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THE ENZYMIC CLEAVAGE OF THE CARBON–PHOSPHORUS BOND: PURIFICATION AND PROPERTIES OF PHOSPHONATASE

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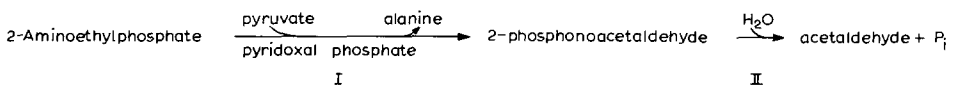
SUMMARY

1. *Bacillus cereus* degrades 2-aminoethylphosphonate by the following pathway:



2. Reaction II above involves the enzymic cleavage of the carbon–phosphorus bond and has not been described previously. The enzyme responsible for this reaction has now been purified and studied; we have suggested that it be named "2-phosphonoacetaldehyde phosphonohydrolase", and that the trivial name be "phosphonatase".

3. Phosphonatase shows optimal activity between pH 8 and 9, and has a K_m for 2-phosphonoacetaldehyde of 40 μM. Apart from 2-phosphonoacetaldehyde, phosphonatase shows virtually no activity with any of a number of phosphonates and phosphate esters tested.

4. The active enzyme is composed of two similar subunits, each of an approximate molecular weight of 33 000–37 000. Mg^{2+} is required for activity, but this probably helps to maintain the enzyme in its aggregated state rather than play a catalytic role at the active site; no other metals have been shown to be necessary for activity.

5. Phosphonatase is inhibited by CN^-, S^{2-} and SO_3^{2-}, which suggests that disulphide bridges may be necessary in maintaining the tertiary structure of the active form of the enzyme.

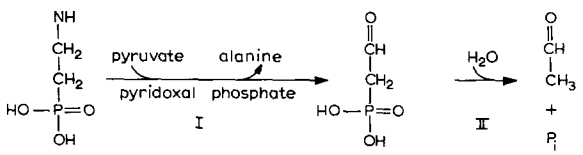
6. Orthophosphite, which has been shown previously to abolish the growth of *B. cereus* on 2-aminoethylphosphonate, inhibits the activity of phosphonatase, but only in the presence of 2-phosphonoacetaldehyde or acetaldehyde. Experiments with [^{32}P]orthophosphite showed that, although the inhibitor was bound tightly to phosphonatase, it did not form a covalent bond with the enzyme.

Abbreviations: TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethane sulphonic acid. Ammediol, 2-amino-2-methyl-1,3-propanediol.

INTRODUCTION

During the last few years, it has become apparent that phosphonates (compounds which possess a carbon–phosphorus bond) occur commonly amongst lower animals, chiefly in the form of 2-aminoethylphosphonate and its derivatives[1–3]. Recently, workers in several laboratories have isolated strains of bacteria which are able to use a variety of phosphonates as sources of phosphorus[3–8]. Since 2-aminoethylphosphonate is extremely resistant to chemical hydrolysis[9,10] and higher animals and plants appear to be unable to cleave the carbon–phosphorus bond[11–15,63], these bacteria perform an important role in the phosphorus cycle in nature.

In this laboratory, we have isolated a strain of *B. cereus* which is able to use 2-aminoethylphosphonate as a sole source of phosphorus. This organism takes up 2-aminoethylphosphonate from the surrounding medium by means of an inducible transport system with a high affinity for 2-aminoethylphosphonate[7]. We have shown[16] that in cell-free extracts prepared from a mutant strain of this organism 2-aminoethylphosphonate is degraded by the following pathway:



2-Aminoethylphosphate 2-Phosphonoacetaldehyde

In this paper we describe the purification and properties of the enzyme which catalyses Reaction II above. Since an enzyme able to cleave the carbon–phosphorus bond has not been reported before, we have suggested "2-phosphonoacetaldehyde phosphonohydrolase" as the systematic name for this enzyme. In the present work we shall refer to the enzyme by the trivial name of "phosphonatase".

MATERIALS AND METHODS

Chemicals

2-Phosphonoacetaldehyde was prepared from 2-acetoxy-2-chlorethylphosphonyldichloride[17]; this, together with 2-aminoethylphosphonate and aminomethylphosphonate were provided by Professor A. F. Isbell of the Texas A and M University. 2-Hydroxy-5-nitrobenzylphosphonate was a gift from Dr. D. R. Trentham of the University of Bristol, and phosphonomycin (1,2-epoxypropylphosphonic acid)[18,19] was a gift from Dr. C. W. Mushett of the Merck Sharp and Dohme Research Laboratories.

Acrylamide, *N,N'*-methylene-bis-acrylamide and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Organic Chemicals. *N*-[Tris(hydroxymethyl)methyl]-2-aminoethane sulphonic acid (TES), *p*-nitrophenylphosphate, protamine sulphate, dithiothreitol, and yeast alcohol dehydrogenase were obtained from Calbiochem. Tris, ovalbumin (Grade V), bovine serum albumin (Fraction V), α-amylase (*Bacillus subtilis*, Type IIA), alkaline phosphatase (calf intestine and *Escherichia coli*, Type III-S) and lysozyme (egg white, Grade I) were obtained from

Sigma. Deoxyribonuclease was from Nutritional Biochemicals Co. Soybean trypsin inhibitor and myoglobin (twice crystallized) were from Mann Research Laboratories. Pepsin (twice crystallized), ribonuclease and trypsin (twice crystallized) were obtained from Worthington; trypsin used for protein digestion was treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone[20] to destroy chymotryptic activity.

All other chemicals were of analytical grade or of the highest purity available.

[^{32}P]Orthophosphite was prepared by neutron irradiation of orthophosphorous acid[21], performed by the Australian Atomic Energy Commission.

Microorganisms and cell-free preparations

Most of the work was carried out using a mutant strain (AI-2) of *B. cereus* which is "constitutive" for both the uptake of 2-aminoethylphosphonate and the enzyme (phosphonatase) which degrades 2-phosphonoacetaldehyde to P_i. Neither of the two functions requires induction by substrate, but both the rate of uptake of 2-aminoethylphosphonate and the specific activity of phosphonatase in cell-free preparations are increased if the cells are first starved of phosphorus. Hence, the following procedure was adopted for the preparation of cell-free extracts from the mutant: Cells were grown overnight at 30° in a medium (referred to as PPYG) which contained (w/v) : 2% Difco proteose peptone, 0.5% Difco yeast extract and 1% glucose. They were harvested at a cell density of $8 \cdot 10^8$/ml, re-suspended in a defined, phosphate-free medium (BXPG)[7], which was modified slightly to contain: 0.25% (w/v) of the amino acid–salt mixture[7], 0.5% (w/v) glucose and 50 mM *N*-ethylmorpholine–HCl buffer (pH 7.5). The cells were then shaken vigorously for 3 h, after which the suspension was centrifuged and the cells re-suspended in 4 vol. of cold 50 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ and 0.1 mM dithiothreitol (referred to as Tris–$MgCl_2$–dithiothreitol buffer). They were broken in a Sorvall Ribi Fractionator at 25 000 lb/inch^2; the homogenate was centrifuged at 4° for 15 min at 38 000 × *g* and the resulting supernatant retained (referred to as the crude cell-free extract).

The wild strain of *B. cereus* (W) was also used. The conditions of growth and the preparation of crude cell-free extracts from this organism were similar to those described above, but have been altered slightly (as described in RESULTS) to allow for the induction of the systems necessary for the catabolism of 2-aminoethylphosphonate.

Enzyme assays

The standard reaction mixture contained: 100 mM 2-amino-2-methyl-1,3-propanediol–HCl (Ammediol–HCl) buffer (pH 8.5), 5 mM $MgCl_2$, 1 mM 2-phosphonoacetaldehyde, and the enzyme preparation (equivalent to 3–5 μg of the purified enzyme per ml of reaction mixture). Incubations were carried out at 25°. The reaction was stopped by the addition of $HClO_4$ to 0.75 M and the P_i released was estimated by the method of HARRIS AND POPAT[22]. Deviations from the standard procedure are indicated in the text. For kinetic studies, the P_i released was estimated by the method of ITAYA AND UI[23]; the acetaldehyde released was estimated spectrophotometrically by reducing it to ethanol in the presence of excess NADH and alcohol dehydrogenase[24].

One unit of phosphonatase activity is defined as that amount of enzyme which releases 1 μmole of P_i or acetaldehyde from 2-phosphonoacetaldehyde in 1 min.

Protein fractionation procedures

Protein solutions were concentrated by ultrafiltration under a pressure of 3–7 atm of helium, using a Diaflo cell (Amicon Co.) with a UM-10 membrane (retention size 10 000 daltons). Unless otherwise stated, protein was estimated by the method of LOWRY *et al.*[25], using bovine serum albumin as a standard.

A linear gradient[26] of NaCl in Tris–$MgCl_2$–dithiothreitol buffer was applied to elute protein from DEAE-Sephadex columns, and the actual salt gradient obtained was determined by measuring the conductance of the effluent in comparison with a standard solution of NaCl in Tris–$MgCl_2$–dithiothreitol buffer.

Polyacrylamide gel electrophoresis

Gel electrophoresis was carried out as described by DAVIS[27] in the pH range 9–10, or as modified by ORR[28] in the pH range 7–8 using triethanolamine–TES–chloride buffer. In both cases, gels were polymerised with light using riboflavin (13.3 μM) as a catalyst. The stacking gel consisted of 2.5% acrylamide and 0.3% bisacrylamide. Electrophoresis was carried out at 4° and the voltage was maintained at 12.5 V/cm. When $MgCl_2$ (10 mM) was included in the gels, it was added only to the upper (cathode) buffer reservoir and to the gel itself.

Molecular weight determinations

The molecular weight of phosphonatase was determined by gel filtration[29,30] on Sephadex G-150, as well as by two distinct procedures using polyacrylamide gels. The first procedure was a combination of the methods of ZWAAN[31] and PARISH[32] using triethanolamine–TES–chloride buffer[28], and gel concentrations of 8 and 12%. The ratio of the distance migrated by each protein in the two gel concentrations was determined in three separate experiments and the average calculated; the logarithm of this ratio is directly proportional to the molecular weight of the protein[32]. The molecular weight of phosphonatase was also determined under dissociation conditions (in the presence of sodium dodecyl sulphate) by the method of SHAPIRO *et al.*[33] as modified by WEBER AND OSBORN[34].

Peptide mapping

(1) Tryptic digestion. Purified phosphonatase (8 mg) was oxidized with performic acid[35]. The oxidized protein was digested with trypsin (1% of the protein by weight) at 37° in 0.5% (w/v) NH_4HCO_3 (pH 8.0) for 4 h. The digest was lyophilized to remove the NH_4HCO_3, the residue was redissolved in 4 ml of water, and the solution adjusted to pH 4 with glacial acetic acid. After 15 min the heavy precipitate, or insoluble "core", which formed was removed by centrifugation; the precipitate was washed several times with dilute acetic acid (about pH 4), and the washings added to the soluble peptides.

(2) Electrophoresis and chromatography. Samples of the soluble peptides (about 2 mg) were electrophoresed[36] on Whatman 3 MM paper at 40 V/cm in formic acid–acetic acid buffer (pH 1.9)[37] for 45 min, or in pyridine–acetic acid buffer (pH 4.7)[38] for 1 h. A sample of the core peptides was run in 2% (w/v) $(NH_4)_2CO_3$ (pH 8.9) at 40 V/cm with a reference sample of the soluble peptides. The samples were chromatographed in the second dimension[39] using either *n*-butanol–acetic

acid–water (4 : 1 : 5, by vol., upper phase only)[40], or isoamyl alcohol–pyridine–water (35 : 35 : 30, by vol.)[41].

Autoradiographs were prepared as described previously[42].

(3) Specific stains. Each chromatogram was sprayed with ninhydrin reagent: 0.1% (w/v) ninhydrin, 0.2% (v/v) acetic acid and 0.2% (v/v) pyridine in acetone, and stored in the dark for 24 h to allow the colour to develop fully. Larger peptides, which stained poorly, were detected by chlorination followed by starch–iodide reagent[43]. Peptides containing tyrosine or histidine, and phosphorus-containing compounds were detected by specific reagents[44,45].

Amino acid analyses

The acid-hydrolysed protein samples were chromatographed[46] on a Beckman amino acid analyzer, Model 120B. The content of tryptophan in the samples was not estimated. The amino acid composition of the insoluble core was determined after acid hydrolysis of the appropriate material eluted from the chromatogram.

Metal analyses

The samples were digested[47] and the resulting solutions analysed by atomic absorption, using the instrument described by DAVID[48] against iron, cobalt, copper and zinc standards in water; the determination of these elements was free from interference.

RESULTS

Enzyme purification

In the early stages of this work, phosphonatase was found to lose activity, especially during fractionation on Sephadex G-150 columns, unless Mg^{2+} was present. Consequently, the buffer used throughout the purification procedure (referred to as Tris–$MgCl_2$–dithiothreitol buffer) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$* and 0.1 mM dithiotehritol. This resulted in good recoveries from columns and enabled enzyme preparations to be stored at −20° for several months with only a

TABLE I

PURIFICATION OF PHOSPHONATASE FROM A CRUDE, CELL-FREE EXTRACT FROM *B. cereus* (AI-2)

Purification step	*Vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Recovery (%)*	*Purification*
1. Crude extract	390	5080	350	0.07	100	1.0
2. Removal of nucleic acids	450	4220	345	0.08	98	1.1
3. $(NH_4)_2SO_4$ precipitation*	47	990	228	0.23	65	3.3
4. DEAE-Sephadex column	144	29	187	6.4	53	90
5. Sephadex G-150 column	42	12.7	178	13.9	51	200

* The fraction precipitating between 0.4 and 0.7 saturation was collected.

* Recent work had indicated that 10 mM $MgCl_2$ is more effective than 5 mM $MgCl_2$ in maintaining the activity of phosphonatase.

gradual fall in activity; however, solutions which were repeatedly frozen and thawed rapidly lost activity.

The purification of phosphonatase from the crude cell-free extract of the mutant AI-2 is shown in Table I. All operations were carried out at 4°. Most of the nucleic acids were removed from the crude extract by adding 0.2 vol. of a 0.5% (w/v) solution of protamine sulphate (Step No. 2). Any nucleic acids remaining in solution were digested by overnight incubation at 4° with ribonuclease and deoxyribonuclease (1 μg/ml each), and nucleotides so formed were removed in the subsequent $(NH_4)_2SO_4$ precipitation and dialysis which followed (Step No. 3). The dialysed solution, equilibrated in Tris–$MgCl_2$–dithiothreitol buffer containing 0.3 M NaCl, was applied to a column of DEAE-Sephadex (45 cm × 2.5 cm) also equilibrated with this buffer. The protein was eluted using a linear gradient (0.3–0.4 M) of NaCl (Step No. 4). The effluent fractions were assayed for enzyme activity and protein content. Phosphonatase absorbed strongly to DEAE-Sephadex and was eluted well towards the end of the protein profile (at about 0.36 M NaCl). The fractions containing the activity were pooled, concentrated to a volume of 5 ml, and applied to a column of Sephadex G-150 (96 cm × 3 cm) equilibrated in Tris–$MgCl_2$–dithiothreitol buffer (Step No. 5). The effluent fractions comprising the peak of activity were pooled, concentrated and stored at −20°.

Behaviour on polyacrylamide gels

Freshly isolated enzyme (from Step No. 5, Table I) migrated as a single band in polyacrylamide gels with the triethanolamine–TES–chloride buffer system (pH 7–8, Fig. 1). If the enzyme was kept at 4° for 24 h, subsequent electrophoresis

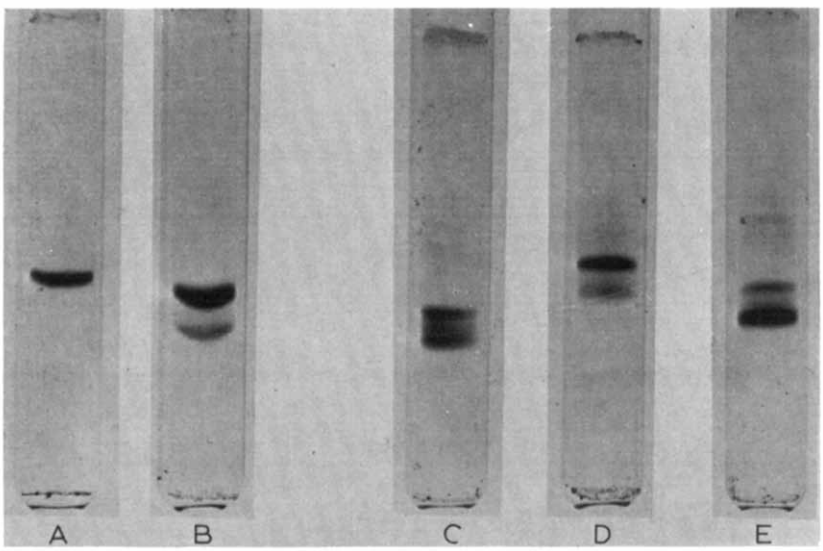


Fig. 1. Electrophoresis of phosphonatase in polyacrylamide gel. Purified phosphonatase (from Step 5, Table I) was electrophoresed on polyacrylamide gels, using either the triethanolamine–TES–chloride system (Tubes A and B) or the Tris–glycine system (Tubes C, D and E) as described in MATERIALS AND METHODS. A. Freshly purified enzyme. B. 24-h-old enzyme. C. 24-h-old enzyme. D. 24-h-old enzyme with 10 mM $MgCl_2$ included in the gel and upper reservoir buffer. E. Enzyme dialysed overnight against water.

showed that a second, faster-moving component had appeared; however, only the slower band possessed enzymic activity. When the enzyme was electrophoresed at the higher pH (pH 9–10), diffused protein appeared between the two bands. The relative intensity of the slow (active) band was increased when 10 mM $MgCl_2$ was included in the system, whereas that of the fast band was increased if enzyme, dialysed overnight at 4° against water, was used. These results indicated that the enzyme was probably composed of subunits and that Mg^{2+} was directly involved in their association.

The effect of Mg^{2+}

At 25°, the addition of 1 mM NaF (without Mg^{2+} added to the assay system) had no effect on the activity during the period of assay (10–15 min), indicating that the enzyme does not react with a Mg^{2+}–substrate complex. However, when phosphonatase was incubated in the absence of Mg^{2+} at 35° before the addition of substrate, there was a rapid loss in activity. Mg^{2+} protected the enzyme against this loss of

TABLE II

THE EFFECT OF VARIOUS COMPOUNDS ON THE RATE OF INACTIVATION OF PHOSPHONATASE AT 35°

Purified enzyme (5 μg/ml), in either 50 mM Ammediol–HCl buffer (pH 8.5) or in 50 mM KOH–TES buffer (pH 6.5), was incubated at 35° for 15 min in the presence of a number of compounds, but without substrate. The samples were cooled, 1 mM 2-phosphonoacetaldehyde added, and the activity assayed at 25° by measuring the release of P_i as described in MATERIALS AND METHODS.

Treatment	*Activity remaining (units/mg protein)*	
	Enzyme at pH 8.5	*Enzyme at pH 6.5*
Control:		
Untreated enzyme with 5 mM $MgCl_2$	3.40	0.91
Enzyme heated without $MgCl_2$	0.44	0.47
Enzyme heated with:		
0.75 mM $MgCl_2$	1.39	—
2 mM $MgCl_2$	2.00	—
5 mM $MgCl_2$	2.54	0.78
10 mM $MgCl_2$	2.83	—
Enzyme heated with:		
2 mM EDTA	0	—
2 mM $CaCl_2$	0.21	0.04
5 mM $MnCl_2$	—*	0.60
5 mM $ZnCl_2$	—*	0.06

* Testing was prevented by solubility difficulties.

activity, and the degree of protection was related to the concentration of Mg^{2+} present (Table II). Inactivation in the absence of Mg^{2+} was aggravated at higher pH, or when either EDTA or Ca^{2+} was also added. At pH 6.5, Mn^{2+} showed a slight protective effect, but Zn^{2+} and Ca^{2+} were antagonistic. As mentioned above, the dissociation of phosphonatase was greater in polyacrylamide gels run in the buffer system with the pH range of 9–10, than that of pH 7–8. A gradual restoration of activity of the heat-treated enzyme occurred following incubation with 5 mM Mg^{2+}. The concentration of the inactive component remaining (probably the dissociated

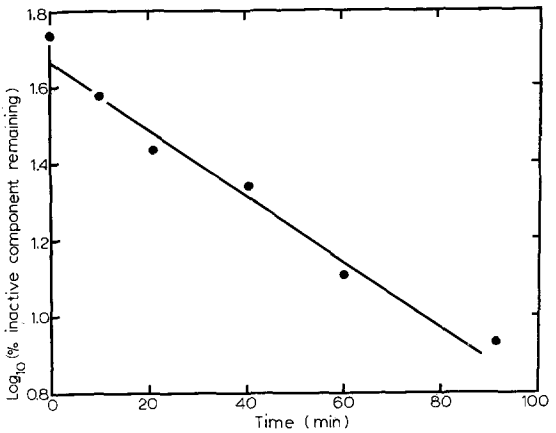


Fig. 2. Re-activation of heat-treated phosphonatase by incubation with $MgCl_2$. Purified enzyme (5 μg/ml) in 50 mM Ammediol–HCl buffer (pH 8.5) was incubated at 35° for 15 min. 5 mM $MgCl_2$ was then added, the temperature lowered to 25° and samples of the enzyme were withdrawn at times shown for assay. The amount of P_i released after 5 min was estimated as described in MATERIALS AND METHODS and the results plotted (by reference to a control heated in the presence of 5 mM $MgCl_2$) as the log 10 (% inactive component remaining) *vs.* time of preincubation with $MgCl_2$.

form of the enzyme) decreased exponentially with time (Fig. 2); 91% of the activity of a sample heated in the presence of 5 mM Mg^{2+} was recovered after 90 min.

Molecular weight determinations, peptide mapping and amino acid analyses

The molecular weight of the active form of phosphonatase was estimated to

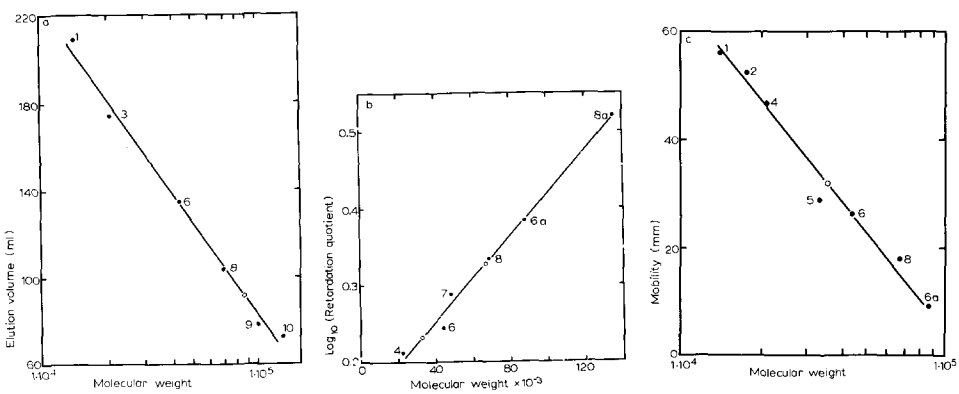


Fig. 3. Determination of the molecular weight of phosphonatase. The molecular weight of phosphonatase was estimated using (a) Sephadex G-150, (b) polyacrylamide gels run at two different gel concentrations, (c) polyacrylamide gels run in the presence of sodium dodecyl sulphate. Details of the methods are given in MATERIALS AND METHODS. The proteins used as standards (●) were as follows (the molecular weight of each protein is given in parentheses): 1, lysozyme (13 900)[49]; 2, myoglobin (17 200)[50]; 3, trypsin (20 700)[49]; 4, soybean trypsin inhibitor (21 000)[51]; 5, pepsin (35 000)[49]; 6, ovalbumin (44 000)[49], 6a, ovalbumin dimer; 7, α-amylase (48 000)[51]; 8, bovine serum albumin (69 000)[50], 8a, bovine serum albumin dimer; 9, alkaline phosphatase from calf intestine (100 000)[53]; 10, yeast alcohol dehydrogenase (129 000)[54]. The molecular weight of phosphonatase (○) was estimated to be (a) 83 000, (b) 68 000 for the active enzyme and 33 000 for the subunit, and (c) 35 000 for the subunit.

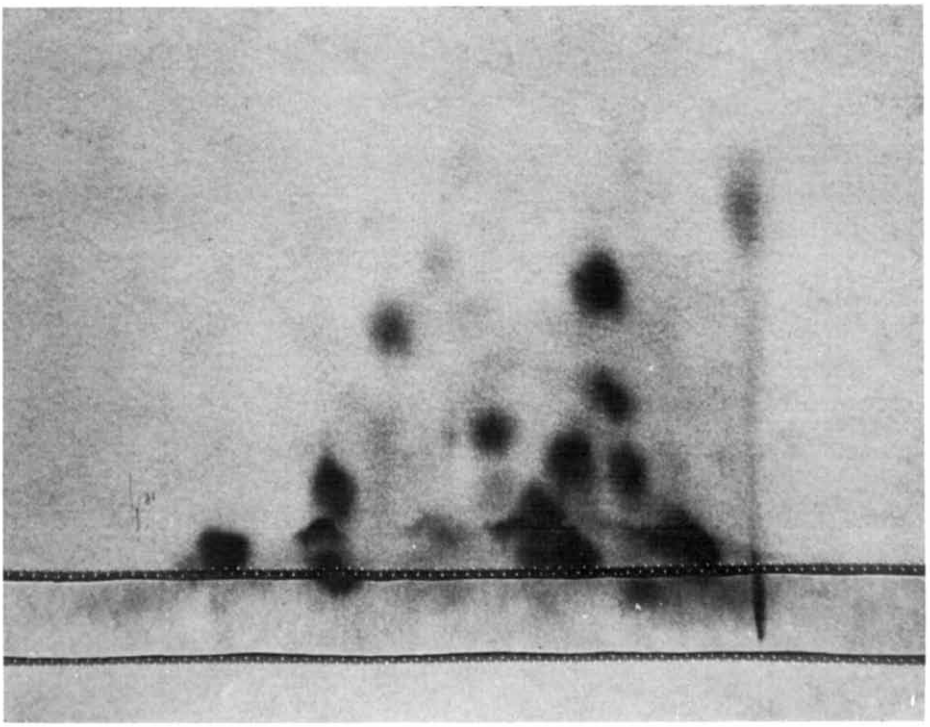


Fig. 4. Peptide map of the soluble fraction of a tryptic digest of phosphonatase. The digest was electrophoresed in the first dimension at pH 4.7 and then chromatographed in the second dimension in *n*-butanol–acetic acid–water as described in MATERIALS AND METHODS. The chromatogram, shown above, was then sprayed with ninhydrin.

be about 83 000 from studies using Sephadex G-150 (Fig. 3a), and about 68 000 from polyacrylamide gels (Fig. 3b). The difference in the values obtained by these two methods may reflect the effect on the shape of the enzyme of alterations in the pH and ionic environment (*cf.* Table IV).

The purified enzyme was digested with trypsin and prepared for peptide mapping as described in MATERIALS AND METHODS. The soluble peptides were separated in three alternative systems (see MATERIALS AND METHODS), one of which is shown in Fig. 4. The core fraction was treated separately (Fig. 5). From a comparison of these, the total number of peptides (including those of the core) was 31–41.

For a mean molecular weight of 75 000, phosphonatase contains 37 lysine and 37 arginine residues (Table III), therefore 75 tryptic peptides would be expected. Instead, between 31 and 41 peptides were found, thus suggesting that phosphonatase is composed of two similar subunits. The molecular weight of the dissociated enzyme, estimated by two different methods, was found to lie between 33 000 and 35 000 (Figs. 3b and 3c); this result is in agreement with the suggestion that the active enzyme is composed of two similar subunits. Using specific reagents (see MATERIALS AND METHODS), we detected a total of 7–8 tyrosine-containing peptides and 6–7 histidine-containing peptides; this result is also consistent with the suggestion that phosphonatase is composed of two similar subunits, since from amino acid analyses

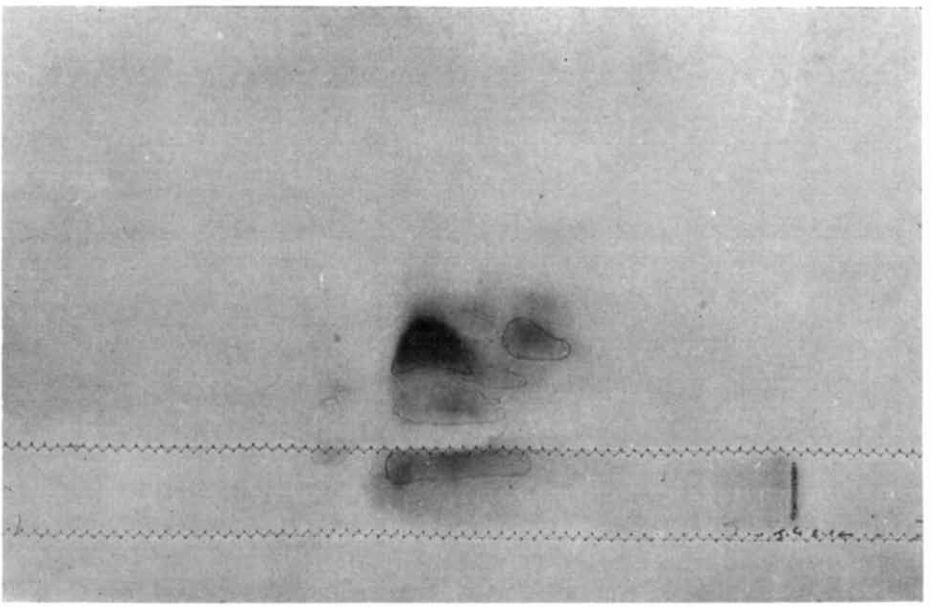


Fig. 5. Peptide map of the core fraction of a tryptic digest of phosphonatase. The material was electrophoresed in the first dimension at pH 8.9 and then chromatographed in the second dimension in pyridine–isoamyl alcohol–water. The peptides staining with ninhydrin were marked lightly with a pencil before chromatogram was developed with the chlorine–starch–iodide reagent as described in MATERIALS AND METHODS.

TABLE III

THE AMINO ACID COMPOSITION OF PHOSPHONATASE

Amino acid	*Residues calculated for a molecular weight of 75 000*	
	*I**	*II***
Lys	37.0	—
His	15.4	—
Arg	37.3	—
Asp	54.7	55.0
Thr	36.0	36.3
Ser	25.4	28.0
Glu	104.8	101.8
Pro	31.6	21.8
Gly	49.1	49.8
Ala	52.4	54.0
Val	53.2	49.8
Met	32.4	27.0***
Ile	48.5	43.6
Leu	47.8	47.8
Tyr	17.5	18.7†
Phe	29.9	27.0
Cys	—	—
Cysteic acid		5.5§

* Single 22-h hydrolysate; 2 mg purified enzyme.

** Duplicate 22-h hydrolysates of 75 μg of performic acid-oxidized material, estimated using the expanded scale. This was a different preparation from I.

*** As the methionine sulphone.

† Determined from an unoxidized sample of II run in parallel.

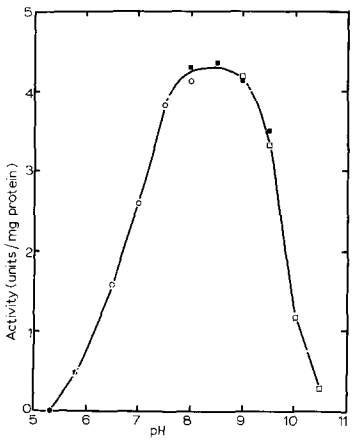
§ The unoxidized material consistently has an unidentified peak corresponding to about 3.5 residues of cysteic acid per mole.

(Table III) the number of tyrosine and histidine residues in a molecular weight of 75 000 is 18 and 15, respectively.

Amino acid analysis of the main core peptide (not shown) revealed that it contained no basic amino acids. It also contained a large amount (about 32 mole%) of glutamic acid which explains why the peptide had a limited solubility at pH 4 but migrated well on electrophoresis at pH 8.9 (Fig. 5).

Some properties of phosphonatase

Phosphonatase showed optimal activity between pH 8 and 9 (Fig. 6). The reaction rate was directly proportional to the concentration of enzyme over the range tested (Fig. 7). The Michaelis constant (K_m) for 2-phosphonoacetaldehyde, estimated by measuring the release of either P_i or acetaldehyde, was about 40 μM (Fig. 8),



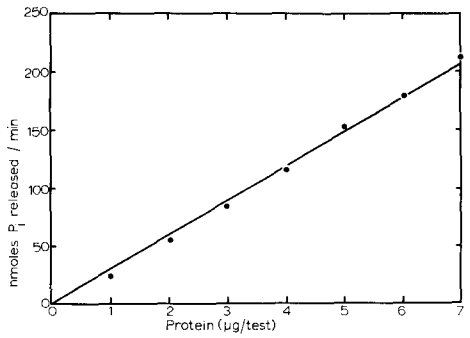


Fig. 6. The effect of pH on the activity of phosphonatase. The assay conditions were as those described in MATERIALS AND METHODS, except that the pH of the reaction mixture was varied using the following buffers, all at 50 mM; ●—●, sodium acetate; ○—○, TES–HCl; ■—■, Ammediol–HCl; □—□, glycine–KOH.

Fig. 7. The effect of enzyme concentration on the activity of phosphonatase. The assay conditions and the method for estimating the amount of P_i released from 2-phosphonoacetaldehyde are described in MATERIALS AND METHODS.

which is comparable with that of alkaline phosphatase at dilute substrate concentrations[55,56]. However, phosphonatase differs from alkaline phosphatase in other respects. Thus, the activity of phosphonatase is not markedly affected by high concentrations of P_i (100 mM P_i produced only 15% inhibition in the rate of release of acetaldehyde from 0.5 mM 2-phosphonoacetaldehyde, see DISCUSSION), but is inhibited by increasing concentrations of NaCl (Table IV); in contrast, the activity of alkaline phosphatase is markedly affected by P_i (K_i = 3.8 μM, ref. 55), and is stimulated by increasing ionic strengths[57]. Apart from 2-phosphonoacetaldehyde, phosphonatase released P_i from only one of a number of phosphorus-containing compounds tested (Table V), namely, *p*-nitrophenylphosphate; the rate of hydrolysis of this compound, however, was considerably lower than that of 2-phosphonoacetal-

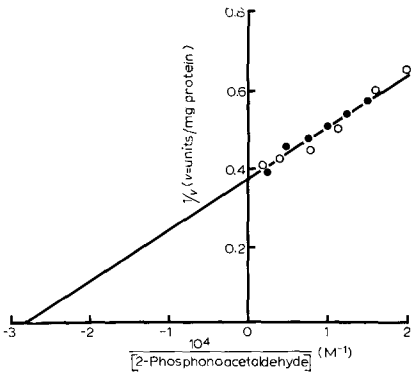


Fig. 8. Determination of the apparent Michaelis constant (K_m) of phosphonatase for 2-phosphonoacetaldehyde. The conditions of assay, and the methods used for determining the amount of P_i (●—●) or acetaldehyde (○—○) released from 2-phosphonoacetaldehyde are described in MATERIALS AND METHODS. The K_m was estimated to be about 40 μM.

dehyde. *E. coli* alkaline phosphatase did not degrade 2-phosphonoacetaldehyde, even after incubation for 1 h with the compound.

Phosphonatase is heat-labile as, even in the presence of 5 mM Mg^{2+}, its optimal activity is at 45° (Fig. 9). The activation energy, calculated from an Arrhenius plot of the data for Fig. 9, is 9.5 kcal/mole. The turnover number for freshly purified enzyme, under optimal conditions, is close to 1000 molecules of 2-phosphonoacetaldehyde cleaved per enzyme molecule per min at 25°. This is approximately one-third the turnover number of alkaline phosphatase under similar conditions[55].

The isoelectric point was estimated roughly by adding a small sample of the purified enzyme to a series of buffers, ranging, in 0.5-unit intervals, from pH 3.5 to 7.5. A precipitate formed within 10 min in the buffer at pH 4.5, and a slight precipitate formed overnight at pH 5.0, indicating that the isoelectric point is slightly higher than pH 4.5.

Although none of the commonly used heavy metal chelators inhibited the activity of phosphonatase, CN^- (1 mM) had a marked effect (Table VI). Estimation

TABLE IV

THE EFFECT OF THE IONIC ENVIRONMENT ON THE ACTIVITY OF PHOSPHONATASE

NaCl was added to the standard assay system (see MATERIALS AND METHODS) at varying concentrations up to 1.0 M at which level it was found not to interfere with either P_i or acetaldehyde estimation.

NaCl (M)	*Enzyme activity (units/mg) assayed as*	
	P_i release	*Acetaldehyde release*
0	2.7	2.29
0.05	2.45	1.56
0.125	2.18	1.29
0.25	1.65	1.29
0.50	0.90	0.83
1.00	0.40	0.66

TABLE V

SUBSTRATE SPECIFICITY OF PHOSPHONATASE

Phosphonatase (4 μg/ml) was incubated at 25° in 50 mM Ammediol–HCl buffer (pH 8.5) containing 5 mM $MgCl_2$ and various test substrates (1 mM) shown below. The amount of P_i released was determined as described in MATERIALS AND METHODS. The action of *E. coli* alkaline phosphatase (5 μg/ml), is shown for comparison. Samples which showed no activity were incubated for 1 h.

Enzyme tested	*Compound*	*Activity (units/mg)*
Phosphonatase	2-Phosphonoacetaldehyde	4.64
	p-Nitrophenylphosphate	0.15
	α-Glycerophosphate	0
	β-Glycerophosphate	0
	Ethanolamine phosphate	0
	2-Aminoethylphosphonate	0
	Aminomethylphosphonate	0
	2-Hydroxy-5-nitrobenzyl phosphonate	0
	Phosphonomycin	0
E. coli alkaline phosphatase	β-Glycerophosphate	7.7
	2-Phosphonoacetaldehyde	0

by atomic absorption spectroscopy of the metal content of phosphonatase showed that the enzyme contained no cobalt, trace amounts of iron, and significant amounts of zinc and copper; however, the latter two metals corresponded to a metal content of only 0.4 and 0.3 g atom, respectively, per molecular weight of 75 000. Since SO_3^{2-} and S^{2-} were also found to inhibit enzymic activity, the observed effect of CN^- is more likely to result from the cleavage of essential disulphide bridges. Surprisingly, preincubation with dithiothreitol and mercaptoethanol did not inhibit the enzyme; perhaps this is because cleavage of disulphide bridges by these reagents does not result in the introduction of an extra group which could cause steric hindrance.

The effect of orthophosphite on the activity of phosphonatase

We have shown elsewhere[58] that 0.05 mM orthophosphite abolished the growth of *B. cereus* on media where 2-aminoethylphosphonate was the sole source of phos-

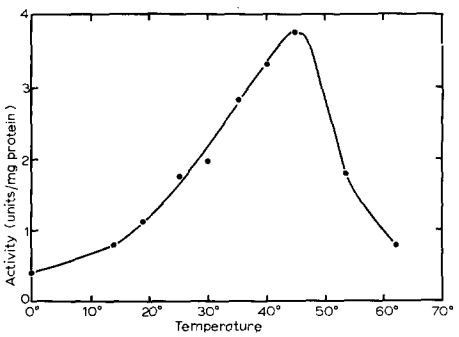
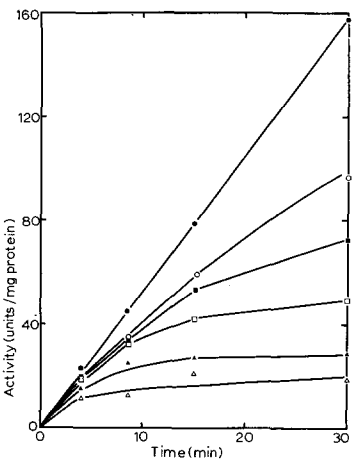


Fig. 9. The effect of temperature on the activity of phosphonatase. The standard assay conditions (see MATERIALS AND METHODS) were used with the enzyme present at 5 μg/ml. Initial reaction rates were measured over a period not exceeding 10 min.

phorus, but not when other sources of phosphorus were used. We found that the locus of biochemical lesion was the enzyme phosphonatase. Thus, the addition of orthophosphite to phosphonatase caused a progressive decline in the reaction rate until the complete cessation of activity. The rate of decline depended on orthophosphite concentration (Fig. 10) and the overall pattern suggested destruction of active enzyme. Preincubation of phosphonatase with 2.5 mM orthophosphite, for up to 60 min before the addition of 2-phosphonoacetaldehyde had no effect on subsequent activity, but preincubation for 30 min with 25 mM acetaldehyde together with 2.5 mM orthophosphite resulted in complete inhibition of the activity of the enzyme (Fig. 11); acetaldehyde alone did not inhibit the reaction, except for a slight effect seen after 20 min. A similar experiment, in which the release of acetal-



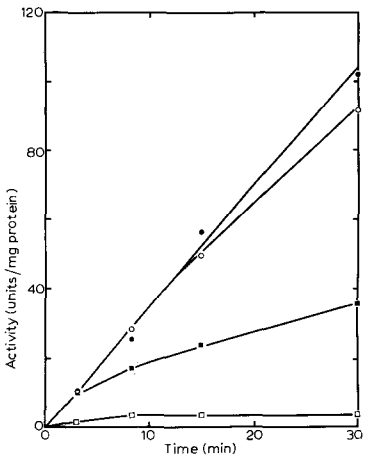


Fig. 10. The effect of varying concentrations of orthophosphite on the activity of phosphonatase. Orthophosphite was added to the reaction mixture, and the release of P_i from 2-phosphonoacetaldehyde (2 mM) estimated as described in MATERIALS AND METHODS. The concentrations (mM) of orthophosphite used were: ●—●, 0; ○—○, 0.5; ■—■, 1; □—□, 2; ▲—▲, 4; △—△, 8.

Fig. 11. The effect on the activity of phosphonatase of preincubation with various compounds. The assay conditions described in MATERIALS AND METHODS were used. Phosphonatase was incubated for 30 min with the following additions: ●—●, none; ○—○, 25 mM acetaldehyde; ■—■, 2.5 mM orthophosphite; □—□, 25 mM acetaldehyde *plus* 2.5 mM orthophosphite. At zero time, 2 mM 2-phosphonoacetaldehyde was added and the release of P_i followed.

dehyde instead of P_i was followed, showed that preincubation of phosphonatase with 25 mM P_i together with 2.5 mM orthophosphite did not inactivate the enzyme. Thus, orthophosphite can act as an inhibitor only in the presence of 2-phosphonoacetaldehyde or acetaldehyde. Prolonged dialysis (22 h) of orthophosphite-inactivated phosphonatase resulted in the recovery of only 30–40% of the activity (Table VII).

[^{32}P]Orthophosphite-inactivated enzyme was dialysed overnight against 3 × 500 vol. of distilled water. A portion of this enzyme was passed through a column of Sephadex G-25; the radioactivity was not retarded, but emerged with the Blue Dextran marker at the buffer front. A second portion, after lyophilization, was heated with 6 M HCl at 100° for 30 min, or digested with trypsin (pH 8) or pepsin (pH 2). The resulting solutions were electrophoresed at pH 1.9, 3.5 and 8.9 and

TABLE VI

THE EFFECT OF METAL CHELATORS AND DISULPHIDE REAGENTS ON THE ACTIVITY OF PHOSPHONATASE

The test compound and enzyme (4 μg) were incubated together as indicated below. The assay conditions and method for estimating the amount of P_i released from 2-phosphonoacetaldehyde are as described in MATERIALS AND METHODS, except that, after incubation the first four reagents were extracted into ether as they were found to interfere with P_i estimation.

Treatment	*Inhibition (%)*
2 h preincubation at 0° with:	
1 mM mercaptoethanol	0
1 mM 8-hydroxyquinoline-5-sulphonic acid	0
1 mM 2,2′-dipyridyl	0
1 mM 1,10-phenanthroline	0
1 mM neocuproine	0
1 mM dithiothreitol	0
Added at zero time:	
1 mM NaCN, Na_2S or Na_2SO_3	>95
0.1 mM NaCN, Na_2S or Na_2SO_3	82–88
0.01 mM NaCN, Na_2S or Na_2SO_3	30–40
0.001 mM NaCN, Na_2S or Na_2SO_3	0

TABLE VII

RECOVERY OF PHOSPHONATASE ACTIVITY FOLLOWING DIALYSIS AFTER EXPOSURE TO VARIOUS COMPOUNDS

Purified enzyme (7 μg per test) was incubated for 30 min at 25° in 50 mM Ammediol–HCl buffer (pH 8.5) containing 5 mM $MgCl_2$, with additional compounds as indicated below. The samples were dialysed for 22 h at 4° against 3 × 200 ml of Tris–$MgCl_2$–dithiothreitol buffer. They were then assayed for activity as described in MATERIALS AND METHODS, with linear initial rates measured over 10 min.

Treatment	*Activity of dialysed enzyme (units/mg)*	*% Control*
None (control)	2.9	100
5 mM orthophosphite	3.1	107
25 mM acetaldehyde	3.2	110
5 mM orthophosphite + 2 mM 2-phosphonoacetaldehyde	0.9	31
5 mM orthophosphite + 25 mM acetaldehyde	1.2	42

TABLE VIII

PHOSPHONATASE ACTIVITY IN THE WILD STRAIN (W) OF *B. cereus*

The activity was assayed in crude, cell-free extracts of the wild strain directly after growth in PPYG medium and after starvation in phosphate-free media with or without the additions shown below. Protein was measured by the biuret method.

Treatment	*Specific activity of phosphonatase (μmole P_i released/min per mg protein)*
PPYG-grown cells	0
2-h starved cells, treated for a further 2 h as follows:	
no additions	0.0018
0.5 mM 2-aminoethylphosphonate	0.0061
0.5 mM aminomethylphosphonate	0.0048

autoradiographs prepared (see MATERIALS AND METHODS). In all cases, the predominant radioactive component present (estimated to be greater than 90% of the total) was shown to be orthophosphite by comparison with a standard. These results indicate that orthophosphite does not bind covalently to phosphonatase.

Phosphonatase activity in the wild strain (W) of B. cereus

The wild strain (W) of *B. cereus* transports 2-aminoethylphosphonate by means of an inducible system[7]; the formation of the transport system, but not its function, is repressed by extracellular P_i. Following the identification of 2-phosphonoacetaldehyde as an intermediate in the degradation of 2-aminoethylphosphonate to P_i, and the purification of phosphonatase from the mutant strain (AI-2), we examined the wild strain for phosphonatase activity. Our results (Table VIII) show that no activity was present in cells grown in a rich medium (PPYG); after prolonged phosphorus deprivation (4 h), some activity appeared, and this was increased 3–4-fold by the presence of 2-aminoethylphosphonate or aminomethylphosphonate (a gratuitous inducer for the 2-aminoethylphosphonate transport system)[7]. Thus, phosphonatase was found to be inducible in the wild strain, but the specific activity of the enzyme after induction was considerably lower than that obtained from the constitutive mutant AI-2 (*cf.* Table I).

DISCUSSION

An enzyme able to cleave the carbon–phosphorus bond has not been described before. We have found that phosphonatase resembles the alkaline phosphatase of *E. coli* in a number of its properties: for instance, both have maximum activity in alkaline pH (ref. 55), are composed of two subunits[59], and are protected by Mg^{2+}, inactivated by EDTA[55,60], but not by F^- (refs. 56, 61). However, phosphonatase does not degrade phosphate esters which are readily degraded by alkaline phosphatase; of those tested, the one exception is *p*-nitrophenylphosphate which is degraded at a considerably slower rate than 2-phosphonoacetaldehyde. We do not understand why this reaction occurs, especially since phosphonatase does not cleave a phosphonate with a structure similar to *p*-nitrophenylphosphate (2-hydroxy-5-nitrobenzylphosphonate). Alkaline phosphatase attacks a wide range of phosphate esters[55], whereas the range of substrates degraded by phosphonatase seems to be very limited. It would be of interest to compare the active sites of the two enzymes. Our work indicates that phosphonatase differs from alkaline phosphatase in one important respect: it is not a heavy metal metalloenzyme. The function of Mg^{2+} is not clear. We do not know whether Mg^{2+} is present at the active site, but the failure of F^- to inhibit the activity of phosphonatase suggests that the enzyme does not react with a Mg^{2+}–substrate complex. The high concentration of Mg^{2+} required, and the behaviour of the enzyme on polyacrylamide gels, suggest that Mg^{2+} helps to maintain the aggregated form of the enzyme.

Earlier, we found that orthophosphite inhibited the breakdown of 2-aminoethylphosphonate by *B. cereus* both within whole cells[58] and in cell-free extracts[16]. It is clear from the present work that it does so by inhibiting the activity of phosphonatase. The structural similarity of orthophosphite to the phosphonate moiety of 2-phosphonoacetaldehyde suggests that it acts at the active site of phosphonatase,

particularly as it acts as an inhibitor only in the presence of the substrate (2-phosphonoacetaldehyde) or one of the products (acetaldehyde). One explanation of this is that the release of products is ordered: only 2-phosphonoacetaldehyde or acetaldehyde can react with the free enzyme. When 2-phosphonoacetaldehyde is the substrate, P_i is the first product to be released, leaving acetaldehyde attached to the enzyme surface. The conformational state of the enzyme–acetaldehyde complex is different from that of the free enzyme, and orthophosphite, by its structural analogy to P_i, may combine with this form of the enzyme to produce an irreversible complex (*cf.* ref. 62).

$$PA + E \rightleftharpoons PA\text{–}E \xrightarrow[H_2O]{P_i} A\text{–}E^* \rightarrow A\text{–}E \rightarrow A + E$$

orthophosphite inhibits here

where E = phosphonatase, E* = altered form of the enzyme, PA = 2-phosphonoacetaldehyde and A = acetaldehyde. We have found that, although orthophosphite binds tightly to phosphonatase, it is not bound covalently, and some activity is recovered after prolonged dialysis. As yet, we have been unable to test by kinetic means whether or not the release of products is ordered as suggested above. The reaction is not readily reversible and concentrations of up to 0.1 M acetaldehyde or P_i have to be used for any product inhibition to be observed. It is difficult to assess the side effects that such concentrations could be having on the activity of the enzyme, especially since phosphonatase is adversely affected by high ionic strengths.

The ability to derive phosphorus from 2-aminoethylphosphonate is presumably an advantage under certain circumstances. However, in the wild strain (W) of *B. cereus*, the formation of the transport system is repressed by external P_i (ref. 7); the existence of a means for preventing 2-aminoethylphosphonate entering the cell when P_i is available suggests that it is metabolically costly for the cell to use 2-aminoethylphosphonate as a source of phosphorus.

Finally, it is tempting to suggest that the pathway for the degradation of 2-aminoethylphosphonate to P_i in *B. cereus* occurs in all organisms which are able to grow on 2-aminoethylphosphonate. However, since other workers have shown that bacteria can degrade a variety of synthetic phosphonates other than aminoalkylphosphonates[4,5,8], it is evident that alternative pathways in which the carbon–phosphorus bond is cleaved must exist in nature.

Phosphonatase does not degrade phosphonomycin[18,19], a recently discovered antibiotic with a carbon–phosphorus bond. Thus, it does not seem likely that organisms which degrade 2-aminoethylphosphonate to P_i *via* the 2-phosphonoacetaldehyde pathway will necessarily be resistant to the action of this drug.

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