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CALF THYMUS ALKALINE PHOSPHATASE

I. PROPERTIES OF THE MEMBRANE-BOUND ENZYME

P.L. EY * and E. FERBER

Max-Planck-Institut für Immunbiologie, D-78 Freiburg/Br. (G.F.R.)

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Summary

A membrane fraction from calf thymocytes was used to investigate molecular and catalytic properties of membrane-bound alkaline phosphatase (orthophosphoric-monoester phosphohydrolase EC 3.1.3.1). The principal findings were:

1. Solubilization of membranes with the non-ionic detergent Triton X-100 increases alkaline phosphatase activity by 30—40%. The enzyme activity elutes in a single peak (Stokes' radius = 7.7 nm) after chromatography in Sepharose 6B in the presence of Triton X-100. The activity also sediments as a single component of approx. 6.4 S during centrifugation in sucrose gradients containing Triton X-100.

2. Ion-exchange chromatography and isoelectric focusing in the presence of Triton X-100 indicate substantial charge heterogeneity. Two overlapping bands, a peak at pH 5.92 with a pronounced shoulder at pH 5.29, are apparent by isoelectric focusing.

3. The pH optimum for hydrolysis of *p*-nitrophenylphosphate (pNPhP) by the undissolved enzyme(s) is 9.57. Half-maximal activity occurs at pH 8.65 and pH 10.45. Triton X-100 has no effect on the pH profile.

4. Catalytic activity is affected by amines, especially analogues of ethanolamine. Diethanolamine exerts a unique stimulatory effect, but does not change the pH dependency. Increasing the concentration of diethanolamine from 0 to 1 M causes a 6-fold increase in K_m and a 10-fold increase in the rate of hydrolysis of pNPhP. Glycine is inhibitory.

5. EDTA causes an irreversible loss of activity with $t_{1/2}$ (1 mM EDTA, pH 8.2, 23° C) = 3.5 h. Optimal activity is achieved in 0.1—1.0 mM Mg^{2+}, although

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* Present address: Department of Microbiology and Immunology, The University of Adelaide, G.P.O. Box 498, Adelaide, S.A. 5001, Australia.

Abbreviations: 2′,3′-cyclic AMP, adenosine cyclic 2′,3′-monophosphate; 3′,5′-cyclic AMP, adenosine cyclic 3′,5′-monophosphate.

this does not cause the degree of activation reported to occur with the purified enzymes. Other divalent ions are inhibitory. Concentrations required to reduce activity to 50% of control are: Zn^{2+}, 4.0 μM (no added Mg^{2+}) and 30 μM (in the presence of 1 mM Mg^{2+}); Mn^{2+}, 0.25 mM (± Mg^{2+}); Ca^{2+}, 20 mM (± Mg^{2+}).

6. Monovalent cations have little effect on activity. In the absence of added Mg^{2+}, 50—150 mM Na^+ is partially inhibitory, but markedly less so in the presence of 1 mM Mg^{2+}. K^+ has no significant effect.

7. Of the substrates tested, pNPhP (K_m = 44 μM) was most rapidly hydrolyzed. Other substrates (rate relative to pNPhP) were α-naphthylphosphate (0.79), 2′-AMP (0.80), 5′-AMP (0.70), 3′-AMP (0.63), α-glycerophosphate (0.47) and glucose 6-phosphate (0.35). Phosphodiesterase activity was $\leqslant$10% of the phosphomonoesterase activity (for pNPhP) as evidenced by the lack of hydrolysis of bis(*p*-nitrophenyl)-phosphate and cyclic 3′,5′-AMP. The ability of these substances to inhibit hydrolysis of pNPhP reflected their capacity as substrates, i.e. the most inhibitory were the most rapidly hydrolyzed.

Introduction

Although "non-specific" alkaline phosphatase activity (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) is found in variety of mammalian tissues, the functional significance of this (these) enzyme(s), as well as the identity of the natural substrate(s), remains unclear [1]. Alkaline phosphatase activity is associated with particulate material and in subcellular fractionation it sediments largely with the microsomes [2—4]. Furthermore, histochemical and biochemical evidence indicates that, in thymocytes at least, a large proportion of the total cellular activity resides in the plasma membrane [4,5].

Enzymes exhibiting alkaline phosphatase activity have recently been purified from rat liver [6], human placenta [7], porcine and bovine kidney [8,9] and mouse duodenum [10]. They exhibit high turnover numbers (e.g. 2600 s^{-1}; ref. 8) and only small absolute amounts of enzyme can be obtained from large quantities of tissue ($\leqslant$8 mg/kg of tissue; refs. 6 and 8). The purified enzyme has been shown to be a glycoprotein with a molecular weight of 150 000—170 000 [6,8,9], an $s_{20,w}$ of between 6 and 7 [8,9] and a carbohydrate content of 15—20% [8,9]. Various forms, which can be distinguished by ion-exchange chromatography or electrophoresis [3,9—11], have been shown to contain variable amounts of neuraminic acid [8,11]. The enzyme contains Zn^{2+} which is essential for activity and has binding sites for Mg^{2+}, which is stimulatory [6,9,12—14]. Incubation with EDTA or at low pH results in a rapid, usually irreversible loss of activity [3,6,14].

Recently, increasing attention has been given to the regulatory role of lipids and other membrane components in the activity of membrane-bound enzymes [15—17]. The findings that (a) alkaline phosphatase purified from pig kidney undergoes a phase transition at 25°C [14] and (b) that a sterol and small hydrophobic peptides have been found associated with alkaline phosphatase purified from mouse duodenum [10] raised the possibility that this enzyme might also be regulated by its membrane micro-environment. Nearly all previous work on alkaline phosphatase has been done on enzymes extracted with

organic solvents (e.g. butanol, acetone) and purified by salt fractionation and chromatography [6—10]. The characteristics of such enzymes, separated from most if not all of the lipid with which they are normallly associated, may differ substantially from those of the native, membrane-bound enzyme.

The purpose of this study was 2-fold. Firstly, to partially characterize the alkaline phosphatase of calf thymus, for which there is as yet no published data. Secondly, to compare the properties of this membrane-bound enzyme to those reported by other laboratories for alkaline phosphatases purified by extraction with organic solvents. The properties of the particulate enzyme were investigated after it had been established that the activity being measured in a purified plasma membrane fraction was not that of a complex mixture of different enzymes.

Materials and Methods

Chemicals. Tris(hydroxymethyl)aminomethane (Tris), Triton X-100 (Scintillation grade) and sodium α-naphthylphosphate were obtained from Serva (Heidelberg). Sepharose 6B and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden) and Ampholines from LKB. Bis(*p*-nitrophenyl)-phosphate, *p*-nitrophenylphosphate, 2′,3′-cyclic AMP, 3′,5′-cyclic AMP, 2′-,3′- and 5′-AMP, ADP, ATP, α-glycerophosphate and glucose 1- and 6-phosphate were purchased from Boehringer (Mannheim). Mono-, di- and triethanolamine and choline chloride were from E. Merck (Darmstadt). $Na^{125}I$, carrier-free, 20—140 Ci/l was obtained from Buchler Amersham (Braunschweig). All other reagents were of reagent or analytical grade.

Proteins. Bovine serum albumin was obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Rat IgM and rabbit IgG were purified from the respective sera by precipitation with 45% saturated $(NH_4)_2SO_4$ followed by chromatography on Sephadex G-200 and DEAE-cellulose [18].

Preparation of thymocyte low density microsomal MS(U) membranes. The details for preparing thymocyte suspensions, cell disruption and subellular fractionation have been described elsewhere (ref. 19; Ey, P.L. and Ferber, E., to be submitted). Briefly, cell suspensions of thymii from freshly slaughtered calves were treated by the "nitrogen cavitation" method [20,19] and the microsomes isolated by differential centrifugation of the "homogenate". The microsomal material was then further fractionated according to particle density by centrifugation in discontinuous sucrose density gradients (45%/35%, w/v buffer; Beckman Ti60 rotor, 50 000 rev./min, 5° C, 2 h). The membranous material banding between the buffer and 35% sucrose layer (d = 1.132) was washed with and resuspended in 10 mM NaCl/25 mM KCl/10 mM Tris · HCl, pH 8.2, containing 15 mM NaN_3 (Buffer A). This material (the "upper" microsomal band, termed MS(U) membrane) contained 1.01 ± 0.11 μmol of phospholipid and 0.73 ± 0.12 μmol of cholesterol per mg of protein (mean ± S.D.) (Ey, P.L. and Ferber, E., in preparation) and was stored unfrozen at 0° C. No changes in total alkaline phosphatase activity or specificity could be detected under these conditions, even after several months. No evidence was found to suggest that the enzyme was in any way degraded or altered during storage. However, a new preparation was made each week and fresh preparations were used in most experiments.

Iodination of marker proteins. Small amounts (10—20 μg) of protein were trace labelled with ^{125}I using chloramine-T as described elsewhere [21].

Analytical methods. Protein was determined by the ninhydrin method [22]. Inorganic and total phosphate (ashed with 10% $Mg(NO_3)_2$ in 95% ethanol) were assayed by the method of Ames and Dubin [23].

Alkaline phosphatase assay. Because the assay conditions were in many experiments altered to investigate the properties of the enzyme, exact conditions are described in each figure and table legend. Except where substrate specificity was measured (Table II), pNPhP was used as substrate, routinely at 2.5 mM. Routine assays, such as on fractions from gel chromatography, were done in 1 mM $MgCl_2$/0.5% Triton X-100/0.1 M diethanolamine · HCl, pH 9.5. Except where specifically stated, Triton X-100 was not used in the assay. Assay mixtures of 1.0 ml were stopped with 0.2 ml of 5 M NaOH and activity determined from absorbance at 405 nm, using an extinction coefficient for *p*-nitrophenol of 18 200 $l \cdot mol^{-1} \cdot cm^{-1}$. The activity at 37° C was constant for at least 90 min, providing <5% of the substrate was hydrolyzed.

Isoelectric focusing. This was performed at 4° C in 4% polyacrylamide gel rods (0.5 × 9 cm; cross-linked 2.5%) containing 2% Ampholines (pH range 3.5—10), 10% sucrose and 1% Triton X-100 as described by Bhakdi et al. [24], but with water substituted for urea. Membrane samples were made 1% with Triton X-100 and 20% with sucrose and 0.1-ml aliquots applied to each gel. Focusing was performed for 16 h at 250 V (constant). Each gel, including blanks, was then cut into 5-mm slices which were mixed with 0.5 ml of deaerated water and left for 90 min at room temperature. The pH of each tube was measured using a small electrode, after which 1.0 ml of 2 mM $MgCl_2$/0.5 M diethanolamine · HCl (pH 9.5) was added. After a further 60 min at 25° C, 0.1 ml of 25 mM pNPhP was added and hydrolysis allowed to proceed for 90 min at 25° C.

DEAE-cellulose chromatography. MS(U) membranes (0.6 ml; 0.5 mg protein) were mixed with 1.4 ml of 25 mM Tris · HCl, (pH 8.2) containing 0.1% Triton X-100 and 5 mM NaN_3 (starting buffer). The clear solution was dialyzed overnight at 4° C against 200 ml of starting buffer and then applied at room temperature to a 1.0 × 45 cm column of DEAE-cellulose equilibrated with starting buffer. The column was eluted at 0.22 ml/min with a linear salt gradient (0—0.6 M NaCl) containing 25 mM Tris, pH 8.2.

Results

Size of membrane-bound alkaline phosphatase

Before determining the kinetic properties of membrane-bound alkaline phosphatase, it was necessary to ascertain that the activity being measured in the purified membranes did not result from a number of quite different enzymes. The molecular heterogeneity of the membrane-associated enzyme(s) was therefore investigated.

Membranes were first dissolved in Triton X-100 and chromatographed on Sepharose 6B. It can be seen in Fig. 1 that essentially all of the alkaline phosphatase activity applied to the column eluted in a single, symmetrical peak between IgG and IgM. Using the relationship between $(-\log_{10} K_{av})^{1/2}$ and Stokes' radius (r) [25,26], the position of this peak corresponded to a molecule

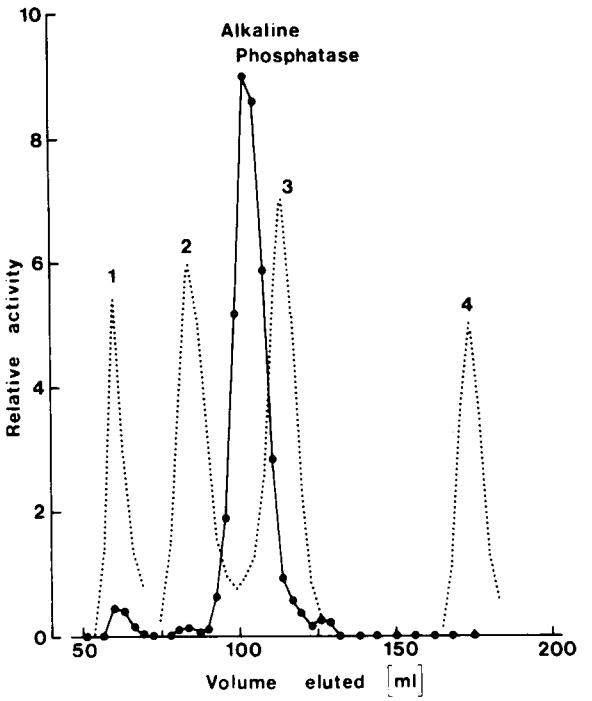

Fig. 1. Sepharose 6B chromatography of Triton X-100-solubilized microsomal alkaline phosphatase. A 2.5 ml sample containing MS(U) membranes (0.65 mg protein), Blue Dextran 2000 (0.4 mg/ml), ^{125}I-labelled IgM (16 000 cpm), IgG (21 000 cpm), *p*-nitrphenol (0.2 mM) and Triton X-100 (5%) in buffer A was applied to a 1.5 × 90 cm column of Sepharose 6B equilibrated with buffer A containing 0.05% Triton X-100. Elution was performed at room temperature with the latter solution at a flow rate of 5.32 ml/h. Fractions of 3.0 ml were collected and aliquots assayed for alkaline phosphatase, radioactivity and absorbance at 620 and 405 nm. The dotted peaks indicate the positions of Blue Dextran (1), IgM (2), IgG (3) and *p*-nitrophenol (4).

of $r = 7.7$ nm. Only a small proportion (<5%) of the applied enzyme activity remained aggregated to any degree, as evidenced by the minor peak eluting in the void volume (60 ml). In contrast, when undissolved membrane was applied to the column and the latter eluted in the absence of detergent, all of the applied enzyme activity eluted in the void volume.

Samples of Triton X-100-dissolved membrane were also analyzed by velocity sedimentation in sucrose gradients (Fig. 2). Again, all alkaline phosphatase activity was recovered in one peak with an apparent sedimentation coefficient (relative to intrinsic radioactive markers) of 6.3—6.5 S. No heterogeneity in the size of the enzyme(s) present in the membrane could therefore be detected.

Charge heterogeneity of Triton X-100-solubilized alkaline phosphatase

Efforts were also made to distinguish different membrane-bound alkaline phosphatases on the basis of their charge. Thus, Triton X-100-dissolved membranes were applied to acolumn of DEAE-cellulose which was eluted at pH 8.2 in the presence of Triton X-100 using a salt gradient. Approx. 60% of the total enzyme activity eluted as a sharp peak in the range 0.12—0.16 M NaCl. The remaining activity was released over a broad concentration range (0.16—0.34

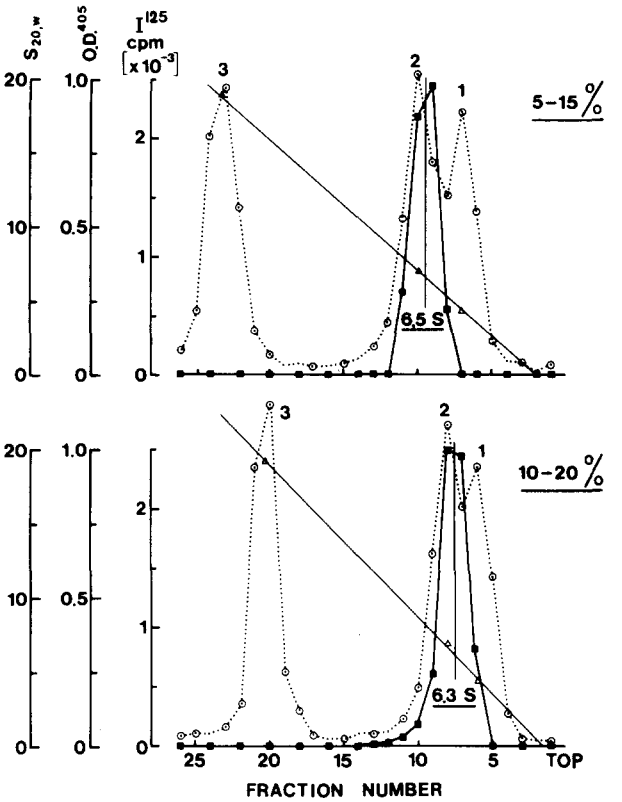

Fig. 2. Velocity sedimentation of Triton X-100-solubilized alkaline phoshatase. MS(U) membrane material (0.3 ml; 0.65 mg protein) in buffer A was mixed with 100 µl of 20% Triton X-100 and 30 µl each of ^{125}I-labelled bovine serum albumin, IgG and IgM (approx. 15 000 cpm each). A 0.2 ml aliquot of this mixture was layered over each of two precooled linear sucrose density gradients (5—15% and 10—20%, w/v, respectively, containing 0.05% Triton X-100 in buffer A). The gradients were centrifuged in a Beckman SW56 rotor at 40 000 rev./min for 11.5 h at 5° C, after which each was fractionated by upward displacement with a 30% sucrose solution. Fractions were tested for radioactivity and alkaline phosphatase activity. The position of the radio-labelled markers bovine serum albumin (1), IgG (2) and IgM (3) are indicated and these have been plotted against the sedimentation coefficients of these proteins [27].

M), indicating a substantial degree of charge heterogeneity among a significant although minor proportion of enzyme molecules.

When solubilized membranes were electrofocused in the presence of Triton X-100, two overlapping bands were obtained. The larger part (>60%) of the enzyme activity focused with apeak pK_i of 5.92. This band had a pronounced shoulder on its acidic side, indicating an overlapping peak of pK_i 5.29. This finding was consistent with that from DEAE-cellulose chromatography (above), where the larger fraction of enzyme molecules was less acidic than the remainder. Purified alkaline phosphatases from other sources have been shown to consist of populations of identical enzyme molecules to which different amounts of neuraminic acid are covalently linked and which can be separated on DEAE-cellulose [8,9,11]. The alkaline phosphatase activity of the purified thymus membranes was therefore considered to be due to molecules of a single type of enzyme which were similarly heterogeneous with respect to their neuraminic acid content.

Effect of pH

The pH profile of the particulate enzyme was determined using different concentrations of diethanolamine buffer. The overall activity was markedly enhanced by diethanolamine, which has been reported to stimulate serum alkaline phosphatase activity [28,29], but the pH dependency remained unchanged. Inclusion of Triton X-100 in the assay solution also had no effect on the pH profile, although overall stimulation of activity resulted. The pH optimum was 9.57, with half-maximal activity at pH 8.65 and at pH 10.45.

Influence of amine derivatives

To investigate which chemical groups are involved in the stimulation of alkaline phosphatase by diethanolamine, other chemically related derivatives were tested. The results are shown in Fig. 3A. The various analogues had significantly different effects on the enzyme, but diethanolamine was unique in its ability to stimulate activity so greatly. All of the substances whose effects are depicted in Fig. 3A have an ethanolamine ($NH_2CH_2CH_2OH$) base. Ethanolamine itself had the least effect, enhancing activity by approx. 70% at (relatively) low concentration (50—100 mM) but having no effect at concentrations $\geqslant$0.4M. Choline ($(CH_3)_3N^+CH_2CH_2OH$) and triethanolamine were more effective, especially at higher concentrations ($\geqslant$0.5 M), enhancing activity maximally by about 150%. Tris (not shown), which is also an ethanolamine analogue ($NH_2C(CH_2OH)_2CH_2OH$), gave a curve almost identical to that of tri-

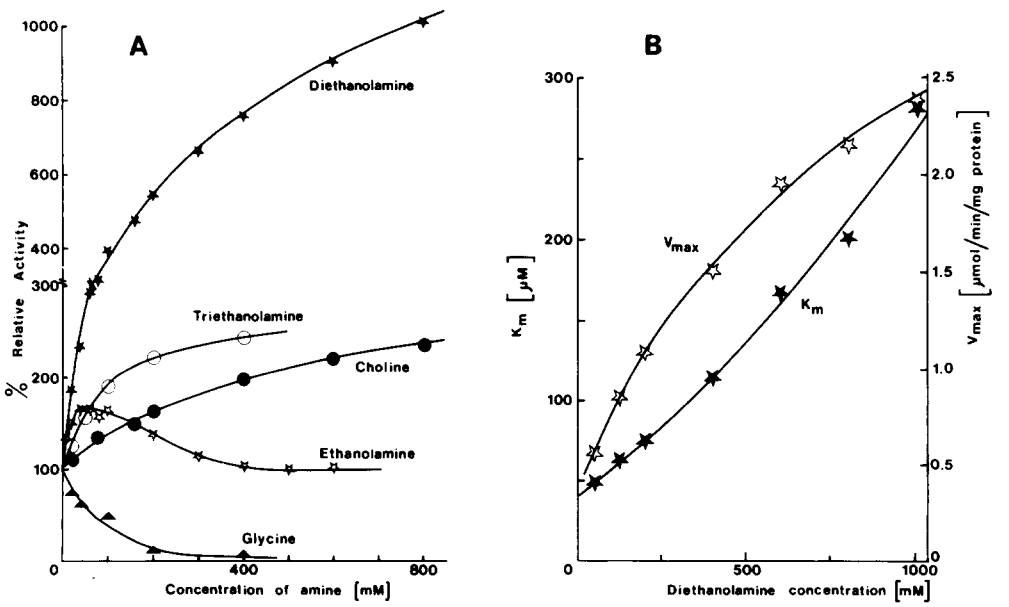

Fig. 3 (A) Effect of ethanolamine analogues on the rate of hydrolysis of pNPhP by alkaline phosphatase. Each duplicate assay contained the indicated amine (adjusted beforehand to pH 9.5 with HCl; glycine with NaOH) in 25 mM sodium barbitone (pH 9.5), 10 mM $MgCl_2$ and 2.5 mM pNPhP. (B) Effect of diethanolamine on K_m and V of alkaline phosphatase at pH 9.5. For each concentration of diethanolamine, K_m and V were calculated from a double reciprocal plot [30] after determining the rate of hydrolysis of pNPhP at 10 different concentrations (0.06—1.25 mM) in the presence of 1 mM $MgCl_2$.

ethanolamine. In contrast, glycine (NH_2CH_2COOH), which structurally is ethanolamine with a carbonyl oxygen attached to the C1(hydroxy) atom, was a powerful inhibitor which caused nearly 100% inhibition at 0.2 M.

The effect of diethanolamine was clearly distinct from these other related substances, causing greater enhancement of activity at all concentrations (total activity 4-fold at 0.1 M; 10-fold at 0.8 M). It can be seen from Fig. 3B that V and K_m (pNPhP) both increased non-linearly as the concentration of diethanolamine was raised. The K_m for pNPhP (44 μM in the absence of diethanolamine) increased to 281 μM in 1 M diethanolamine and was accompanied by a 10-fold increase in V.

Sensitivity to inactivation by EDTA or low pH

The results of incubating microsomal membranes in solutions containing EDTA or of different pH are shown in Table I. EDTA inactivated membrane-bound alkaline phosphatase by a concentration-dependent process ($t_{1/2}$: 37 h, 0.1 mM; 3.5 h, 1 mM) whose rate was marginally increased when the membranes were solubilized in Triton X-100 (9.5 h, 0.1 mM; 3.3 h, 1 mM). If EDTA (1 mM) was substituted for Mg^{2+} in the assay solution, substrate hydrolysis was inhibited by >95%. The enzyme was found quite resistant to inactivation in solutions of low pH, with $t_{1/2} \geqslant 40$ h at pH 4.0. This stability did not seem to be affected by dissolution in Triton X-100.

Effect of inorganic cations

Mg^{2+} exerted a slight stimulatory effect on the activity of membrane-bound alkaline phosphatase which was maximal in the concentration range 0.1—1.0

TABLE I

SENSITIVITY OF MEMBRANE-BOUND AND TRITON X-100-SOLUBILIZED ALKALINE PHOSPHATASE ACTIVITY TO EDTA AND LOW pH

Fixed amounts of MS(U) membrane material (28 μg protein/ml) were incubated at room temperature (23° C) as 0.1 ml aliquots in 50 mM NaCl/10 mM Tris · HCl, pH 8.2/15 mM NaN_3 containing Triton X-100 or EDTA as indicated. For incubations at acid pH, the same buffer was adjusted beforehand to the desired pH with 1 M acetic acid. At selected intervals, samples were mixed with 0.8 ml of 1.25 m $MgCl_2$/0.625% Triton X-100/125 mM diethanolamine · HCl (pH 9.5), incubated 2 min at 37° C and then assayed for alkaline phosphatase activity after the addition of 0.1 ml of 25 mM pNPhP. Half-lives were calculated by plotting $\log_{10}(N_0/N_t)$ against time, where N_0, initial activity; N_t, activity at time t.

Incubation conditions		Half-life at 23° C (h)	
pH	EDTA	No Triton X-100	1% Triton X-100
8.2	0.1 mM	37.2	9.5
8.2	0.25 mM	4.6	4.1
8.2	1.00 mM	3.5	3.3
4.0	—	40.3	n.d.
5.0	—	n.d.	n.d. (≤10 min; ref. 8) *
5.5	—	—	— (2 min; ref. 9) *
6.0	—	n.d.	n.d.
8.2	—	n.d.	n.d.

n.d., not detectable (>95% of activity remaining after 25 h; $t_{1/2} > 330$ h).

* Purified enzyme.

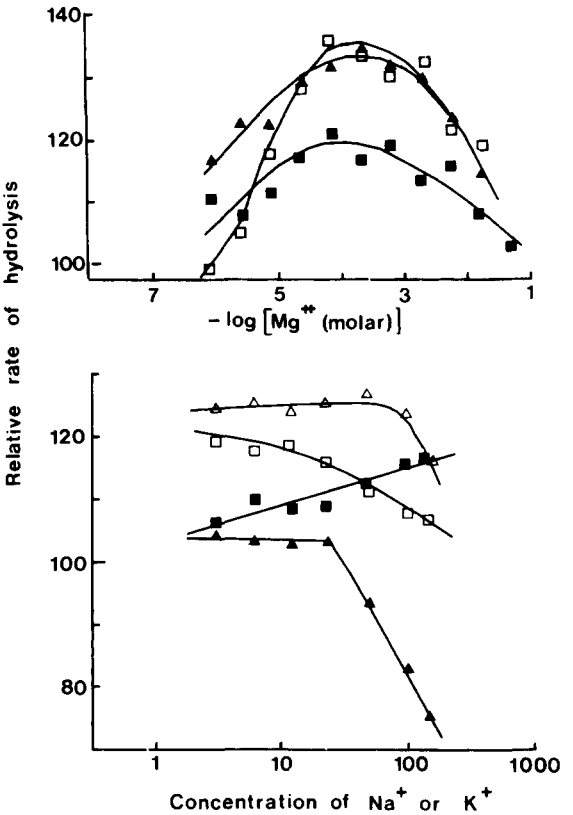

Fig. 4. Top. Effect of Mg^{2+} on alkaline phosphatase activity. Hydrolysis rates for 2.5 mM pNPhP in 0.2 M diethanolamine · HCl buffer (pH 9.5) by 0.78 μg protein in 1 ml at 37°C. For these measurements, the membrane fraction was washed with and resuspended in 50 mM NaCl/10 mM Tris · HCl (pH 8.2)/15 mM NaN_3 and subsequently diluted with distilled water for the assay (final dilution, 1 : 2000). Control rate (100) = rate of hydrolysis with no added cations. Symbols: ■, no other added cations; □, including 50 mM NaCl; ▲, including 50 mM KCl. Bottom. Effect of Na^+ and K^+ on alkaline phosphatase activity. Assay conditions as described above. Symbols: △, ▲, increasing NaCl; □, ■, increasing KCl. Closed symbols: activity with no other added cations. Open symbols: activity in presence of 1 mM $MgCl_2$.

mM (Fig. 4, top). The presence of either Na^+ or K^+ (50 mM) had no significant influence on the effect of Mg^{2+}.

In the absence of added Mg^{2+}, Na^+ was partially inhibitory at physiological concentrations (25% at 0.15 M; Fig. 4, bottom). However, in the presence of Mg^{2+} (1 mM) this effect was substantially reduced. K^+ had no noticeable effect at any concentration with or without added Mg^{2+}.

Divalent cations other than Mg^{2+} were inhibitory. As can be seen from Fig. 4 (top), Mg^{2+} was not inhibitory, even at 50 mM. In contrast, Zn^{2+}, which had no effect at ≤ 0.2 μM, was at higher concentrations a potent inhibitor, resulting in 50% inhibition at 4.0 μM, 85% at 31 μM and 93% at 100 μM (Fig. 5). The inhibitory effect of Zn^{2+} at low concentrations (≤ 6 μM) could be completely overcome by Mg^{2+} (1 mM), in the presence of which the enzyme was slightly activated (30% in 4 μM Zn^{2+}) with respect to the control. However, higher Zn^{2+} concentrations were potent even in 1 mM Mg^{2+}, 30 and 100 μM resulting in

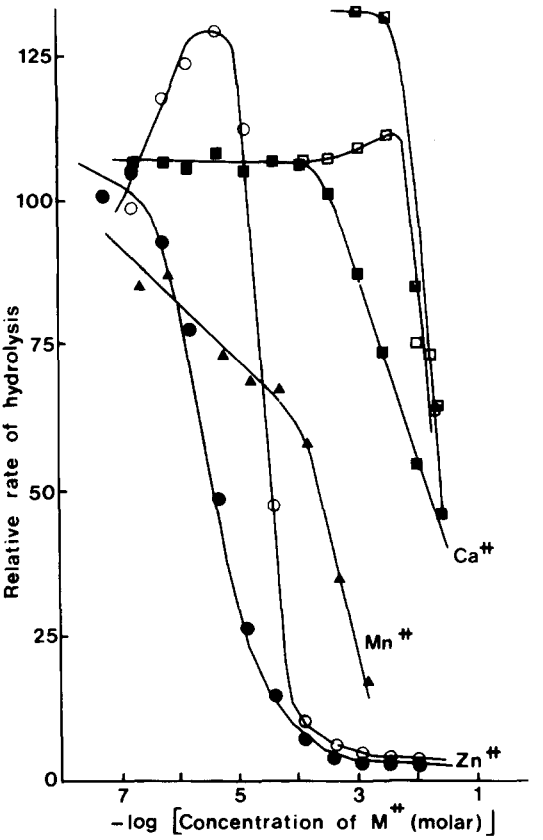

Fig. 5. Effect of divalent cations on activity of alkaline phosphatase. Assay conditions as described in Fig. 4. Symbols. Closed, no added cations other than the one indicated; open, including 1 mM $MgCl_2$; half-closed (Ca^{2+} only), including 1 mM $MgCl_2$ and 2.5 μM $ZnCl_2$. Each point represents the mean of two experiments.

50- and 89% inhibition, respectively. Mn^{2+} was a less effective inhibitor and exhibited a biphasic inhibition curve, 0.25 mM being required for 50% inhibition. Mg^{2+} (1 mM) had almost no effect on Mn^{2+} inhibition.

Ca^{2+} was a very poor inhibitor of alkaline phosphatase. For 50% inhibition, 17 mM was required in the absence of other added cations and 22 mM in the presence of either 1 mM Mg^{2+} or of 1 mM Mg^{2+} and 2.5 μM Zn^{2+}. These results suggested an essential role of Zn^{2+} and Mg^{2+} for the hydrolytic activity of alkaline phosphatase, Zn^{2+} being capable of competing with Mg^{2+} for the Mg^{2+}-binding site(s).

Substrate specificity

Purified mammalian alkaline phosphatases hydrolyze orthophosphate and pyrophosphate esters as substrates [6,14,31]. This specificity was verified for the membrane-bound enzyme from calf thymus (Table II). pNPhP was hydrolyzed most rapidly, followed (in order of decreasing rate of hydrolysis) by α-naphthylphosphate, 2'-AMP, 5'-AMP, 3'-AMP, α-glycerophosphate and glucose 6-phosphate. The orthophosphate diesters bis(*p*-nitrophenyl)-phosphate

TABLE II

SUBSTRATE SPECIFICITY OF MEMBRANE-BOUND ALKALINE PHOSPHATASE

Enzyme activity was measured by release of orthophosphate at 37°C using the method of Ames and Dubin [23]. Each 0.5 ml assay contained 200 mM diethanolamine · HCl (pH 9.5), 1 mM $MgCl_2$, 4 mM substrate and 2.1 μg of MS(U) membrane protein. At 5 min intervals, 1.2 ml of phosphate reagent were added to duplicate assays for each substrate and colour was developed by incubation for 20 min at 45°C. Blanks included enzyme without substrate and vice versa. Values represent means of determinations on three preparations.

Substrate (4 mM)	Relative rate of hydrolysis (%)
p-Nitrophenylphosphate	100
α-Naphthylphosphate	78.7
2′-AMP	79.6
3′-AMP	63.2
5′-AMP	70.4
2′,3′-Cyclic AMP	24.0
3′,5′-Cyclic AMP	⩽9.5
Glucose 6-phosphate	34.7
α-Glycerophosphate	46.8
Bis(*p*-nitrophenyl)-phosphate	⩽11.2

and 3′,5′-cyclic AMP were not hydrolyzed to any detectable degree and 2′,3′-cyclic AMP at 24% of the rate for pNPhP.

As can be seen in Table III, the ability of these and other substances to inhibit hydrolysis of pNPhP reflected their capacity as substrates. Bis(*p*-nitrophenyl)-phosphate and the two cyclic AMP compounds caused no appreciable

TABLE III

SUBSTRATE INHIBITION OF MEMBRANE-BOUND ALKALINE PHOSPHATASE

The rate of hydrolysis of pNpHP was determined at 37°C in 1 mM $MgCl_2$ and 50 mM diethanolamine · HCl (pH 9.5) at eight different concentrations of pNPhP (0.1—1.0 mM) in the presence of a fixed concentration of each inhibitor. Inhibitor constants (K_i) and V were calculated from double reciprocal plots [30]. Values represent means of determinations on three preparations. V is compared to uninhibited control (100% = 730 nmol of pNPhP hydrolyzed/min per mg of protein).

Inhibitor	K_i (μM)	Relative V (%)
None	*	100
α-Naphthylphosphate	35	91.8
2′-AMP	87	95.7
3′-AMP	176	62.3
5′-AMP	540	81.5
ADP	317	51.5
ATP	931	45.6
2′,3′-Cyclic AMP	4037	97.0
3′,5′-Cyclic AMP	∞	99.6
Adenosine	∞	100
Glucose 1-phosphate	943	105.1
Glucose 6-phosphate	793	104.9
α-Glycerophosphate	1068	105.7
Orthophosphate	253	99.7
Bis(*p*-nitrophenyl)-phosphate	∞	99.3

* K_m for pNPhP = 44 μM.

inhibition, indicating that orthophosphate diesters are poorly recognized. The inhibition by orthophosphate (product inhibition) and by all the orthophosphate monoesters was purely competitive, whereas that of ADP and ATP was of a mixed type. The glucose 1- and 6-monophosphates and α-glycerophosphate were very poor inhibitors compared to the adenosine monophosphates and α-naphthylphosphate.

Discussion

The uniform molecular size of calf thymus alkaline phosphatase, together with the finding that more than half of these molecules were similar in pK_i and overall charge at pH 8.2, indicated that the enzyme activity of our purified membranes was not due to several phosphatase enzymes differing in molecular and kinetic properties. Moreover, the charge characteristics of the Triton X-100-solubilized enzymes were consistent with the known heterogeneity in neuraminic acid content of purified alkaline phosphatase [3,9—11]. Although the content of neuraminic acid can affect the charge and stability properties of the purified enzyme, it appears to have little influence on the kinetic properties [11].

Using the Stokes' radius of 7.7 nm and the (apparent) sedimentation coefficient of 6.4 determined for the calf thymus enzyme in Triton X-100, the approximate molecular weight and frictional coefficient (f/f_0) of the detergent-solubilized enzyme can be calculated. Thus, assuming $0.73 < v < 0.78$ [8,32], the molecular weight should be 206 600—253 000 with f/f_0 in the range 1.97—1.80 [26]. Note that these figures are for the enzyme in Triton X-100, i.e., for enzyme plus bound Triton X-100 [32—34]. If the molecular weight (150 000—170 000) of purified alkaline phosphatases [6,8,9] is taken to be the real molecular weight range of the calf thymus enzyme, this estimate indicates 37 000—103 000 daltons (57—160 mol) of bound detergent/mol of enzyme.

The marked stimulation of alkaline phosphatase by diethanolamine was in agreement with previous findings [28,29]. This substance changed both K_m and V in a complex manner. The unique action of diethanolamine was shown by the inability of structurally related amines to activate alkaline phosphatase to a similar degree. That some stimulation is, however effected by ethanolamine and Tris (both primary amines), triethanolamine (tertiary) and choline (quaternary) suggests that the nature of the amino substituents, as well as the presence or absence of a positive charge on the amino group, is not critical for enzyme stimulation. The hydroxyl moiety seems important, however, as substitution of the C1 hydrogen atoms in ethanolamine (-CH_2OH) by oxygen, as in glycine (-COOH), results in severe inhibition, as reported previously [28,29]. The inhibition by aromatic and certain other amino acids at much lower concentrations (5—10 mM) would seem to be an independent phenomenon [11, 14].

Diethanolamine causes an increase of both K_m and V of calf thymus alkaline phosphatase, i.e. reduced affinity for substrate and faster formation and/or removal of product (phosphate) from the enzyme. As alkaline phosphatase is known to transphosphorylate to Tris [14] and to glucose [35], diethanolamine might act as an effective phosphate acceptor, accelerating the removal of phos-

phate from the active centre while interfering with the binding of substrate. This possibility has not been investigated.

The effects of divalent cations on calf thymus alkaline phosphatase were qualitatively similar to those found with purified alkaline phosphatases [6,9, 11,14]. Quantitatively however, the addition of Mg^{2+} in optimal amounts did not activate the membrane-bound to the extent found for e.g. purified pig kidney alkaline phosphatase (6-fold; ref. 14), nor was it as sensitive as the latter to inhibition by Zn^{2+} (50% at 4.0 and 0.3 μM, respectively). Furthermore, the membrane-bound enzyme seems significantly more resistant to inactivation by loss of essential metal ions in EDTA or at low pH. For instance, purified alkaline phosphatase from pig kidney has a reported $t_{1/2}$ of 5 min at pH 4.5, 10 min at pH 5.0 [8,14] and 36—210 min at pH 5.5 [11]; that from calf kidney [9], 2 min at pH 5.5. This compared to the membrane-bound calf thymus enzyme which had a $t_{1/2} \geqslant 40$ h at pH $\geqslant$4.0. These observations suggest that the purified enzyme may be more susceptible to loss of essential Zn^{2+}. Furthermore, as the membrane enzyme is also less sensitive to inhibition by Zn^{2+}, it may be able to retain its Mg^{2+} more effectively than the purified enzyme. Conformational restrictions applied by neighbouring membrane components or physical protection of labile portions of the enzyme by membrane lipids [31] may be important in this respect. Solubilization in Triton X-100, which probably "coats" lipophilic parts of the solubilized enzyme which are normally buried within the lipid region of the membrane, does not greatly increase the lability of the enzyme to metal ion loss.

In other respects, there seems little difference between the membrane-bound calf thymus enzyme which may have tightly bound sterols and phospholipids [10], and those purified from other tissues. The broad substrate specificity, with preference for pNPhP, poor cleavage of pyrophosphate esters and a complete inability to hydrolyze orthophosphate diesters seems common to alkaline phosphatases from most sources [3,6,14,31]. The major difference seems to be one of stability.

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References

1 Kaplan, M.M. (1972) Gastroenterology 62, 452—468
2 Michell, R.H., Karnovsky, M.J. and Karnovsky, M.L. (1970) Biochem. J. 116, 207—216
3 Lee, M.H. and Sartorelli, A.C. (1974) Biochim. Biophys. Acta 358, 69—81
4 Schmidt-Ullrich, R., Ferber, E., Knüfermann, H., Fischer, H. and Wallach, D.F.H. (1974) Biochim. Biophys. Acta 332, 175—191
5 Ruuskanen, O.J., Pelliniemi, L.J. and Kouvalainen, K.E. (1975) J. Immunol. 114, 1611—1615
6 Ohkubo, A., Langerman, N. and Kaplan, M.M. (1974) J. Biol. Chem. 249, 7174—7180

7 Doellgast, G.J. and Fishman, W.H. (1974) Biochem. J. 141, 103—112
8 Wachsmuth, E.D. and Hiwada, K. (1974) Biochem. J. 141, 273—282
9 Cathala, G. and Brunel, C. (1975) J. Biol. Chem. 250, 6040—6045
10 Nayudu, P.R.V. and Hercus, F.B. (1974) Biochem. J. 141, 93—101
11 Hiwada, K. and Wachsmuth, E.D. (1974) Biochem. J. 141, 293—298
12 Ahlers, J. (1974) Biochem. J. 141, 257—263
13 Cathala, G. and Brunel, C. (1975) J. Biol. Chem. 250, 6046—6053
14 Hiwada, K. and Wachsmuth, E.D. (1974) Biochem. J. 141, 283—291
15 Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Biochemistry 13 5501—5507
16 Cater, B.R., Trivedi, P. and Hallinan, T. (1975) Biochem. J. 148, 279—294
17 Coleman, R. (1973) Biochim. Biophys. Acta 300, 1—30
18 Methods in Immunology and Immunochemistry (1967) (Williams, C.A. and Chase, M.W., eds,) Vol. I Chapter 3, Academic Press, London
19 Ferber, E., Resch, K., Wallach, D.F.H. and Imm, W. (1972) Biochim. Biophys. Acta 266, 494—504
20 Wallach, D.F.H. and Kamat, V.B. (1964) Proc. Natl. Acad. Sci. U.S. 52, 721—728
21 Greenwood, F.C., Hunter, W.M. and Gover, J.S. (1963) Biochem. J. 89, 114—123
22 Moore, S. and Stein, W.G. (1948) J. Biol. Chem. 176, 367—388
23 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769—775
24 Bhakdi, S., Knüfermann, H. and Wallach, D.F.H. (1975) Biochim. Biophys. Acta 394, 550—557
25 Laurent, T.C. and Killander, J. (1964) J. Chromatogr. 14, 317—330
26 Siegel, L.M. and Monty, K.J. (1966) Biochim. Biophys. Acta 112, 346—362
27 Handbook of Biochemistry; Selected Data for Mol. Biol. (1968,1970) (Sober, H.A., Ed.), 2nd edn. The Chem. Rubber Co., Cleveland, Ohio, U.S.A.
28 Hausamen, T.U., Helger, R., Rick, W. and Gross, W. (1967) Clin. Chim. Acta 15, 241—245
29 Rick, W., Fritsch, W.P. and Szasz, G. (1972) Dtsch. Med. Wschr. 97, 1828—1834
30 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666
31 Fernley, H.N. and Walker, P.G. (1967) Biochem. J. 104, 1011—1018
32 Clarke, S. (1975) J. Biol. Chem. 250, 5459—5469
33 Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656—3661
34 Tanford, C., Nozaki, Y., Reynolds, J.A. and Makino, S. (1974) Biochemistry 13, 2369—2376
35 Morton, R.K. (1953) Nature 172, 65—68