Hydrolysis of Phosphonate Esters Catalyzed by 5′-Nucleotide Phosphodiesterase†

Susan J. Kelly, David E. Dardinger, and Larry G. Butler*

ABSTRACT: 4-Nitrophenyl and 2-naphthyl monoesters of phenylphosphonic acid have been synthesized, and an enzyme catalyzing their hydrolysis was resolved from alkaline phosphatase of a commercial calf intestinal alkaline phosphatase preparation by extensive ion-exchange chromatography, chromatography on L-phenylalanyl-Sepharose with a decreasing gradient of $(NH_4)_2SO_4$, and gel filtration. Detergent-solubilized enzyme from fresh bovine intestine was purified after $(NH_4)_2SO_4$ fractionation by the same technique. The purified enzyme is homogeneous by polyacrylamide gel electrophoresis and sedimentation equilibrium centrifugation. It has a molecular weight of 108,000, contains approximately 21% carbohydrate, and has an amino acid composition considerably different from that reported from alkaline phosphatase from the same tissue. The homogeneous intestinal enzyme, an efficient catalyst of phosphonate ester hydrolysis but not of phosphate monoester hydrolysis, was identified as a 5′-nucleotide phosphodiesterase by its ability to hydrolyze 4-nitrophenyl esters of 5′-TMP but not of 3′-TMP. Also consistent with this identification was the ability of the enzyme to hydrolyze 5′-ATP to 5′-AMP and PP_i, NAD^+ to 5′-AMP and NMN, TpT to 5′-TMP and thymidine, pApApApA to 5′-AMP, and only the single-stranded portion of tRNA from the 3′-OH end. Snake venom 5′-nucleotide phosphodiesterase also hydrolyzes phosphonate esters, but 3′-nucleotide phosphodiesterase of spleen and cyclic 3′,5′-AMP phosphodiesterase do not. Thus, types of phosphodiesterases can be conveniently distinguished by their ability to hydrolyze phosphonate esters. As substrates for 5′-nucleotide phosphodiesterases, phosphonate esters are preferable to the more conventional esters of nucleotides and bis(4-nitrophenyl) phosphate because of their superior stability and ease of synthesis. Furthermore, the rate of hydrolysis of phosphonate esters under saturating conditions is greater than that of the conventional substrates. At substrate concentrations of 1 m*M* the rates of hydrolysis of phosphonate esters and of nucleotide esters are comparable and both superior to that of bis(4-nitrophenyl) phosphate.

We have shown (Kelly and Butler, 1975; Kelly et al., 1975) that an enzymic activity capable of hydrolyzing monoesters of phosphonic acids[1] is broadly distributed throughout nature. Several properties of this enzyme obtained from calf intestine resemble those of intestinal alkaline phosphatase, raising the possibility that both activities are properties of the same protein. In this paper we describe the resolution of phosphonate esterase activity from alkaline phosphatase, the purification to homogeneity, from the commercial preparations as well as from fresh bovine intestine, of the enzyme responsible for phosphonate esterase activity, and its identification as an exonuclease capable of hydrolyzing single-stranded nucleic acids to nucleoside 5′-phosphates (5′-nucleotide phosphodiesterase).

Materials and Methods

Triton X-100 (Surfactol-100) was obtained from Huron Chemicals. Ammonium sulfate, "Ultra Pure" grade, was obtained from the Schwarz/Mann Division of Becton, Dickinson and Company. 2-Naphthyl phosphate, 4-nitrophenylthymidine 3′-monophosphate (4-Nph-3-TMP)[2] 5′-AMP, mixed 3′(2′)-AMP, NAD^+, and adenosine 3′,5′-cyclic phosphoric acid were obtained from Sigma Chemical Company. 4-Nitrophenylthymidine 5′-monophosphate was obtained from Boehringer Mannheim Corporation. 4-Nitrophenyl phenylphosphonate was synthesized by two slightly different methods. (A) Phenylphosphonic dichloride (Aldrich Chemical Company), 30 ml (212 mmol), was reacted with 10 g (72 mmol) of 4-nitrophenol in dry pyridine for 30 min at room temperature. After hydrolysis of the remaining chlorides with water the phosphonate ester was extracted into benzene, evaporated to dryness under reduced pressure, recrystallized from boiling water, and lyophilized to dryness. Yield was 20%. Anal. Calcd for $C_{12}H_{10}NO_5P$: C, 51.62; H, 3.61; N, 5.01; P, 11.09; FW, 279.1. Found: C, 51.42; H, 3.62; N, 4.91; P, 11.00; FW, 280. (B) The synthesis was also carried out with dichloride (10 ml) and 4-nitrophenol (10 g) in 1:1 ratio. Extraction of the ester into benzene was promoted by the addition of concentrated HCl to the water layer. The benzene was removed under reduced pressure and the dry product taken up in a minimum amount of acetone. Concentrated NH_4OH was added in an amount equivalent to 100% theoretical yield of ester. The resultant ammonium salt of 4-nitrophenyl phenylphosphonate was filtered, washed with fresh acetone, and dried in a

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[1] As used here, "phosphonate esterase" refers solely to hydrolysis of monoesters, not diesters, of phosphonic acids.

[2] Abbreviations used are: 4-Nph-3-TMP, the 4-nitrophenyl ester of thymidine 3′-monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; 4-Nph-5′-TMP, the 4-nitrophenyl ester of thymidine 5′-monophosphate.

Table I: Purification of Phosphonate Esterase.

	Protein Total (mg)	Phosphonate Esterase				Alkaline Phosphatase
		Total Activity[a] (units)	Specific Activity (units mg^{-1})	Purification	Yield (%)	Total Activity[a] (units)
Commercial phosphatase	2000	710	0.36	1.0	100	1834
First DEAE-cellulose column	69	214	3.10	8.6	30	50.8
Second DEAE-cellulose column	17.4	100.5	12.10	33.6	14	2.4
Phenylalanyl-Sepharose	2.4	80.4	33.5	94.4	11	1.0
Gel filtration	1.2	59.2	47.7	134.4	8	0.2

[a] Determined under standard conditions.

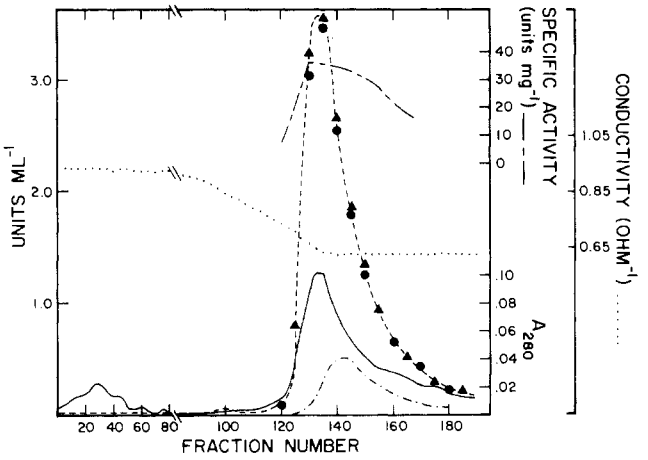

FIGURE 1: Chromatography on L-phenylalanyl-Sepharose. Fractions from the 2nd DEAE-cellulose column were concentrated by ultrafiltration to a volume of 100 ml, adjusted to 1.5 *M* $(NH_4)_2SO_4$, and applied to a 1.0 × 32 cm column of L-phenylalanyl-Sepharose which had been equilibrated with 1.5 *M* $(NH_4)_2SO_4$ in buffer, then eluted with a 200-ml linear gradient of $(NH_4)_2SO_4$ down to 0.65 *M* in the same buffer. Fractions of 3.2 ml were collected and assayed under standard conditions for protein (—), phosphonate esterase activity (●- -●), alkaline phosphatase (graphed × 10, - · - · -) and phosphodiesterase (graphed × 16, ▲- -▲). Conductivity (···) and specific activity (- - -) are also shown.

vacuum desiccator. 2-Naphthyl phenylphosphonate and its ammonium salt were prepared by procedures analogous to the preparation of 4-nitrophenyl phenylphosphonate.[3] Bis(4-nitrophenyl) phosphate (Aldrich Chemical Company) was purified by hydrolysis of contaminating 4-nitrophenyl phosphate (<1%) catalyzed by purified *Escherichia coli* alkaline phosphatase, extraction of the remaining diester into benzene, and isolation as the ammonium salt.

Calf intestinal alkaline phosphatase (Lot PA-2) was from P-L Biochemicals; bovine intestines were obtained fresh from a local slaughterhouse. Bovine spleen 3′-nucleotide phosphodiesterase obtained from Worthington Biochemical Corporation, further purified according to Sulkowski and Laskowski (1971), was the gift of Dr. P. T. Gilham. Beef heart 3′,5′-cyclic nucleotide phosphodiesterase from Sigma Chemical Company was obtained through the generosity of Dr. W. C. Lake.

Standard assays of phosphonate esterase, alkaline phosphatase, and phosphodiesterase were carried out spectrophotometrically at 30°C using a 1 m*M* solution of 4-nitrophenyl phenylphosphonate, 4-nitrophenyl phosphate, and bis(4-nitrophenyl) phosphate, respectively, in 0.1 *M* Tris, pH 8 (phosphonate esterase) or pH 9 (others) (Kelly and Butler, 1975). One unit of enzymic activity is defined as the amount of enzyme which will catalyze the production of 1 μmol of product per min at 30°C under standard conditions. Protein concentration was determined by absorption at 280 nm; using the Lowry et al. (1951) method and bovine serum albumin as standard, a value of 0.98 was determined for A_{280}(0.1%) for the purified bovine intestinal enzyme.

Electrophoresis was carried out in 7% acrylamide gels at pH 8.3 according to the procedure of Davis (1964) omitting the use of a stacking gel. Phosphonate esterase activity was located after incubation of the gel in standard buffer containing 1 m*M* 2-naphthyl phenylphosphonate and 0.1% Fast Garnet GBC Salt (Sigma) by the appearance of a red precipitant band due to the adduct of the freed 2-naphthol with Fast Garnet (*o*-aminoazotoluene diazonium salt). Alkaline phosphatase activity was detected either by a similar assay substituting 1 m*M* 2-naphthyl phosphate for substrate (Menten et al., 1944), or with the standard assay, by precipitating the liberated phosphate as triethylammonium phosphomolybdate according to the procedure of Tono and Kornberg (1967) as modified by Bennett et al. (1973). Proteins were detected by the method of Malik and Berrie (1972). Carbohydrate was detected by staining with fuchsin after periodate oxidation (Zacharius et al., 1969).

Results

Purification of Phosphonate Esterase Activity from Commercial Preparations of Calf Intestinal Alkaline Phosphatase. Partially purified calf intestinal alkaline phosphatase was chromatographed on DEAE-cellulose in 0.03 *M* Tris (pH 7.6) and eluted by increasing the buffer concentration to 0.05 *M*, and then with a linear gradient of NaCl from 0 to 0.6 *M* in the 0.05 *M* buffer. The 0.05 *M* buffer eluted over half of the phosphonate esterase activity and only about 10% of the alkaline phosphatase activity; the remainder of both activities was eluted by the NaCl gradient. After concentration, the phosphonate esterase activity eluted by 0.05 *M* buffer was rechromatographed on DEAE-cellulose, with a linear gradient of Tris (pH 7.6) from 0.04 to 0.06 *M*. The first half of the peak of phosphonate esterase activity was very low in alkaline phosphatase activity; this material was subjected to chromatography on L-phenylalanyl-Sepharose (a gift of Dr. G. J. Doellgast) in buffer containing 1.5 *M* $(NH_4)_2SO_4$, with elution by a linear *decreasing* gradient of $(NH_4)_2SO_4$ from 1.5 to 0.6 *M* (Doell-

[3] Phosphonate esters are available from Regis Chemical Company, Morton Grove, Ill.

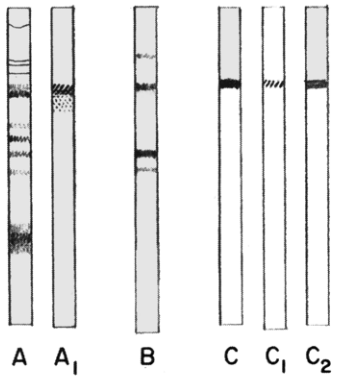

FIGURE 2: Polyacrylamide gel electrophoresis. (A) Unfractionated intestinal preparation; (B) the effluent from the first DEAE column; and (C) the effluent from the Sephadex G-150 column. No subscript indicates gels stained for protein. Subscript 1 indicates gels stained for activity: Phosphonate esterase, hatch marks, and alkaline phosphatase, dotted pattern. Subscript 2 indicates gels stained for carbohydrate. Approximate quantities of protein applied are: A (200 μg); A_1 (20 μg); B (10 μg); C (50 μg); C_1 (1 μg); C_2 (50 μg).

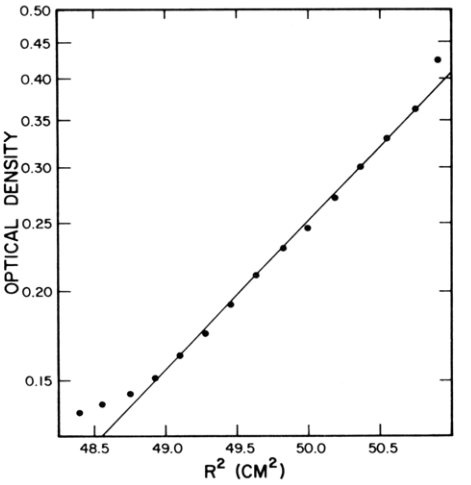

FIGURE 3: Homogeneity of phosphonate esterase on sedimentation equilibrium. The enzyme solution (0.5 mg/ml) was dialyzed against 0.05 *M* Tris (pH 7.6) containing 0.1 *M* KCl and centrifuged at 9000 rpm for 24 hr at 294.4 K in a Spinco Model E ultracentrifuge equipped with absorption optics.

gast and Fishman, 1974). As shown in Figure 1, the phosphonate esterase activity elutes in a pattern slightly different from the small amount of remaining alkaline phosphatase. Similar purification can be obtained on columns of cellulose containing nonspecific hydrophobic substituents such as phenoxyacetyl (Dardinger, 1974) and lauryl[4] groups, suggesting that although the enzyme is specifically inhibited by L-phenylalanine (Kelly and Butler, 1975), purification is due to differences in nonspecific affinity for hydrophobic materials; a similar technique has been reported by Rimerman and Hatfield (1973).

After concentration by ultrafiltration, the enzyme was subjected to gel filtration on Sephadex G-150 in 0.05 *M* Tris (pH 7.6); this procedure removes a lower molecular weight inactive protein. At this stage the protein catalyzing the hydrolysis of phosphonate esters was found to be essentially homogeneous (see below); its specific activity was 50 $\pm$ 5 units mg^{-1}, measured with 1 m*M* 4-nitrophenyl phenylphosphonate in 0.1 *M* Tris (pH 8.0). The enzyme is stable for months in the refrigerator in 0.05 *M* Tris (pH 7.6). A summary of the purification procedure is presented in Table I.

Purification from Fresh Bovine Intestine. No significant differences in the enzyme were observed in preparations obtained from the intestine of animals of different age, breed, and sex. Large amounts of activity are present in the intestinal contents, as well as the mucosa. The enzyme is largely particulate; Triton X-100 more effectively solubilizes it than does solvent extraction. At the slaughterhouse approximately 60 ft of fresh small intestine was cut away from the surrounding tissue and rapidly transported at room temperature to the laboratory. The intestine was divided into approximately 10-ft sections, the contents were expressed and combined with an equal volume of 0.1 *M* Tris buffer (pH 7.6) containing 0.1% Triton X-100, and approximately 500 ml of the same buffered detergent was added to each section of intestine. After several hours at room temperature, the extract was removed from the intestine, combined with the buffered intestinal contents, and centrifuged 30 min at 25,000*g*.

To the supernatant layer was added solid $(NH_4)_2SO_4$ to a concentration of 35% saturation (Dawson et al., 1969). After the mixture was stirred for 3 hr at 4°C the material was centrifuged for 30 min at 30,000*g*; the pellet was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 70% saturation. After stirring for 3 hr the suspension was centrifuged for 30 min at 30,000*g*; under these conditions the insoluble protein sediments upward. The floating layer of protein was removed, dissolved in a minimum volume of 0.01 *M* Tris (pH 7.6), and dialyzed against water and against five changes of the above buffer (5 l. each time). From this point onward the purification paralleled that described above for the enzyme from commercial preparations of calf intestinal alkaline phosphatase. It was usually necessary for the enzyme solution to be further diluted before it would bind to the first DEAE-cellulose column.

Evidence for Homogeneity. Figure 2 represents polyacrylamide gel electrophoretic patterns of protein and enzymatic activity at different stages in the purification. The phosphonate esterase activity routinely migrated slightly slower (R_f 0.22–0.23) than the alkaline phosphatase activity (R_f 0.24–0.26). The nonidentity of the electrophoretic mobilities of the two activities was demonstrated by assaying for both activities on the same gel. The final product migrates as a single protein band in both the absence (Figure 2C) and presence (not shown) of sodium dodecyl sulfate. The phosphonate esterase activity coincides with the protein band. In some preparations only the first half of the protein and activity peak from the last step was homogeneous, due to incomplete resolution from an inactive lower molecular weight protein. Physicochemical characterization was attempted only on those fractions which were found to be homogeneous on polyacrylamide gel electrophoresis. On sedimentation equilibrium ultracentrifugation (Figure 3), the linearity of the plot of concentration vs. r^2 indicated the presence of only one major protein component.

Judging from activity measurements extrapolated to maximum velocity conditions and corrected for differences in turnover number, contamination of the purified enzyme by alkaline phosphatase could amount to no more than 0.05% of the protein present.

Physicochemical Properties of the Purified Enzyme. The

[4] D. Dardinger and L. Butler, unpublished observations.

Table II: Amino Acid Composition.[a]

Amino Acid	Bovine Phosphonate Monoesterase		Calf Intestinal Alkaline Phosphatase	Human Placental Alkaline Phosphatase
Lysine	51[b]	61[c]	42[c,d]	44[c,e]
Histidine	27	32	26	28
Arginine	34	41	82	59
Aspartic acid	88	105	102	99
Threonine	50	59	60	62
Serine	40	49	38	47
Glutamic acid	83	99	82	101
Proline	54	64	49	51
Glycine	35	41	72	90
Alanine	30	36	96	111
Half-cystine[f]	6	7	8	13
Valine	44	52	70	63
Methionine	13	17	21	22
Isoleucine	27	32	24	34
Leucine	60	72	59	79
Tyrosine	49	58	33	33
Phenylalanine	55	66	25	35
Tryptophan[g]	ND	ND	10	2

[a] Specific activity of the sample was 52.4 units/mg. Analysis was carried out on a Beckmann Model 120C amino acid analyzer on samples hydrolyzed for 24, 48, and 72 hr in a 200-fold excess of HCl under a N_2 atmosphere. Averages were taken for the abundances of the amino acids as determined in the three runs except where a decrease in abundance of the amino acid with time was observed, in which case the data from the 24-hr run was used or a zero time value was extrapolated. [b] As moles per 83,920 g, the protein molecular weight of the enzyme. [c] As moles per 100,000 g. [d] From Fosset et al. (1974). [e] From Harkness (1968). [f] As cysteic acid. [g] Determined spectrophotometrically; ND, not determined.

Table III: Kinetic Parameters for Hydrolysis of Nucleotide Esters and Related Compounds by the Purified Intestinal Enzyme.[a]

Compound	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)
4-Nitrophenyl phenylphosphonate	744	11.1
Bis(4-nitrophenyl) phosphate	24	0.85
4-Nph-5′-TMP	270	0.043
4-Nph-3′-TMP	1	
NAD^+	10.6	0.03
5′-ADP	6.4	
5′-ATP	4.6	$\leq$0.2
3′,5′-cAMP	7.0	<0.1

[a] Production of 4-nitrophenol was measured continuously at 400 nm. Hydrolysis of NAD^+ and 3′,5′-cAMP was coupled with *E. coli* alkaline phosphatase (Sperow and Butler, 1971); hydrolysis of 5′-ATP was coupled with yeast inorganic pyrophosphatase (Ridlington et al., 1972). For these substrates the inorganic phosphate produced was measured by a modification (Simmons and Butler, 1968) of the method of Fiske and SubbaRow (1925). The assay conditions were 0.1 M Tris-acetate (pH 8.0), 30°C. The K_m values for 5′-ATP and 3′,5′-cAMP were obtained from measurements at only two substrate concentrations and represent an upper limit.

molecular weight as determined by sedimentation equilibrium (Figure 3) is 108,000. This is in good agreement with the value of 107,000 obtained by gel filtration (Kelly and Butler, 1975). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate yielded an apparent subunit molecular weight of 90,000; this value is anomalously high (Segrest et al., 1971) due to the glycoprotein nature of the enzyme (see below). Thus the enzyme likely consists of at least two subunits, which must be identical or of similar molecular weight because only a single protein band is observed on polyacrylamide gel electrophoresis in the presence of dodecyl sulfate.

The amino acid analysis is summarized in Table II and compared with the composition of human placental alkaline phosphatase (Harkness, 1968), and calf intestinal alkaline phosphatase (Fosset et al., 1974). The data for protein having phosphatase esterase activity is expressed both as moles of amino acid per mole of protein, using 83,920 as the molecular weight of the protein portion of the enzyme (see section on carbohydrate content below), and as moles per 100,000 protein as are the other two enzymes. While the amino acid composition of the two alkaline phosphatases are similar, that of the phosphonate esterase enzyme has about half as much glycine and alanine and twice as much tyrosine and phenylalanine as the other two enzymes.

The partial specific volume of the enzyme was calculated from the analysis for amino acids (McMeekin and Marshall, 1952) and carbohydrate (see next section) (Robbins et al., 1965). The value obtained, 0.716 ml/g, was used to determine the molecular weight in sedimentation equilibrium experiments (see preceding section).

The glycoprotein nature of the enzyme was established by the close correlation between catalytic activity and carbohydrate content, as measured by the anthrone reaction (Spiro, 1966) in the fractions eluted from the L-phenylalanyl-Sepharose column (Figure 1, data not shown), and by the coincidence of a strong fuchsin-positive band with the single band of protein and catalytic activity on polyacrylamide gel electrophoresis of the homogeneous enzyme preparation (Figure 2). According to the anthrone assay, the purified enzyme contained 21% total carbohydrate, as determined by comparison to a sucrose standard.

Identification as 5′-Nucleotide Phosphodiesterase. Hydrolytic activity toward 4-nitrophenyl phenylphosphonate coincided with activity toward bis(4-nitrophenyl) phosphate, a nonspecific phosphodiesterase substrate, in eluates from columns of DEAE-cellulose (not shown) and L-phenylalanyl-Sepharose (Figure 1), suggesting that a single enzyme is responsible for both activities. We therefore compared the kinetic parameters of the purified enzyme toward both these substrates and toward two specific artificial phosphodiesterase substrates, the 4-nitrophenyl esters of thymidine 5′-monophosphate (5′-TMP) and of thymidine 3′-monophosphate (3′-TMP). The ester of 5′-TMP, but not of 3′-TMP, was cleaved at a significant rate (Table III).

Further investigations revealed that the enzyme also catalyzes the hydrolysis of ATP to AMP and PP_i, of ADP to AMP and P_i, of NAD^+ to AMP and NMN, and 3′,5′-cAMP to AMP. Moreover, 5′-AMP, but not 3′-AMP, is an excellent competitive inhibitor of the hydrolysis of 4-nitrophenyl phenylphosphonate (K_i = 1.2 μM). The K_m value for 4-nitrophenyl phenylphosphonate is considerably higher than for nucleotide substrates. The enzyme hydrolyzes the dinucleotide, thymidylylthymidine 5′-monophosphate, to thymidine and 5′-TMP, and tetrapoly(A) (pApApApA) to 5′-AMP; it cleaves only the 3′-OH single-stranded portion of tRNA and has no endonucleolytic activity (P. T. Gilham, personal communication). The enzyme hydrolyzes single-stranded regions of either deoxyribo- or ribonucleic acids from the 3′-terminus to produce 5′-nucleotides. The specificity of the bovine intestinal enzyme is thus similar to that of snake venom 5′-nucleotide phosphodiesterase (Dolapchiev, 1970). We will employ this name for the enzyme, rather than the alternative type I phosphodiesterase suggested by Razzell (1967).

Table IV: Comparison of the Purified Intestinal Phosphodiesterase with Several Purified Phosphodiesterases for Hydrolytic Activity toward Phosphodiesterase Substrates.

Type of Enzyme	pH	Activity (nmol min^{-1} mg^{-1})[a]							
		4-Nph-3′-TMP		4-Nph-5′-TMP		Bis(4-nitrophenyl) Phosphate		4-Nitrophenyl Phenylphosphonate	
(Presence of 2 m*M* Mg^{2+}; EDTA absent throughout)		–	+	–	+	–	+	–	+
5′-Nucleotide phosphodiesterase (Bovine intestine)	8.0	569	452	65700	70700	2380	2450	38900	34000
(Snake venom)	8.0	1.9	2.7	4570	6150	70.9	82.3	1230	4180
3′-Nucleotide phosphodiesterase (Bovine spleen)	8.0	168	182	6.9	3.9	0.8	0.8	1.0	1.5
cAMP phosphodiesterase (Bovine heart)	8.0	0.8	0.2	1.1	0.9	0.3	0.1	1.1	0.9
(Presence of 2 m*M* EDTA; Mg^{2+} absent throughout)		–	+	–	+	–	+	–	+
5′-Nucleotide phosphodiesterase (Bovine intestine)	5.5	415	46.1	9160	273	2880	34.6	18900	23
(Snake venom)	5.5	0.3	0.4	232	1.8	4.3	0.2	342	6
3′-Nucleotide phosphodiesterase (Bovine spleen)	5.5	256	256	6.5	6.9	1.5	1.5	1.0	1.1
cAMP phosphodiesterase (Bovine heart)	5.5	1.9	0.3	0.4	0.3	0.2	0.2	0.2	0.1

[a] Determined at substrate concentrations of 1 m*M*.

Phosphonate Esterase Activity of Several Purified Phosphodiesterases. To determine if the ability to hydrolyze phosphonate esters is a general property of phosphodiesterases, commercial preparations of 5′-nucleotide, 3′-nucleotide, and cAMP phosphodiesterases were assayed with artificial phosphodiesterase substrates as well as 4-nitrophenyl phenylphosphonate; the results are shown and compared in Table IV to those for the purified bovine intestinal phosphodiesterase.

Both 5′-nucleotide phosphodiesterases hydrolyze 4-nitrophenyl phenylphosphonate at a rate comparable to the rate at which they hydrolyze 4-nitrophenyl 5′-TMP, and much greater than the rate of hydrolysis of bis(4-nitrophenyl) phosphate. 3′-Nucleotide phosphodiesterase does not hydrolyze 4-nitrophenyl phenylphosphonate, 4-Nph-5′-TMP, or bis(4-nitrophenyl) phosphate at an appreciable rate, but does strongly hydrolyze 4-Nph-3′-TMP, as expected. cAMP phosphodiesterase hydrolyzed 3′,5′-cAMP at a rate of 75 nmol min^{-1} mg^{-1}, but it has little activity against any of the substrates tested here. It thus appears that phosphonate esterase activity is a characteristic of 5′-nucleotide phosphodiesterase enzymes, but not of other types of phosphodiesterases.

The ratio of activities of 5′-nucleotide phosphodiesterases against 4-nitrophenyl phenylphosphonate and 4-Nph-5′-TMP differ somewhat for the two enzymes, and in the various conditions for the same enzyme. This is probably at least partially because the concentration of the 4-nitrophenyl phenylphosphonate employed was well below its K_m value (Table III), which may vary with the conditions. Both enzymes are little affected by Mg^{2+} at pH 8 and are more effective at pH 8 than pH 5.5; the intestinal enzyme is less affected by lowering the pH. Both are severely inhibited by EDTA, which has been shown to inactivate the intestinal enzyme (Kelly and Butler, 1975).

Discussion

Aromatic esters of phenylphosphonic acid, organophosphorus compounds having no naturally occurring isosteric phosphate analog, are excellent substrates for 5′-nucleotide phosphodiesterases. This paper confirms the suggestion (Kelly and Butler, 1975) that the enzyme catalyzing this reaction is neither limited to phosphonate metabolism nor due to an alkaline phosphatase. Moreover, only the 5′-nucleotide phosphodiesterases, but not 3′-nucleotide phosphodiesterase nor cAMP phosphodiesterase, are capable of hydrolyzing these compounds. This difference in substrate specificity toward phosphonate esters may be due to differences in the catalytic mechanism of the phosphodiesterases.

Phosphonate ester hydrolysis provides a convenient and inexpensive method of assaying 5′-nucleotide phosphodiesterases and distinguishing them from enzymes of different specificity. Although the K_m for 4-nitrophenyl phenylphosphonate is relatively high (11 m*M*) when compared with that of phosphodiesters (<1 m*M*), the V_{max} is greater (744 μmol min^{-1} mg^{-1}) than that of 4-Nph-5′-TMP (270 μmol min^{-1} mg^{-1}) and far greater than that for bis(4-nitrophenyl) phosphate (24 μmol min^{-1} mg^{-1}). Phosphonate esters are much easier to prepare[5] than are esters of 5′-TNP. Unlike assays using bis(4-nitrophenyl) phosphate, 5′-nucleotide phosphodiesterase assays using phosphonate esters are free of interference by alkaline phosphatase, which has no activity toward the phosphonate product. The readily prepared 2-naphthyl esters of phosphonates provide a convenient means of assaying 5′-nucleotide phosphodiesterase activity on polyacrylamide gels. These chromogenic phosphonate esters thus provide several advantages over conventional substrates for assay of 5′-nucleotide phosphodiesterase, which may be useful in the clinical diagnosis of hepatoma (Tsou et al., 1973).

Although 5′-nucleotide phosphodiesterase and alkaline phosphatase are both present in large amounts in particulate form in intestinal mucosa and share some properties (Kelly and Butler, 1975), they are separable (Hynie and Zbarsky, 1970) and differ in several other respects, notably amino acid composition. From the data of Table II it can be estimated that 5′-nucleotide phosphodiesterase of bovine in-

[5] Purdum, W. R., Berlin, K. D., Kelly, S. J., and Butler, L. G., manuscript in preparation.

testine differs from alkaline phosphatase from the same source by at least 50 amino acid residues per molecule; recent divergence from a common ancestor enzyme seems unlikely.

As much as 1.5 g of 5′-nucleotide phosphodiesterase is present in the unfractionated contents of one bovine intestine. With the yield reported here, approximately 120 mg of homogeneous enzyme can be isolated from a single animal and more can be recovered by working up rejected fractions. Because of its general availability, bovine intestine is an excellent source of this enzyme, and with the proper care to remove alkaline phosphatase, should be a better choice than snake venom for large-scale preparations.

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