmine) and enzyme grade ammonium sulfate were obtained from Schwarz/Mann.

Alkaline phosphatase from Escherichia coli was obtained from either Sigma or Worthington. Proteins obtained from the usual commercial sources served as molecular weight standards in sodium dodecyl sulfate polyacrylamide gel electrophoresis.

All other chemicals were the best grade commercially available.

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† Predoctoral Trainee of the National Institutes of Health. Present address, Laboratory of Chemical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014. Address effective July 1, 1975. Department of Chemistry, Yale University, New Haven, Connecticut 06520.

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Bacterial Methods

Enterobacter aerogenes (ATCC 13048) was grown on minimal salts medium, free from inorganic phosphate, with the following composition: 0.08 M NaCl, 0.02 M KCl, 0.02 M NH₄Cl, 0.001 M MgCl₂, 2 X 10⁻⁴ M CaCl₂, 5 X 10⁻⁴ M Na₃SO₄, 2 X 10⁻⁴ M CoCl₂, 2 X 10⁻⁴ M FeCl₃, 2 X 10⁻⁴ M MnCl₂, 0.12 M Tris base, 0.012 M dextrose, and 1.0 X 10⁻⁴ M sodium dimethyl phosphate, adjusted to pH 7.5 with concentrated HCl. Deionized water was used to prepare the medium.

After inoculation with 50 ml stationary phase cultures, 2-liter batches of bacteria were grown for 18 to 20 hours at 37°C on a rotary shaker. The cell paste (about 1.5 g/liter) obtained by continuous centrifugation was used for inorganic phosphate analysis (6).

Hydrolysis of bis-p-Nitrophenyl Phosphate—Enzyme activity was determined by spectrophotometric measurement of the rate of production of p-nitrophenolate from 1 mM sodium bis-p-nitrophenyl phosphate in 0.10 M Tris-HCl, pH 8.0, at 30°C. An extinction coefficient of 15.7 (μmol/ml)⁻¹ at 400 nm was used.

Hydrolysis of Trimethylene Phosphate in Presence of Alkaline Phosphatase—Phosphodiesterase activity was measured by the release of inorganic phosphate from trimethylene phosphate catalyzed by rate-limiting phosphohydrolase and excess alkaline phosphatase. The 1.0 ml reaction mixture contained 1 mM trimethylene phosphate, phosphohydrolase, and 0.05 mg of alkaline phosphatase. The 1.0 ml reaction mixture contained 1 mM triethylene phosphate, phosphohydrolase, and 0.05 mg of alkaline phosphatase. The enzyme activity was measured by spectrophotometric determination of the rate of hydrolysis from trimethylene phosphate to monoester per min.

Measurements of the pH of all buffers were made at room temperature. Specific activities were measured with the bis-p-nitrophenol phosphate assay. A summary of the purification is presented in Table I. A comparison by gel electrophoresis in sodium dodecyl sulfate of a sample from each purification step is presented in Fig. 1.

Crude Extract—Washed cells from 48 liters of culture (72 g) were suspended evenly in 216 ml of 0.01 M Tris-HCl, pH 7.6, and disrupted by a single pass through a French pressure cell (Amicon) at 16,000 psi. The suspension was centrifuged at 34,800 x g for 45 min. Osmotic shock did not release detectable enzyme activity.

Protamine Sulfate Treatment—A 1% protamine sulfate solution (47.6 ml) adjusted to pH 7.5 with NaOH was added dropwise to the cloudy supernatant (238 ml) of the previous centrifugation. The suspension was stirred for 30 min prior to centrifugation at 34,800 x g for 45 min.

Heat Treatment—The slightly turbid supernatant (271 ml) was rapidly heated to 60°C (less than 3 min) in a 90°C water bath and then maintained between 60 and 63°C for 10 min. The suspension was rapidly cooled to 15°C and then centrifuged at 34,800 x g for 25 min. The supernatant was centrifuged again at 34,800 x g for 15 min to remove all particulate matter.

### RESULTS AND DISCUSSION

Enzyme activity for the hydrolysis of alkyl diesters was not detectable in extracts of cells which were grown in minimal medium containing 1 mM inorganic phosphate or in nutrient broth.

All operations were performed at 0-4°C unless otherwise stated. Measurements of the pH of all buffers were made at room temperature. Specific activities were measured with the bis-p-nitrophenol phosphate assay. A summary of the purification is presented in Table I. A comparison by gel electrophoresis in sodium dodecyl sulfate of a sample from each purification step is presented in Fig. 1.

### 31P NMR Spectroscopy

31P NMR spectra were obtained with a Varian XL-100-15 NMR spectrometer operating at 40.5 MHz for 31P, locked on the 1H resonance of the solvent (50% D₂O). The spectrometer is equipped for Fourier transform accumulation of data. Chemical shifts were measured relative to an external capillary of 85% phosphoric acid. Samples were prepared in 2.0 ml of 0.50 mM sodium carbonate buffer, pH 10.0, containing 5 mM EDTA to eliminate paramagnetic broadening of the resonances. The spectra were recorded at the ambient temperature of the probe, 27 ± 2°C.

### Sedimentation Equilibrium Ultracentrifugation

Sedimentation equilibrium experiments were performed at 16,200 rpm in a Spinco model E ultracentrifuge equipped with a photoelectric scanner and ultraviolet optics. The temperature was maintained at 20°C. Prior to centrifugation, the phosphohydrolase was dialyzed for 48 hours against several changes of 0.1 m potassium phosphate, pH 6.8, containing 0.1 m potassium chloride. The data were analyzed by the method of Yphantis (9).
FIG. 1. Sodium dodecyl sulfate electrophoresis of a protein sample from each step in the purification of the phosphohydrolase. Approximately 50 µg of protein were subjected to electrophoresis in 7.5% gels.

FIG. 2. DEAE-cellulose chromatography of partially purified phosphohydrolase. A detailed description of this purification step is presented in the text. The linear NaCl elution gradient was applied at Fraction 50. Phosphodiesterase activity (O--O); absorbance at 280 nm (●—●).

**DEAE-cellulose Chromatography**—The bright yellow supernatant was applied to a column (3.6 x 13 cm) of Whatman DE52 equilibrated in 0.01 M Tris-HCl, pH 7.6. After sample application, the column was washed for 6 hours with the Tris buffer containing 0.15 M NaCl. A 4.0-liter linear gradient of 0.15 to 0.55 M NaCl in the Tris buffer was then applied for elution of the phosphohydrolase. The flow rate was maintained at about 200 ml/hour, and fractions containing approximately 30 ml were collected throughout the procedure.

Fractions in the region of Number 90 contain the enzyme (Fig. 2). Fractions with a specific activity greater than 2.0 were combined and concentrated with an Amicon ultrafilter employing a PM-10 membrane.

**Ammonium Sulfate Precipitation**—The concentrated enzyme solution (24.8 ml) was brought to 60% saturation in ammonium sulfate by the addition of 9.59 g of the solid enzyme grade salt. The suspension was stirred for 30 min before centrifugation at 34,800 x g for 20 min. The precipitate was dissolved in about 4 ml of 0.01 M potassium phosphate, pH 6.8 (equimolar mixture of the mono- and dibasic salts), and centrifuged for 34,800 x g for 20 min.

**Sephadex G-200 Gel Filtration**—The supernatant was applied to a column (2.6 x 90 cm) of Sephadex G-200 equilibrated in the potassium phosphate buffer and run ascending at a flow rate of 15 ml/hour.

The enzyme eluted after about 260 ml had passed through the column. Fractions with a specific activity greater than 11 were combined and concentrated with an Amicon ultrafilter employing a PM-10 membrane.

**Hydroxylapatite Chromatography**—The concentrated enzyme solution (18.5 ml) was applied to a column (2.6 x 7.5 cm) of Bio-Gel HT hydroxylapatite equilibrated in the 0.01 M potassium phosphate buffer. After sample application, the column was washed for 1 hour with the phosphate buffer prior to application of a 400-ml linear gradient of 0.01 to 0.10 M potassium phosphate, pH 6.8. The enzyme was eluted at a flow rate of 22 ml/hour.

The activity eluted after about 190 ml of the gradient had passed through the column. Fractions containing enzyme activity were combined and concentrated with the Amicon ultrafilter.

**Crystallization**—The concentrated enzyme solution (18.7 ml with 2 mg/ml) was clarified by centrifugation and then carefully adjusted to 35% saturation in ammonium sulfate by addition of the solid enzyme grade salt. This amount of ammonium sulfate was such that the solution became slightly turbid upon standing for several minutes. The suspension was set aside in a refrigerator where crystallization proceeds slowly (Fig. 3).

**Criteria of Purity**—The purity of the phosphohydrolase was assessed with polyacrylamide gel electrophoresis. Under native (8) and denaturing (7) conditions, the enzyme migrated as a single species. (Although ultracentrifugal methods are less sensitive to impurities, neither sedimentation velocity nor equilibrium experiments gave any evidence of heterogeneity.) That the phosphomonoesterase and phosphodiesterase activities which accompany the purified protein (see “Substrate Specificity”) are catalyzed by the single protein species detectable in the Hedrick and Smith electrophoresis system was demonstrated as follows. Gels run in triplicate were separately stained for protein and incubated in p-nitrophenol phosphate and bis-p-nitrophenyl phosphate solutions. Single bands with identical mobility were detected by each staining procedure. Results were similar whether electrophoresis was conducted in 6, 8, or 10% gels.

**Molecular Weight**—From sedimentation equilibrium centrifugation and assuming a partial specific volume of 0.73, based on the amino acid composition (Table II), the molecular weight...
of undissociated enzyme was determined to be 173,000. When the phosphohydrolase was subjected to electrophoresis with appropriate molecular weight standards in polyacrylamide gels containing sodium dodecyl sulfate (7), a single species of molecular weight 29,000 was observed. These data indicate that the enzyme is a hexamer of subunits with identical molecular weight.

**Extinction Coefficient**—The extinction coefficient, E_{15}, estimated as described under “Methods,” is 15.1.

**Substrate Specificity**—In addition to the diesters used to follow the purification procedure (trimethylene and bis-p-nitrophenyl phosphates), the enzyme catalyzes the hydrolysis of other phosphate esters (Table III).

**Catalytic Properties**—The enzymatic activity for hydrolysis of both bis-p-nitrophenyl and p-nitrophenyl phosphate was maximal at about pH 5, with the ratio of the two maximal velocities at pH 4.9 being about 4 (Fig. 4).

Lineweaver-Burk plots for bis-p-nitrophenyl phosphate hydrolysis were often nonlinear, with Hill plots of the same data having a slope (n) less than unity. The apparent K_m was calculated from such data by a graphical procedure (12). Phosphodiester undergo no change in protonation, from pH 5 to 8.5, and the apparent K_m for bis-p-nitrophenyl phosphate is independent of pH in this range (Fig. 4); the K_m for p-nitrophenyl phosphate which has a pK_a of 5.4 (13) increases 40-fold as the pH increases over this same range. This behavior suggests that a phosphoric acid ester is recognized as a substrate on the basis of its single negative charge. Incubation of the enzyme with several chelators did not cause any significant inactivation. Of various divalent metal ions tested, none produced significant stimulation of activity, but several did produce partial inactivation.

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Dependence of V_max and K_m for bis-p-nitrophenyl phosphate (●—●) and p-nitrophenyl phosphate (○—○) on pH. Buffers used were sodium acetate, pH 4.6, sodium cacodylate, pH 4.9, sodium Pipes, pH 5.9 and 6.9, Tris-HCl, pH 8.4. The buffer concentrations were 0.05 m. The assays were performed at 30°C by following the production of p-nitrophenolate at 400 nm.

though nucleases and other phosphohydrolases frequently require metal ions for activity, the lack of pronounced activation with added metal ions or inhibition by chelators suggests that this phosphohydrolase is not a metal-dependent enzyme.

**Hydrolysis Products**—The possible hydrolysis products of methyl α-D glucopyranoside cyclic 4:6-phosphate (14), methyl β-D-ribofuranoside cyclic 3:5-phosphate (5), and cyclic AMP were distinguished by the splitting pattern in 31P NMR spectra produced by 3P-H coupling. The signal from the phosphorus atom of esters of a secondary alcohol, which have 2 hydrogen on the carbon with a primary hydroxyl group are 

![Diagram](http://www.jbc.org/)

- At very high enzyme concentrations, hydrolysis of mononucleotides (and alkyl monooesters) is detectable (5).

| **Table II** Amino acid composition |
|-----------------------------------|
| Protein samples were hydrolyzed in 6 N HCl for 24, 48, and 72 hours in the presence of phenol to prevent destruction of tyrosine (10), with norleucine added to serve as an internal standard. Throneine and serine values were obtained by extrapolation to zero time, and half-cystine was determined after performic acid oxidation (11). Amide ammonia and tryptophan were not determined. |
| Amino acid | Residues/subunit |
|------------|------------------|
| Lysine     | 4                |
| Histidine  | 12               |
| Arginine   | 16               |
| Aspartic acid | 28           |
| Threonine  | 13               |
| Serine     | 17               |
| Glutamic acid |               |
| Proline    | 20               |
| Glycine    | 17               |
| Alanine    | 21               |
| Half-cystine | 6             |
| Valine     | 12               |
| Methionine | 6                |
| Isoleucine | 12               |
| Leucine    | 31               |
| Tyrosine   | 12               |
| Phenylalanine |             |

| **Table III** Substrate specificity |
|-----------------------------------|
| Rates measured either by inorganic phosphate production in the presence of excess alkaline phosphatase or by production of p-nitrophenolate in 0.1 m Tris-HCl, pH 8.0, at 30°C. |
| | Relative activity |
| Substrates |
| Ethylene phosphate | 670 |
| Trimethylene phosphate | 100 |
| Cyclic AMP | 130 |
| Cyclic uridine 3':5'-monophosphate | 5.5 |
| 3':AMP (5'-AMP, 3'-UMP, 5'-UMP) | 6.9 |
| bis-p-Nitrophenyl phosphate | <0.025 |
| p-Nitrophenol phosphate | 1100 |
| p-Nitrophenyl thymidine 3'-phosphate | 8.2 |
| p-Nitrophenyl thymidine 5'-phosphate | <0.05 |
| p-Nitrophenyl thymidine 5'-phosphate plus alkaline phosphatase | 2.3 |

| **Note** |
| At very high enzyme concentrations, hydrolysis of mononucleotides (and alkyl monooesters) is detectable (5). |

![Diagram](http://www.jbc.org/)

- EDTA, N-hydroxymethyl ethylenediamine triacetate, diethylenetriamine pentacacetate, 8-hydroxyquinoline-5-sulfonic acid, bis-p-nitrophenyl phosphate, 3'-AMP (5'-AMP, 3'-UMP, 5'-UMP).
- Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}. |
FIG. 5. Determination of the enzymatic hydrolysis product of methyl \( \beta \)-d-ribofuranoside cyclic 3:5 phosphate with \(^{31}P\) NMR spectroscopy. The top left and top right spectra are those of authentic cyclic phosphate and authentic methyl \( \beta \)-d-ribofuranoside 5-phosphate, respectively. The bottom left spectrum is that of cyclic phosphate which was hydrolyzed in barium hydroxide solution. The bottom right spectrum is that of cyclic phosphate which was hydrolyzed with the phosphohydrolase.

Cyclic AMP (Fig. 6) was hydrolyzed by both the phosphohydrolase and barium hydroxide to yield mixtures of 3'- and 5'-AMP. The composition of a mixture can be evaluated from the intensities of resonances in proton-decoupled spectra. The barium hydroxide hydrolysis of cyclic AMP yielded 3'-AMP and 5'-AMP in a ratio of about 9; the reason for the discrepancy between this value and the literature value of 5 (16) is not known. The enzymatic hydrolysis resulted in a 3'-AMP : 5'-AMP ratio of 4.

Relationship to Other Phosphohydrolases—Enterobacteriaceae are known to produce a cyclic 2':3'-nucleotide phosphodiesterase (17). The enzyme isolated in this study appears to be distinct from the previously reported enzymes since the latter are synthesized in the presence of inorganic phosphate, are released by osmotic shocks, have different substrate specificities, and require added metal ions for activity. Also, the phosphohydrolase from Enterobacter aerogenes appears to be the first isolated which is known to cleave simple aliphatic phosphodiesters, although it is not unusual for phosphodiesterases to cleave bis-p-nitrophenyl phosphate or other esters in which a p-nitrophenyl residue is a leaving group (18).

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REFERENCES
1. Greengard, P., Rudolph, S. A., and Sturtevant, J. M. (1969) J. Biol. Chem. 244, 4798–4800
2. Rudolph, S. A., Johnson, E. M., and Greengard, P. (1971) J. Biol. Chem. 246, 1271–1273
3. Wolfenden, R., and Spence, G. (1967) Biochim. Biophys. Acta 146, 290–298
4. Gerlt, J. A., and Westheimer, F. H. (1973) J. Am. Chem. Soc. 95, 5106–5115
5. Gerlt, J. A., Westheimer, F. H., and Sturtevant, J. M. (1975) J. Biol. Chem. 260, 5059–5067
6. Ames, B. N. (1966) Methods Enzymol. 8, 115–118
7. Weber, K., and Osborn, M. (1961) J. Biol. Chem. 244, 4406–4412
8. Hedrick, J. I., and Smith, A. J. (1968) Arch. Biochem. Biophys. 126, 155–164
9. Yphantis, D. A. (1964) Biochemistry 3, 297–317
10. Benshek, W. F., Rappert, M. A., and Cole, R. D. (1967) Biochemistry 6, 3789–3790
11. Moore, S. (1965) J. Biol. Chem. 239, 235–237
12. Dunn, C. P., Gerlt, J. A., Rabinowitz, K. W., and Wood, W. A. (1973) J. Biol. Chem. 248, 8189–8199
13. STURTEVANT, J. M. (1955) J. Am. Chem. Soc. 77, 255-258
14. BADDELEY, J., BUCHANAN, J. G., AND SZABO, L. (1954) J. Chem. Soc. 3821-3832
15. SZABO, P., AND SZABO, L. (1960) J. Chem. Soc. 3762-3768
16. SMITH, M., DRUMMOND, G. I., AND KHORANA, H. G. (1961) J. Am. Chem. Soc. 83, 698-700
17. DRUMMOND, G. I., AND YAMAMOTO, M. (1971) in The Enzymes (BOYER, P. D., ed) 3rd Ed, pp. 355-371, Academic Press, New York.
18. BERNARDI, A., AND BERNARDI, G. (1971) in The Enzymes (BOYER, P. D., ed) 3rd Ed, pp. 329-336, Academic Press, New York.
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