Separation and Purification of the Alkaline Phosphatase and a Phosphodiesterase from *Halobacterium cutirubrum*

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1. *Halobacterium cutirubrum* alkaline phosphatase is associated in crude extracts with a phosphodiesterase. 2. The enzymes were stabilized in buffers containing both $(\text{NH}_4)_2\text{SO}_4$ and 10 mM-Mn^{2+} . 3. Adsorption chromatography on Sepharose 6B/agarose-gel columns in the presence of $1.4\text{ M-(NH}_4)_2\text{SO}_4$ gave a phosphatase-free phosphodiesterase and the alkaline phosphatase associated with some phosphodiesterase activity. 4. Further chromatography of the separated enzymes gave a good recovery of >600-fold purified phosphodiesterase and >3000-fold purified alkaline phosphatase. 5. The requirements of these enzymes and their relationship to each other was examined. 6. A detailed study showed that the alkaline phosphatase was adsorbed at least partially to agarose and dextran columns at all $(\text{NH}_4)_2\text{SO}_4$ concentrations from 0.25 to 2 M. 7. In contrast, no adsorption of the enzyme or protein standards was evident in 2.5 M-KCl/1 M-NaCl or 0.25 M-KCl/0.1 M-NaCl, in agreement with previous studies by Louis, Peterkin & Fitt [(1971) *Biochem. J.* **121**, 635–641], thus confirming the validity of gel filtration in 2.5 M-KCl/1 M-NaCl as a method for determining the approximate molecular weights of extreme-halophile proteins.

Our previous studies (Fitt & Peterkin, 1976) showed that *Halobacterium cutirubrum* contains an alkaline phosphatase of unusually small molecular size that is highly stimulated by Mn^{2+} . The enzyme was purified about 20-fold, but its instability under the conditions used at that time prevented its extensive purification. The phosphatase was associated in crude extracts (P. S. Fitt, unpublished work) with an enzyme capable of catalysing the hydrolysis of bis-*p*-nitrophenyl phosphate, the standard phosphodiesterase substrate (Laskowski, 1966), but this enzyme was completely inactivated during the dialysis to low ionic strength used before the $(\text{NH}_4)_2\text{SO}_4$ precipitation in our published purification procedure (Fitt & Peterkin, 1976), and all attempts to stabilize it were unsuccessful.

Subsequently we became aware of work by McParland (1969) in which the purification to electrophoretic homogeneity of a very similar alkaline phosphatase from *Halobacterium salinarum* was described. We attempted to apply this method to the *H. cutirubrum* enzyme, but were unable to reproduce a critical acetone fractionation, which in our hands led invariably to a great loss of activity. However, the buffers used by McParland (1969), which contained both Mn^{2+} and a high concentration of $(\text{NH}_4)_2\text{SO}_4$, proved extremely valuable, since we found that they

stabilized both the alkaline phosphatase and the hitherto intractable phosphodiesterase.

The $(\text{NH}_4)_2\text{SO}_4/\text{Mn}^{2+}$ buffers have made possible the separation and extensive purification of the phosphatase and phosphodiesterase as described in the present paper. The separation of the enzymes depends on the partial adsorption of the alkaline phosphatase to agarose and dextran gels in the presence of $(\text{NH}_4)_2\text{SO}_4$. However, we have previously used gel filtration at high ionic strength in buffers containing 2.5 M-KCl/1 M-NaCl for the determinations of the approximate molecular weights of several *H. cutirubrum* enzymes (Louis *et al.*, 1971; Fitt *et al.*, 1975; Fitt & Peterkin, 1976; Kim & Fitt, 1977), and it was necessary to confirm that the adsorption effects observed in the presence of $(\text{NH}_4)_2\text{SO}_4$ were indeed peculiar to this salt and did not affect our previous results. Accordingly we made a detailed study of the effect of $(\text{NH}_4)_2\text{SO}_4$ on the behaviour of *H. cutirubrum* alkaline phosphatase on gel columns. The results of these experiments are given in the present paper and they confirm that, although gel filtration in the presence of $(\text{NH}_4)_2\text{SO}_4$ is greatly affected by adsorption of proteins to gels at all $(\text{NH}_4)_2\text{SO}_4$ concentrations above 0.25 M (the lowest used), similar effects are not observed in the presence of KCl and NaCl, in agreement with our previous detailed study by gel filtration and density-gradient centrifugation at high ionic strength of three extreme-halophile enzymes (Louis *et al.*, 1971).

Abbreviation used: DNAase, pancreatic deoxy-ribonuclease I.

Experimental

Materials

Chemicals and substrates were from the following suppliers: Schwarz-Mann enzyme-grade $(\text{NH}_4)_2\text{SO}_4$, Becton Dickinson, Mississauga, Ont., Canada; bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl phosphate, BDH Chemicals, Toronto, Ont., Canada; Blue Dextran, Sephadex G-50 and Sepharose 6B, Pharmacia (Canada), Dorval, P.Q., Canada; crystalline bovine serum albumin, ovalbumin grade V, α -chymotrypsinogen A type II (ox pancreas), cytochrome *c* type VI (horse heart), DNAase and Tris (Trizma base), Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were from Canadian Laboratory Supplies, Ltd., or Fisher Scientific Co., Ottawa, Ont., Canada.

Buffers

Buffers containing $(\text{NH}_4)_2\text{SO}_4$ were prepared by dilution with 0.1 M-Tris/HCl, pH 8, to the appropriate final concentration of a stock 4M- $(\text{NH}_4)_2\text{SO}_4$ /0.1 M-Tris/HCl (pH 8) buffer that had been passed through a Millipore filter (pore size 0.45 μm ; Millipore Corp., Bedford, MA, U.S.A.) and stored at approx. 25°C, the temperature at which the 4M solution is close to saturation and cannot be cooled without crystallization occurring. MnCl_2 and 2-mercaptoethanol were added as required during dilution of the stock concentrated buffer. $(\text{NH}_4)_2\text{SO}_4$ was found to retard the oxidation of Mn^{2+} markedly when present at a concentration of at least 1.2M, but the presence of 2-mercaptoethanol was essential at lower $(\text{NH}_4)_2\text{SO}_4$ concentrations, so it was included routinely in all the working buffers.

Cell culture

Halobacterium cutirubrum N.R.C. 34001 cells were grown in Gochnauer & Kushner's (1969) complex medium as previously described (Fitt *et al.*, 1975; Peterkin & Fitt, 1971).

Separation and purification of *H. cutirubrum* phosphodiesterase and alkaline phosphatase

All operations were done at 0–5°C.

The freshly harvested bacteria were suspended in 3M-KCl/10mM- MnCl_2 /0.1M-Tris/HCl (pH 8) (3ml/g wet wt. of bacteria). The suspension was stirred magnetically for 15 min and 2 vol. of 10mM- MnCl_2 /0.1M-Tris/HCl (pH 8) containing 1 mg of DNAase/ml were then added. Cell lysis was complete within 10 min. Stirring was continued for a further 50 min and the mixture was then centrifuged at 45 000g_{max} for 15 min to give the crude extract (Table 1). The latter was cooled in ice and 4M- $(\text{NH}_4)_2\text{SO}_4$ /10mM- MnCl_2 /0.1M-Tris/HCl (pH 8) (5.86 vol.) was added with stirring. The mixture was stirred for 30 min and

then centrifuged at 27 000g_{max} for 15 min. The pellet was stirred with 1.3M- $(\text{NH}_4)_2\text{SO}_4$ /10mM- MnCl_2 /0.1M-Tris/HCl (pH 8) (3ml/g wet wt. of bacteria) for 30 min and the suspension was then centrifuged at 27 000g_{max} for 15 min. The insoluble protein was discarded, and the supernatant containing both activities was dialysed overnight against 60 vol. of 1.4M- $(\text{NH}_4)_2\text{SO}_4$ /10mM- MnCl_2 /10mM-2-mercaptoethanol/0.1M-Tris/HCl (pH 8) (Buffer I) to give the $(\text{NH}_4)_2\text{SO}_4$ fraction (Table 1). The two enzymes were then separated by adsorption chromatography on a column (2.5 cm $\times$ 40 cm) of Sepharose 6B that had been kept in 1.4M- $(\text{NH}_4)_2\text{SO}_4$ /0.1M-Tris/HCl (pH 8) for at least 2 days. The column was washed with Buffer I, which was also used for application of the $(\text{NH}_4)_2\text{SO}_4$ fraction. Elution was performed with Buffer I at 26 ml/h. Fractions (10 ml) were collected and assayed for protein content and for both phosphodiesterase and alkaline phosphatase activities. The fractions containing the two peaks of activity were combined to give the Sepharose bulk fractions and each bulk fraction was concentrated by ultrafiltration against an Amicon PM10 membrane to give PM10 supernatant fractions (Sephadex 6B I and II, Table 1). Each of the latter fractions was then purified further by chromatography on a column (2.5 cm $\times$ 90 cm) of Sephadex G-50 prepared and run as described above for the Sepharose 6B column, except that the buffer was 1.2M- $(\text{NH}_4)_2\text{SO}_4$ /10mM- MnCl_2 /10mM-2-mercaptoethanol/0.1M-Tris/HCl (pH 8) at all times. The active fractions were combined to give the bulk Sephadex G-50 fractions. The dilute, purified phosphodiesterase (Sephadex G-50 I, Table 1) could not be concentrated without almost total loss of activity, but the corresponding alkaline phosphatase fraction was concentrated successfully by ultrafiltration against an Amicon YM10 membrane to give the Sephadex G-50-II fraction (Table 1). The highly purified Sephadex G-50 I and II fractions were stored at 4–5°C and lost about 5% of their activity per week under these conditions.

Enzyme assays

Assay I. *H. cutirubrum* alkaline phosphatase was assayed by a modification of the method of Fitt & Peterkin (1976). The standard assay medium (0.5 ml) contained: 30mM-Tris/HCl buffer, pH 8.3; 1mM-EDTA (disodium salt); 0.8M-KCl; 5mM- MnCl_2 ; 4mM-*p*-nitrophenyl phosphate; enzyme. An amount of enzyme was used that would give $\Delta A_{400} \leq 0.5/15$ min. Incubation was at 37°C for 15 min and the reaction was stopped by addition of 0.5 ml of aq. 5% (w/v) K_2HPO_4 (cf. Schlesinger, 1966). The mixture was centrifuged at top speed (approx. 2000g) in an IEC model CL clinical centrifuge for 5 min, and the A_{400} of the supernatant was determined spectrophotometrically by using a control lacking enzyme as blank.

Assay II. *H. cutirubrum* phosphodiesterase was assayed as described above with 2 mM-bis-*p*-nitrophenyl phosphate as substrate instead of *p*-nitrophenyl phosphate.

In both cases, a unit of activity was defined as the amount of enzyme that catalysed the formation of 1 nmol of *p*-nitrophenol/min under the conditions of the standard assay. The ϵ_{400} of *p*-nitrophenol was taken to be 18 300 (Dawson *et al.*, 1969).

Protein assay

Protein was assayed either spectrophotometrically (Warburg & Christian, 1942) or by Peterson's (1977) modification of the Lowry *et al.* (1951) procedure, with bovine serum albumin as standard. The spectrophotometric method was found to be unreliable at low protein concentrations, owing to the presence of non-dialysable, u.v.-absorbing material in the preparation, possibly arising from the bacteria themselves, but this source of interference was eliminated in Peterson's (1977) method, which involves a preliminary precipitation of the protein with sodium deoxycholate and trichloroacetic acid.

Results and Discussion

Purification of the enzymes

The results of a typical preparation of the two enzymes by the method described in the Experimental section are shown in Table 1. The success of the method is due to the comparatively good stability of the alkaline phosphatase and phosphodiesterase in buffers containing both $(\text{NH}_4)_2\text{SO}_4$ and Mn^{2+} . The $(\text{NH}_4)_2\text{SO}_4$ itself was at least as effective as KCl or NaCl in stabilizing the enzymes when used at the same ionic strength, and therefore at one-third the concentration, as the two alkali-metal chlorides. In addition, it prevents the oxidation of Mn^{2+} at pH 8–8.6 when used at a concentration of 1.4 M, provided that before the addition of the Mn^{2+} the $(\text{NH}_4)_2\text{SO}_4$ solutions are filtered through fine Millipore membranes to remove brown contaminants present even in 'enzyme' grades of this salt. At $(\text{NH}_4)_2\text{SO}_4$ concentrations of 1.2 M or

lower the addition of 2-mercaptoethanol was also necessary to avoid a slow oxidation of the Mn^{2+} , so the thiol was routinely added to all Mn^{2+} -containing buffers. Preliminary experiments showed that, in these buffers, Mn^{2+} plays an essential role in stabilizing the two enzymes, which are both stimulated specifically by it (see below). The combination of $(\text{NH}_4)_2\text{SO}_4$ with Mn^{2+} was therefore very effective in overcoming the instability that had previously prevented extensive purification of the alkaline phosphatase (Fitt & Peterkin, 1976) or any purification at all of the phosphodiesterase (P. S. Fitt & P. I. Peterkin, unpublished work). It seems possible that buffers containing a combination of $(\text{NH}_4)_2\text{SO}_4$ with an essential bivalent cation may be valuable in work with other unstable enzymes from extreme halophiles.

Separation of the two enzymes was achieved readily by adsorption chromatography on Sepharose 6B in the presence of 1.4 M- $(\text{NH}_4)_2\text{SO}_4$ (Fig. 1). McParland (1969) observed that *H. salinarium* alkaline phosphatase was partially adsorbed to Sephadex G-50 in concentrated $(\text{NH}_4)_2\text{SO}_4$ solutions, and we adapted his method in order to separate *H. cutirubrum* alkaline phosphatase and phosphodiesterase on agarose gels, which give more stable and faster-running columns under these conditions. More recently, Mevarech *et al.* (1976, 1977) were able to purify dehydrogenases from an extreme halophile by adsorption to Sepharose 4B columns in the presence of 2.6 M- $(\text{NH}_4)_2\text{SO}_4$ followed by elution with a gradient of decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration. The two *H. cutirubrum* enzymes discussed in the present paper were completely adsorbed to Sepharose 6B in 2 M- $(\text{NH}_4)_2\text{SO}_4$, and could be eluted with more dilute solutions, but the recovery of activity was better, the procedure more rapid and the purification at least as good when the whole process was performed by using a single $(\text{NH}_4)_2\text{SO}_4$ concentration (1.4 M) chosen to give optimum separation of the two enzymes without their total adsorption to the gel.

The final step in the purification of the two enzymes involved a combination of gel filtration and adsorp-

Table 1. *Purification of Halobacterium cutirubrum* alkaline phosphatase and phosphodiesterase

H. cutirubrum alkaline phosphatase and phosphodiesterase were purified from 6.7 g (wet wt.) of cells, and their activities were measured by the standard assays as described in the Experimental section.

Fraction	Volume (ml)	Total protein (mg)	Alkaline phosphatase		Phosphodiesterase	
			Activity (units)	Sp. activity (units/mg)	Activity (units)	Sp. activity (units/mg)
Crude extract	62	284	74 214	260	8771	31
$(\text{NH}_4)_2\text{SO}_4$ fraction	22	68.7	44 488	647	15 991	232
Sepharose 6B I	0.8	3.8	—	—	2830	745
Sepharose 6B II	4.3	1.25	21 676	17 304	1117	894
Sepharose G-50 I	40	<0.20	—	—	3718	>19 000
Sepharose G-50 II	6.0	<0.03	28 274	>940 000	162	>5000

Fig. 1. Separation of *Halobacterium cutirubrum* alkaline phosphatase and phosphodiesterase activities by $(\text{NH}_4)_2\text{SO}_4$ /agarose adsorption chromatography

Adsorption chromatography on a column ($2.5\text{ cm} \times 40\text{ cm}$) of Sepharose 6B was performed in 1.4 M $(\text{NH}_4)_2\text{SO}_4$ /10 mM-MnCl₂/10 mM-2-mercaptoethanol/10 mM-Tris/HCl (pH 8.0), and the alkaline phosphatase and phosphodiesterase activities located by Assays I and II as described in the Experimental section. ○—○, Alkaline phosphatase activity; ●---●, phosphodiesterase activity.

tion chromatography on Sephadex G-50 in buffers containing 1.2 M $(\text{NH}_4)_2\text{SO}_4$.

Both enzymes could be successfully concentrated by ultrafiltration against Amicon PM10 membranes after the first gel-filtration/adsorption-chromatography step on Sepharose 6B columns, although both were almost completely inactivated in the presence of Amicon UM10 membranes. However, after the final chromatographic steps on Sephadex G-50, when the protein concentration of each of the two purified bulk fractions was below detectability, both enzymes were either totally inactivated by or irreversibly adsorbed to PM10 and UM10 membranes. In the case of the alkaline phosphatase, this problem was successfully overcome by using the new Amicon YM10 membranes, but the phosphodiesterase activity was still lost totally with this membrane. Other methods of concentration, such as dialysis against Ficoll solutions, total adsorption to Sepharose columns at $(\text{NH}_4)_2\text{SO}_4$ followed by elution at lower ionic strength etc., were not successful, and the purified phosphodiesterase had to be stored and used as the combined, dilute fractions from the Sephadex G-50 columns.

The final, purified fractions of both enzymes contained undetectable amounts of protein, and the recoveries of alkaline phosphatase and phosphodiesterase activity were 38 and 42% respectively. The specific activities of the Sephadex G-50 fractions (Table 1) are based on a protein concentration of

$5\text{ }\mu\text{g/ml}$ [the approximate limit of detectability by the Peterson (1977) method], and are minimum values given merely to emphasize that both enzymes were highly purified.

A part of the phosphodiesterase activity remained associated with the alkaline phosphatase peak after both the Sepharose 6B and Sephadex G-50 columns (Fig. 1 and Table 1). It was unstable during the final concentration of the bulk Sephadex G-50 II fraction against the Amicon YM10 membrane under conditions where the recovery of alkaline phosphatase activity was excellent, so the two activities in this fraction are probably due to separate enzymes.

The main peak of phosphodiesterase from the Sepharose 6B column was totally devoid of phosphatase activity, and its substrate specificity and bis-*p*-nitrophenyl phosphate saturation curve differed from that of the phosphatase-associated phosphodiesterase (see below).

Enzymic properties of the purified proteins

The general requirements of the highly purified enzymes were examined.

The properties of the alkaline phosphatase did not differ significantly from those previously described for the partially purified enzyme by Fitt & Peterkin (1976) with respect to optimum substrate concentration, pH optimum and salt requirement. However, it was much less stable at low ionic strengths in the absence of Mn^{2+} ; the 20-fold-purified phosphatase retained 5–10% of its activity after 24 h in 10 mM-Tris buffer, pH 8.6, whereas the highly purified enzyme was so unstable in 0.25 M -KCl/0.1 M-NaCl/10 mM-Tris/HCl (pH 8.6) that fractions from the analytical gel filtrations performed in this buffer (Table 2) had to be collected directly into a concentrated $(\text{NH}_4)_2\text{SO}_4$ solution containing the components of the standard assay to permit location of the activity, and mere 10-fold dilution with 10 mM-Tris/HCl, pH 8, of the concentrated enzyme solution [stored in 1.2 M $(\text{NH}_4)_2\text{SO}_4$ /10 mM-Mn²⁺/10 mM-2-mercaptoethanol/10 mM-Tris/HCl (pH 8)] immediately before assay caused total inactivation.

All three activities, namely the phosphodiesterase, the alkaline phosphatase and the alkaline phosphatase-associated phosphodiesterase, had broad pH optima in the range 7–9 and were stimulated by Mn^{2+} rather than dependent on it. The alkaline phosphatase activity increased about 6-fold, and both phosphodiesterase activities were stimulated approximately 50% in the presence of 5 mM -Mn²⁺, the optimum concentration in all three cases, and Ca^{2+} , Co^{2+} , Mg^{2+} and Zn^{2+} could not replace Mn^{2+} . These results confirm and extend our previous observations on the unusual importance of Mn^{2+} in satisfying the bivalent-cation requirement of many extreme-halophile enzymes (Fitt & Peterkin, 1976; Fitt *et al.*, 1975; Louis & Fitt, 1971a,b; Peterkin & Fitt, 1971). In this

connection it should be noted that our previous finding (Fitt *et al.*, 1975) that the bivalent-cation requirement of *H. cutirubrum* DNA-dependent RNA polymerase could, under certain conditions, be satisfied by Mg^{2+} appears to have been due to an artifact;

Fig. 2. Effect of bis-*p*-nitrophenyl phosphate concentration on the activity of the phosphodiesterase and alkaline phosphatase-associated phosphodiesterase of *Halobacterium cutirubrum*

The phosphodiesterase activity of the two Sepharose G-50 fractions (Table 1) was determined at the indicated concentrations of bis-*p*-nitrophenyl phosphate in the conditions of Assay II (see the Experimental section). ●, Sepharose G-50 I fraction (phosphodiesterase); ○, Sepharose G-50 II fraction (alkaline phosphatase).

when it is assayed in the presence of an amount of EDTA sufficient to form a complex with all the bivalent cations present as contaminants in the KCl and NaCl used in high concentration in the incubation medium, this enzyme is absolutely dependent on added Mn^{2+} (P. S. Fitt & P. I. Peterkin, unpublished work).

The bis-*p*-nitrophenyl phosphate saturation curves of the two phosphodiesterases were determined under the conditions of Assay II (Fig. 2) and differed significantly. In particular, the saturation curve of the phosphatase-associated phosphodiesterase was clearly sigmoidal.

A comparison of the ability of the two phosphodiesterases to catalyse the hydrolysis of the two specific substrates thymidine 3'-nitrophenyl phosphate and thymidine 5'-nitrophenyl phosphate was made (Table 2). The alkaline phosphatase-associated phosphodiesterase was essentially inactive with the 5' substrate. In contrast, the principal phosphodiesterase was active with both the thymidine esters and may still have been a mixture of 3'- and 5'-exonucleases, since such enzymes are usually specific with respect to their mode of hydrolysis of the phosphodiester linkage (Adams *et al.*, 1976). The main phosphodiesterase was inactive towards yeast or wheat-germ RNA, but did hydrolyse heat-denatured DNA from calf thymus, salmon sperm or the bacterium *Micrococcus lysodeikticus* slowly to give a ΔA_{260} of 0.05/15 min in the acid-soluble supernatant obtained by addition of an equal volume of 7% (w/v) $HClO_4$ to Assay-II incubation mixtures containing 1 mg of DNA/ml as substrate in place of bis-*p*-nitrophenyl phosphate.

Table 2. Substrate specificity of *Halobacterium cutirubrum* phosphodiesterase and alkaline phosphatase-associated phosphodiesterase

The activities of the phosphodiesterase (Enzyme I) and alkaline phosphatase-associated phosphodiesterase (Enzyme II) were determined with the indicated substrates under the conditions of Assay II (see the Experimental section) with and without Mn^{2+} .

Substrate	Concn. (mm)	Activity ($\Delta A_{400}/15 \text{ min}$)			
		Enzyme I		Enzyme II	
		+ Mn^{2+}	- Mn^{2+}	+ Mn^{2+}	- Mn^{2+}
Thymidine 5'-nitrophenyl phosphate	0.2	0.003	0	0	0
	0.4	0.023	0	0	0
	0.6	0.035	0.040	0	0
	1.0	0.058	0.063	0	0
	2.0	0.097	0.118	0.005	0.005
Thymidine 3'-nitrophenyl phosphate	0.2	0.040	0.036	0	0
	0.4	0.086	0.083	0	0
	0.6	0.114	0.122	0	0
	1.0	0.148	0.161	0.009	0.007
	2.0	0.169	0.187	0.049	0.025
Bis- <i>p</i> -nitrophenyl phosphate	2.0	0.480	0.338	0.159	0.056

A study was made of the effect of temperature on the rate of the reactions catalysed by the alkaline phosphatase and the two phosphodiesterases under the conditions of Assays I and II respectively. The results (Fig. 3) show that the activity of the alkaline phosphatase was essentially constant over the range 20–40°C, but decreased rapidly at higher temperatures. In contrast, both phosphodiesterases were most active at 30°C, but lost activity steadily as the incubation temperature was raised further; the alkaline phosphatase-associated phosphodiesterase appeared to be particularly unstable above 40°C.

From the results discussed above it seems clear that the two phosphodiesterase activities are due to different enzymes, since they are readily separated and their substrate specificities and saturation curves differ. The alkaline phosphatase and its associated phosphodiesterase must also be separate enzymes in view of their difference in stability both during purification and as a function of reaction temperature. The main phosphodiesterase is active against DNA and is therefore a nuclease, or a mixture of nucleases; it is the first exo- or endo-nuclease to be isolated from an extremely halophilic bacterium.

Fig. 3. Effect of incubation temperature on the activity of *Halobacterium cutirubrum* alkaline phosphatase and phosphodiesterase

The activities of *H. cutirubrum* alkaline phosphatase, phosphodiesterase and alkaline phosphatase-associated phosphodiesterase were measured at the indicated incubation temperatures under the conditions of the appropriate standard assay (see Assays I and II, Experimental section). The results are expressed as a percentage of the appropriate activity at 30°C (○). ●, Alkaline phosphatase; □, phosphodiesterase; ▲, alkaline phosphatase-associated phosphodiesterase.

Effect of (NH₄)₂SO₄ and alkali-metal chlorides on the elution volume of the alkaline phosphatase from gel columns

We have previously used gel filtration in buffers containing 2.5M-KCl/1M-NaCl (Louis *et al.*, 1971; Fitt *et al.*, 1975; Fitt & Peterkin, 1976) or 4M-KCl (Kim & Fitt, 1977) for estimation of the approximate molecular weight of several *H. cutirubrum* enzymes. By this method the 20-fold purified alkaline phosphatase was found to have a mol.wt. of only 15 500 (Fitt & Peterkin, 1976). Louis *et al.* (1971) made a detailed study of the molecular-weight values obtained for three highly purified *H. cutirubrum* enzymes by gel filtration and sucrose-density-gradient centrifugation in 2.5M-KCl/1M-NaCl, and proved that gel filtration was not subject to adsorption artifacts under these conditions. However, the fact that halophile proteins are adsorbed to gels from concentrated (NH₄)₂SO₄ solutions made further study of this phenomenon desirable.

Table 3 shows the effect on the elution volumes of *H. cutirubrum* alkaline phosphatase during gel fil-

Table 3. Study of the effect of ionic strength on the gel filtration of *Halobacterium cutirubrum* alkaline phosphatase

Gel filtration was performed as described by Fitt & Peterkin (1976) and Louis *et al.* (1971). All the buffers contained 10mM-Tris/HCl and their pH was adjusted to 8.0; in addition, (NH₄)₂SO₄ buffers contained 10mM-MnCl₂ and 10mM-2-mercaptoethanol. After each change of buffer, the columns were washed with several column volumes of the new buffer before application of the next sample of alkaline phosphatase in the appropriate buffer (1–2ml; total units approx. 150). Elution was performed at 26ml/h and 4ml fractions were collected. The active fractions were located by using the standard Assay I (see the Experimental section). The columns were 2.5 cm in diameter and had the length shown in the Table under the appropriate gel. All elution volumes are the average of two values. (The latter did not differ by more than 4ml, i.e., one fraction.)

Gel	Salt	<i>I</i>	Elution vol. (ml)
Sephadex G-50 (column length 90 cm)	2.5M-KCl/1M-NaCl	3.5	232
	0.25M-KCl/0.1M-NaCl	0.35	232
	1.2M-(NH ₄) ₂ SO ₄	3.6	264
Sephacrose 6B (column length 33 cm)	0.25M-(NH ₄) ₂ SO ₄	0.75	136
	0.50M-(NH ₄) ₂ SO ₄	1.5	144
	1.00M-(NH ₄) ₂ SO ₄	3.0	152
	1.20M-(NH ₄) ₂ SO ₄	3.6	172
	1.30M-(NH ₄) ₂ SO ₄	3.9	180
	1.40M-(NH ₄) ₂ SO ₄	4.2	200
	1.45M-(NH ₄) ₂ SO ₄	4.35	228
	1.50M-(NH ₄) ₂ SO ₄	4.5	276
	1.60M-(NH ₄) ₂ SO ₄	4.8	292
	2.00M-(NH ₄) ₂ SO ₄	6.0	Not eluted

tration of buffers containing different concentrations of either $(\text{NH}_4)_2\text{SO}_4$ or a 2.5:1 (mol/mol) mixture of KCl and NaCl.

Gel filtration of the alkaline phosphatase on a column (2.5 cm $\times$ 90 cm) of Sephadex G-50 in the presence of either 2.5 M-KCl/1 M-NaCl (I3.5) or 0.25 M-KCl/0.1 M-NaCl (I0.35) gave an identical elution volume (232 ml) in both cases. Subsequent calibration of the column with Blue Dextran, cytochrome *c*, myoglobin and α -chymotrypsinogen as previously described (Louis *et al.*, 1971) gave an approx. mol.wt. of 15 100 for the enzyme, in excellent agreement with the value of 15 500 obtained for the partially purified enzyme by Fitt & Peterkin (1976) by using a Sephadex G-100 column run in 2.5 M-KCl/1 M-NaCl. Thus a 10-fold change in the ionic strength of this buffer system did not affect the behaviour of either the halophile enzyme or the non-halophile standards. Further, Fitt & Peterkin (1976) showed that the alkaline phosphatase and non-halophile standards behaved as expected during gel filtration on Sepharose 6B in the presence of 2.5 M-KCl/1 M-NaCl.

In contrast, gel filtration on the same Sephadex G-50 column in the presence of 1.2 M- $(\text{NH}_4)_2\text{SO}_4$ (I3.6) led to a 14% increase in the elution volume (to 264 ml) of *H. cutirubrum* alkaline phosphatase. Further, the three protein standards behaved abnormally, so that the column could not be calibrated under these conditions.

This effect of $(\text{NH}_4)_2\text{SO}_4$ was investigated further by using a Sepharose 6B column (2.5 cm $\times$ 33 cm), since the agarose gel withstands constant changes in ionic strength better than Sephadex G-50. Table 3 shows that there was a steady increase in the elution volume (V_e) of the alkaline phosphatase with increasing $(\text{NH}_4)_2\text{SO}_4$ concentration from 0.25 to 2.0 M [at which point the enzyme could not be eluted without lowering the $(\text{NH}_4)_2\text{SO}_4$ concentration]. The change in ionic strength from 0.75 to 4.8 [0.25 M- to 1.6 M- $(\text{NH}_4)_2\text{SO}_4$] led to a 115% increase in the V_e of the enzyme, and a relatively small increase in ionic strength from 3.0 to 3.6 [1.0 M- to 1.2 M- $(\text{NH}_4)_2\text{SO}_4$] caused a 13% change in V_e , whereas a 10-fold increase in ionic strength of the KCl/NaCl buffer had no effect on the behaviour of the enzyme on the Sephadex G-50 column. Thus gel chromatography in presence of $(\text{NH}_4)_2\text{SO}_4$ is affected by adsorption at all concen-

trations of the salt and cannot be used in analytical work, although the method is of great value for enzyme purification.

In contrast, gel filtration in the presence of KCl and NaCl occurs normally, at least up to an ionic strength of 3.6, in agreement with the results of our previous detailed studies (Louis *et al.*, 1971). It is therefore a valid method for the determination of the approximate molecular weights of extreme-halophile proteins at salt concentrations where they are still comparatively stable.

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