

Mouse Uterine Alkaline Phosphatase: Improved Purification by Affinity Chromatography and Further Characterization of the Enzyme

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Abstract

An improved procedure for the purification of alkaline phosphatase from about 10 g of day 7 pregnant mouse uterine tissue is described. Following homogenization, the procedure involved solubilization and extraction with 0.8% (v/v) Triton X-100 and 20% (v/v) n-butanol, ammonium sulfate precipitation, concanavalin A-Sepharose 4B affinity chromatography, DEAE-cellulose anion-exchange chromatography and Sephacryl S200 gel filtration. On subjecting 2162-fold purified enzyme preparations to polyacrylamide-gel electrophoresis, a single band of protein coincident with the zone of enzyme activity and having an apparent molecular weight of $205\,000 \pm 10\,000$ was identified. Affinity chromatography yielded the largest increase in purity of any step in the procedure and established the glycoprotein nature of the uterine enzyme.

Some metalloenzyme properties of the phosphatase were also studied and it was demonstrated that both Mg^{2+} and Zn^{2+} ions are necessary for hydrolytic activity.

Treatment with neuraminidase retarded the anodal migration of the enzyme during electrophoresis on cellulose acetate membranes but did not influence its activity or catalytic properties. These results suggest that the uterine alkaline phosphatase is a sialoglycoprotein. The sialic acid residues, however, do not appear to constitute part of the active centre of the enzyme.

In addition, the optimum pH for activity depended on substrate concentration and decreased with decreasing substrate concentration. Apparent K_m values also depended on variations in pH and decreased with decreasing pH. Plots of pK_m versus pH revealed a functional group with a pK value of 9.45. The enzyme also hydrolysed a variety of compounds having either phosphomonoester or pyrophosphate linkages and was inactivated after heating at 60°C for 15 min. The activation energy, determined from a linear Arrhenius plot, was 50.1 kJ mol^{-1} .

Introduction

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) in the uterus of the mouse is associated with the induction of the decidual cell reaction and increases in activity during the peri-implantation period of pregnancy (Finn and Hinchliffe 1964; Finn and McLaren 1967; Murdoch *et al.* 1978; Parathasarathy *et al.* 1979). Attempts to elucidate the functional significance of the uterine enzyme during early pregnancy have been hampered by the small size of the mouse uterus, the accompanying small yields of enzyme, and the difficulty of obtaining highly purified preparations of the enzyme, particularly in amounts sufficient for the study of its molecular properties.

Murdoch *et al.* (1979) demonstrated that the classical procedures (Morton 1954; Moss *et al.* 1967) of extracting alkaline phosphatase from animal tissues using n-butanol could not be applied to mouse uterine tissue with any degree of success until the enzyme was first solubilized with Triton X-100. Following solubilization

and extraction, further processing using ammonium sulfate fractionation, ion-exchange chromatography, and gel-filtration techniques substantially purified the enzyme but failed to remove all contaminating proteins from the preparation. Further purification of the enzyme could not be achieved by isoelectric-focusing techniques because considerable activity was lost at the isoelectric point of 5.0 due to denaturation.

Concanavalin A bound to Sepharose 4B has been used to purify several glycoproteins, including alkaline phosphatases from various sources (see Trépanier *et al.* 1976). Since concanavalin A has a specific affinity for glycoproteins containing α -D-glucopyranosyl and α -D-mannopyranosyl residues (Goldstein *et al.* 1965*a*, 1965*b*), alkaline phosphatases containing these carbohydrates bind to immobilized concanavalin A in columns and, on elution, are separated from all non-glycoproteins.

The present study describes a procedure which leads to an improved purification of mouse uterine alkaline phosphatase. The procedure is based on previously reported solubilization and extraction methods (Murdoch *et al.* 1979) and includes affinity chromatography on concanavalin A-Sepharose 4B. Some molecular and catalytic properties of the purified enzyme are also described in order to further characterize the uterine alkaline phosphatase.

Materials and Methods

Materials

Neuraminidase (EC 3.2.1.18, from *Clostridium perfringens*) and the crystalline disodium salt of *p*-nitrophenyl phosphate were obtained from Boehringer Mannheim Australia Pty. Ltd., Mount Waverley, Vic. High molecular weight electrophoresis calibration kits, polyacrylamide gels of graded porosity (type PAA 4/30), Sephacryl S200, and concanavalin A-Sepharose 4B were obtained from Pharmacia South Seas Pty. Ltd., North Ryde, N.S.W. DEAE-cellulose (DE-52) was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Other biochemicals were of the highest purity available and were purchased from the following sources: ethylenediaminetetraacetic acid (EDTA), metal chlorides, glycine, and *n*-butanol from Ajax Chemicals, Sydney, N.S.W.; tris(hydroxymethyl)aminomethane and Triton X-100 from Calbiochem Australia Pty. Ltd., Carlingford, N.S.W.; phosphate esters from Sigma Chemical Co., St. Louis, Mo., U.S.A.; methyl- α -D-mannopyranoside from P. L. Biochemicals Inc., Milwaukee, Wis., U.S.A.; citric acid and sodium citrate from BDH Chemicals Ltd., Poole, U.K. All experiments were carried out using demineralized and twice-distilled water.

Preparation of Alkaline Phosphatase

Uteri from female Quackenbush strain mice were collected on day 7 of pregnancy (Murdoch *et al.* 1979) and processed in batches of about 10 g wet weight. The measurement of specific activity (micromoles of *p*-nitrophenyl phosphate hydrolysed per minute per milligram of protein) was used to follow the preparation of the enzyme in all steps. Procedures were carried out at 4°C unless stated otherwise. All centrifugations were performed at 10000 *g* in a RC2-B Sorvall centrifuge using a type SS-34 rotor.

The uteri were homogenized in 20 volumes of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.1 mM MgCl₂ and 0.02 mM ZnCl₂ in a Sorvall Omnimix homogenizer for 1 min at setting 3, and for a further 1 min at setting 6, and then incubated for 1 h at 37°C with 0.8% (v/v) Triton X-100. Previous studies (Murdoch *et al.* 1978, 1979) showed that the enzyme could be completely solubilized by 0.2% (v/v) Triton X-100 if the tissue was homogenized using a ground-glass Potter Elvehjem homogenizer. Under the present conditions of homogenization, 0.8% (v/v) Triton X-100 was required to maximally solubilize the enzyme. Following incubation, the detergent-treated homogenate was centrifuged for 30 min and the clear supernatant containing the solubilized enzyme was extracted with 20% (v/v) *n*-butanol and then treated with ammonium sulfate as previously described (Murdoch *et al.* 1979).

The enzyme preparation was then applied to a column (2.0 by 5.0 cm) of concanavalin A-Sepharose 4B equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.9% (w/v) NaCl and 0.5 mM each of CaCl_2 , MnCl_2 , and MgCl_2 . The column was washed with the equilibrating buffer until the absorbance at 280 nm fell to low levels and was then eluted with about 200 ml of the same buffer containing 0.05 M α -methyl-D-mannopyranoside.

Following this step, the preparation was subjected to anion-exchange chromatography on a column (2.0 by 5.0 cm) of DEAE-cellulose equilibrated with 0.01 M Tris-HCl buffer, pH 7.4. The column was washed with about 200 ml of the equilibrating buffer and eluted with another 200 ml of the same buffer containing 0.1 M NaCl. Techniques employing a linear gradient of increasing salt concentration offered no advantage over this procedure. The enzyme was then purified further by gel filtration on two successive columns (2.0 by 40 cm) of Sephacryl S200 equilibrated with 0.01 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl.

The effluent from all columns was collected in fractions (about 4 ml per fraction), using a LKB 7000 Ultrarac fraction collector and assayed for enzyme activity and absorbance at 280 nm. Fractions with the highest specific activity were pooled for further processing. All purified enzyme preparations were stored at -20°C in stoppered glass vials.

Gradient Polyacrylamide-gel Electrophoresis

The purity of the enzyme preparations and the molecular weight of alkaline phosphatase were determined by electrophoresis in polyacrylamide gels of graded porosity (Murdoch *et al.* 1979). Pharmacia electrophoresis calibration kits were used to provide protein standards of known molecular weight.

Preparation of Apoenzyme

Apoenzyme preparations were obtained using the method described by Harkness (1968). Purified alkaline phosphatase was dissolved in 10 ml of 32 mM Tris-HCl buffer, pH 8.0, to give a concentration of about 3 $\mu\text{g}/\text{ml}$, and dialysed against 1 litre of 0.05 M acetate buffer, pH 5.0, containing 5 mM EDTA for 18 h at 4°C . EDTA was then removed from the enzyme preparation by dialysis against three changes of 500 ml of 32 mM Tris-HCl buffer, pH 8.0, for 24 h at 4°C . The protein content of the preparation was assayed (Lowry *et al.* 1951) before and after dialysis to determine if there was any change in concentration. No significant change in protein content was detected.

Enzyme Assay Methods and Reconstitution of Apoenzyme

Three methods were employed to measure alkaline phosphatase activity. One was a modification of the method of Bessey *et al.* (1946) in which 0.1 ml of appropriately diluted enzyme preparation was incubated at 37°C with 0.9 ml of 50 mM glycine buffer, pH 10.5, containing *p*-nitrophenyl phosphate (6 mM final concentration unless stated otherwise) and 0.5 mM MgCl_2 . The reaction was stopped after 15 min by the addition of 5 ml of 0.05 M NaOH and the resulting absorbances were immediately read at 405 nm in a spectrophotometer against an appropriate blank. This was the most frequently employed assay for alkaline phosphatase and was used in all experiments with the exception of those involving studies of the apoenzyme and enzyme specificity. However, in the experiment described in Fig. 4 a further modification was necessary and enzyme preparations were incubated for 5 min at 25°C with 0.1 mM EDTA in 0.2 ml of 50 mM glycine buffer, pH 10.5. Various metal chlorides were then added to give final concentrations of 0.25 to 4.0 mM and the mixtures held for a further 5 min at 25°C . After this time substrate was added and enzyme activity was assayed as described above.

The second method of assaying alkaline phosphatase activity involved the direct spectrophotometric measurement of hydrolysis of *p*-nitrophenyl phosphate at 25°C (Harkness 1968). This assay was used to study the kinetics of apoenzyme preparations reconstituted in the presence of metal ions. In this method 0.1 ml of appropriately diluted native enzyme or apoenzyme preparation was incubated at 25°C for 10 min in the presence or absence of various bivalent metal chlorides (1.0 mM final concentration) in a total volume of 0.5 ml of 32 mM Tris-HCl buffer, pH 8.0. Following incubation 2.5 ml of 50 mM glycine buffer, pH 10.5, containing 6 mM (final concentration) *p*-nitrophenyl phosphate were added, and the enzyme activity was measured in the 3.0-ml reaction

mixture in a spectrophotometer at 405 nm wavelength. Activity values were recorded over 10 min at 25°C.

In the third method the amount of orthophosphate (Pi) liberated from various substrates in the presence of the enzyme was measured (Fiske and Subba Row 1925). Each incubation mixture of 0.5 ml contained the enzyme and substrate at appropriate concentrations in 50 mM glycine buffer, pH 10.5, containing 0.5 mM MgCl₂. After incubation at 37°C for 15 min, 0.5 ml of 10% (w/v) trichloroacetic acid was added and the total 1.0 ml assayed for orthophosphate. Mixtures incubated simultaneously without enzyme but containing substrate at the corresponding concentration, and mixtures without substrate but with enzyme served as controls. This assay was used in experiments described in Table 2.

Unless stated otherwise, units of enzyme activity are defined as micromoles of substrate hydrolysed per minute.

Determination of K_m and pK Values

Apparent K_m (Michaelis constant) values were measured by the method of Lineweaver and Burk (1934) using eight substrate concentrations ranging from 125 μ M to 16 mM. The pK values of functional groups involved at the active centre were determined from plots of pK_m versus pH according to the theory described by Dixon and Webb (1964) and as applied by Ahlers (1975).

Buffer Solutions

The effects of pH on alkaline phosphatase activity were measured using 50 mM citrate-citric acid buffer at pH 5.0, 32 mM Tris-HCl buffer in the pH range 7.0–8.75, and 50 mM glycine buffer in the pH range 9.0–11.5. The pH of each buffer was measured with an electrode immediately before use and also, when possible, after the enzyme reaction.

Neuraminidase Treatment and Subsequent Assays

Purified enzyme preparations containing about 50 units of alkaline phosphatase activity were dialysed against three changes of distilled water for 18 h at 4°C, concentrated by lyophilization, and reconstituted in a total volume of 1.0 ml of 0.01 M Tris-HCl buffer, pH 7.4. Half (0.5 ml) of each reconstituted preparation was incubated with 0.5 ml of 50 mM acetate buffer, pH 5.5, containing 0.3 units of neuraminidase, 60 mM NaCl, 4 mM CaCl₂, and 2 mM MgCl₂ for 24 h at 37°C. The remaining 0.5 ml of each reconstituted enzyme preparation acted as a control and was incubated under similar conditions but in the absence of any added neuraminidase. Following incubation, alkaline phosphatase activity was assayed in each preparation. Aliquots (10 μ l) were also taken for electrophoresis of the phosphatase on Sepharose III cellulose acetate membranes as described by Murdoch *et al.* (1979).

The incubation mixtures were then passed through columns (2.0 by 40 cm) of Sephacryl S200. Alkaline phosphatase was eluted and studies were undertaken of its kinetic properties and of its sensitivity to changes in pH. The sensitivity of the enzyme to heat was also studied by incubating 1.0-ml aliquots of the preparations eluted from the Sephacryl S200 columns at temperatures ranging from 35 to 60°C for 15 min. Alkaline phosphatase activity was assayed immediately following incubation.

Replication of Experiments and Statistical Methods

All enzyme assays were performed in duplicate and were repeated at least three times using a different enzyme preparation on each occasion. The activity of each preparation was adjusted to similar levels before use by dilution in the appropriate buffer solution in order to avoid effects attributable to differences in enzyme concentration. Unless stated otherwise, all values given in the text, tables and figures are means $\pm$ standard error of the mean. When necessary, Student's *t*-test was employed to assess the significance of results.

Results

Enzyme Preparation, Purity, and Molecular Weight

Table 1 summarizes the results for a typical purification of mouse uterine alkaline phosphatase. The procedure achieved a 2162-fold purification over that

of the crude homogenate and recovered about 17% of the original enzyme activity together with 80 μ g of protein. The final enzyme preparation, obtained from about 10 g wet weight of day 7 pregnant uterine tissue, had a specific activity of 800 units per milligram of protein.

Table 1. Purification of mouse uterine alkaline phosphatase

Fractionation step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification factor
Crude homogenate	987	369	0.37	100	1
Triton X-100 and n-butanol extract	280	350	1.25	95	3.4
30–80% $(\text{NH}_4)_2\text{SO}_4$	188	326	1.73	88	4.7
Concanavalin A–Sephacryl 4B	12	210	17.50	57	47.3
DEAE-cellulose	5	190	38.00	51	102.7
Sephacryl S200 (1)	0.9	103	114.44	28	309.3
Sephacryl S200 (2)	0.08	64	800.00	17	2162.2

Affinity chromatography on a column of concanavalin A–Sephacryl 4B yielded the largest increase in purity (about 10 times) of any step in the procedure. A typical elution profile is shown in Fig. 1. This step effectively separated alkaline

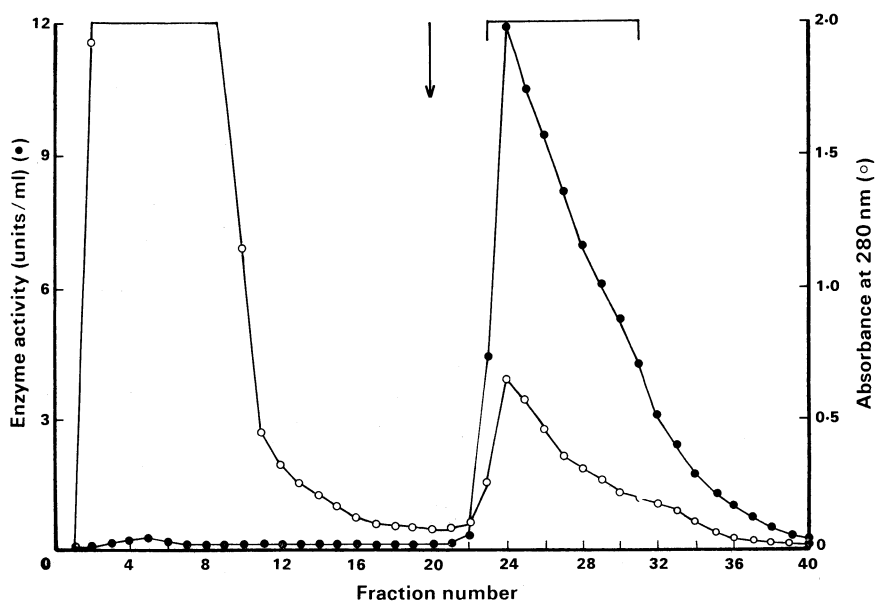


Fig. 1. Elution profile of mouse uterine alkaline phosphatase from concanavalin A–Sephacryl 4B. The arrow indicates the start of 0.05 M methyl- α -D-mannopyranoside. Fractions under the square bracket were pooled for further processing.

phosphatase from the bulk of the protein in the preparation and permitted all non-glycoproteins to be discarded. Alkaline phosphatase and other glycoproteins, which bound to the column, were readily eluted with 0.05 M α -methyl-D-mannopyranoside.

The enzyme also bound to columns of DEAE-cellulose equilibrated in the absence of any added NaCl (Fig. 2). In this case, enzyme activity was recovered as a single peak on elution with the equilibrating buffer containing 0.1 M NaCl. This step produced a further 2.2-fold increase in the purity of the preparation.

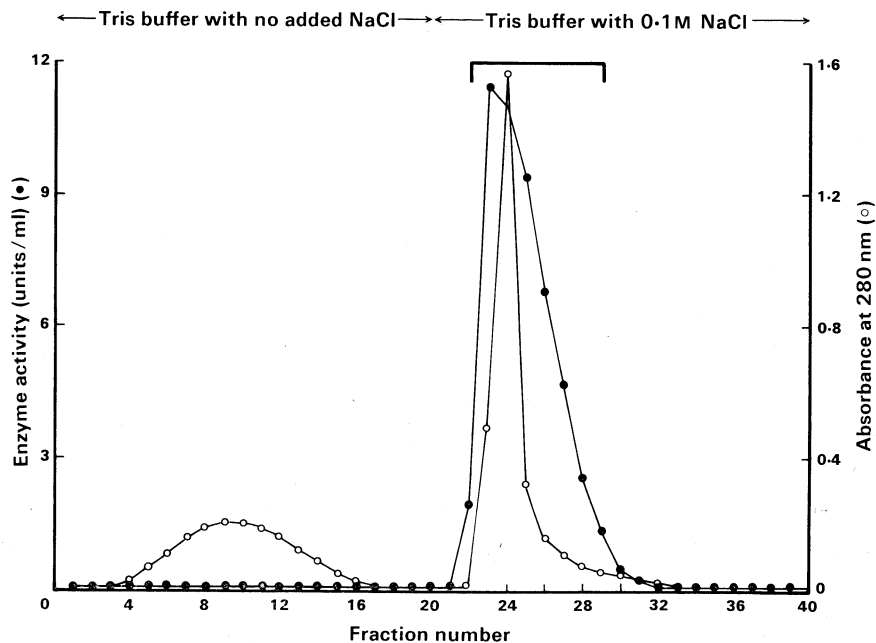


Fig. 2. Elution profile of mouse uterine alkaline phosphatase from DEAE-cellulose. Fractions under the square bracket were pooled for further processing.

Gel filtration successively on two columns of Sephacryl S200 achieved further purification (Fig. 3). The enzyme was eluted as a single symmetrical peak of activity from these columns ahead of a peak of protein. On elution from the second column the absorption at 280 nm of fractions containing high activities of alkaline phosphatase was either not measurable or almost negligible.

Polyacrylamide-gel electrophoresis resolved a single zone of alkaline phosphatase activity in preparations eluted from the second Sephacryl S200 column. Bands in gels were difficult to detect following staining with Amido Black 10B because of the low protein concentration of the purified preparations. However, when the samples were sufficiently concentrated by lyophilization and then subjected to electrophoresis, it was generally possible to detect a single faintly staining protein band in the gels at a position which was coincident with the enzyme activity band.

The apparent molecular weight of the uterine alkaline phosphatase determined by polyacrylamide-gel electrophoresis against standard proteins was found to be $205\,000 \pm 10\,000$.

Metalloenzyme Properties

Incubation of purified preparations of alkaline phosphatase with metal-complexing agents when present at a concentration of 1 mM, including sodium cyanide,

L-cysteine and EDTA, significantly ($P < 0.01$) depressed enzyme activity by 84, 95 and 100%, respectively. The same concentrations of CdCl_2 and ZnCl_2 also depressed activity by 95 and 29%, respectively, while MgCl_2 significantly ($P < 0.01$) enhanced activity by 20%.

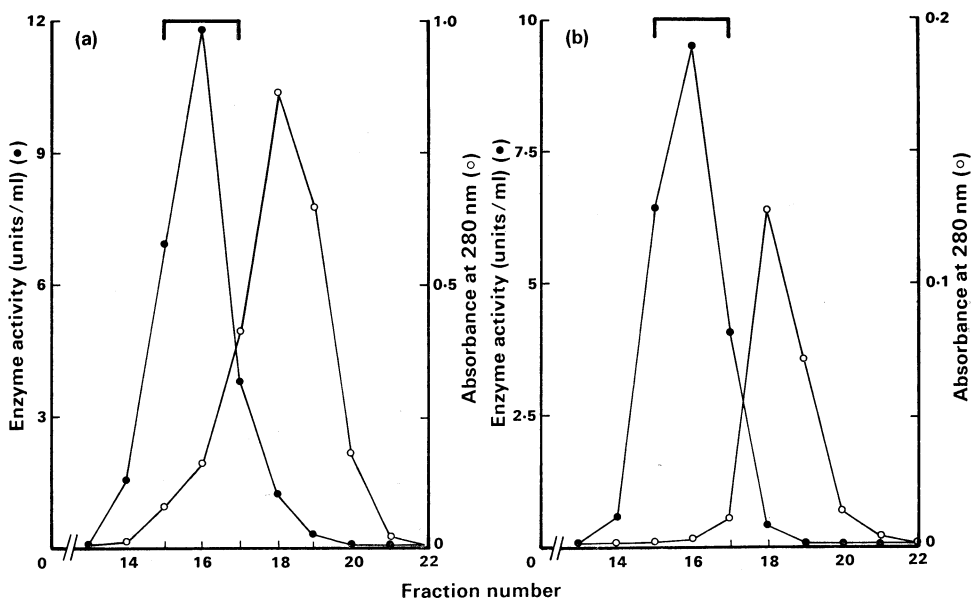


Fig. 3. Gel filtration of mouse uterine alkaline phosphatase on Sephacryl S200. Fractions under the square brackets were pooled for further processing after elution from the first column (a) or for final assay after elution from the second column (b).

Incubation of the enzyme with 0.1 mM EDTA in the absence of added substrate and cations for 10 min at 25°C depressed activity by 73%. The addition of various bivalent cations at 1 mM concentration (results not shown) increased the activity of the EDTA-inhibited enzyme to various extents but only Mg^{2+} or Zn^{2+} ions were effective in restoring it to the original level. Fig. 4 shows that maximal reactivation occurred when Mg^{2+} or Zn^{2+} ions were added at 0.25 mM concentration. Although higher concentrations of the cations displayed inhibitory effects when added separately, a high uniform level of activity was maintained over the concentration range when the two cations were added together, with one of the cations remaining constant at 0.25 mM. The addition of Co^{2+} , which can often replace Zn^{2+} in metalloenzymes because of similar ionic radii (Le Vine *et al.* 1976), also increased the activity of the EDTA-inhibited enzyme. However, the effect in this case was slight and did not mimic the reactivation response obtained with Zn^{2+} .

The apoenzyme of mouse uterine alkaline phosphatase displayed no detectable enzyme activity in the absence of cations and was not reactivated by the separate addition of various bivalent cations when provided at 1 mM concentration. The addition of Zn^{2+} and Mg^{2+} ions together, however, restored activity almost to its original level while Co^{2+} and Mg^{2+} ions together restored about 46% of the original enzymic activity. The effectiveness of other combinations of cations in

activating the apoenzyme were also examined (results not shown) but none gave a significant recovery of activity.

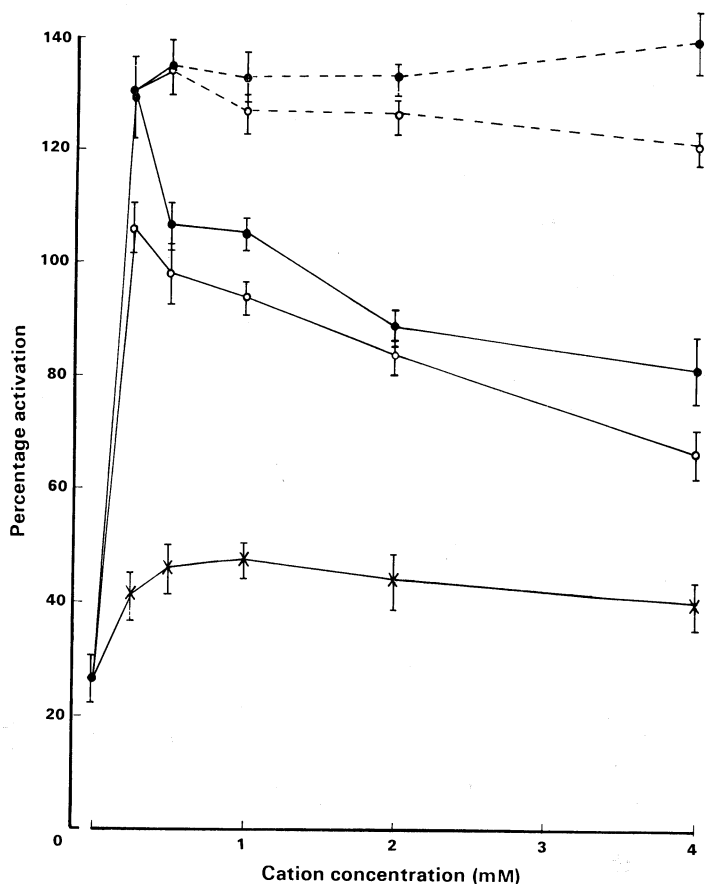


Fig. 4. Effects of Mg^{2+} , Zn^{2+} and Co^{2+} on the activity of EDTA-inhibited alkaline phosphatase. Activity values are expressed as relative to the hydrolysis of substrate in the absence of any added EDTA or cations. The specific activity of the enzyme under the conditions of 100% activation was 620 ± 48 units/mg protein. $\times$ — $\times$ Co^{2+} only added. $\circ$ — $\circ$ Zn^{2+} only added. $\bullet$ — $\bullet$ Mg^{2+} only added. $\circ$ --- $\circ$ Zn^{2+} with 0.25 mM MgCl_2 . $\bullet$ --- $\bullet$ Mg^{2+} with 0.25 mM ZnCl_2 .

The results in Fig. 5 demonstrate that the extent to which the apoenzyme was successfully reactivated by Mg^{2+} and Zn^{2+} ions was a function of time of adding one cation in relation to the other as well as a function of time of adding the cations in relation to the substrate. When the native enzyme was assayed in the absence of any added cations the velocity was linear from time zero. However, when the same amount of apoenzyme was assayed following prior incubation with Mg^{2+} and Zn^{2+} , the initial velocity was low and increased with time to become linear after 4 min. Velocity was also linear from time zero when apoenzyme was assayed after prior incubation with either substrate or Mg^{2+} alone, but never achieved the levels obtained after prior incubation with Mg^{2+} and Zn^{2+} added together. When the apoenzyme was assayed after prior incubation with Zn^{2+} as the only

additive, the initial velocity was again low but increased with time to exceed that which was obtained after prior incubation with Mg^{2+} and Zn^{2+} together.

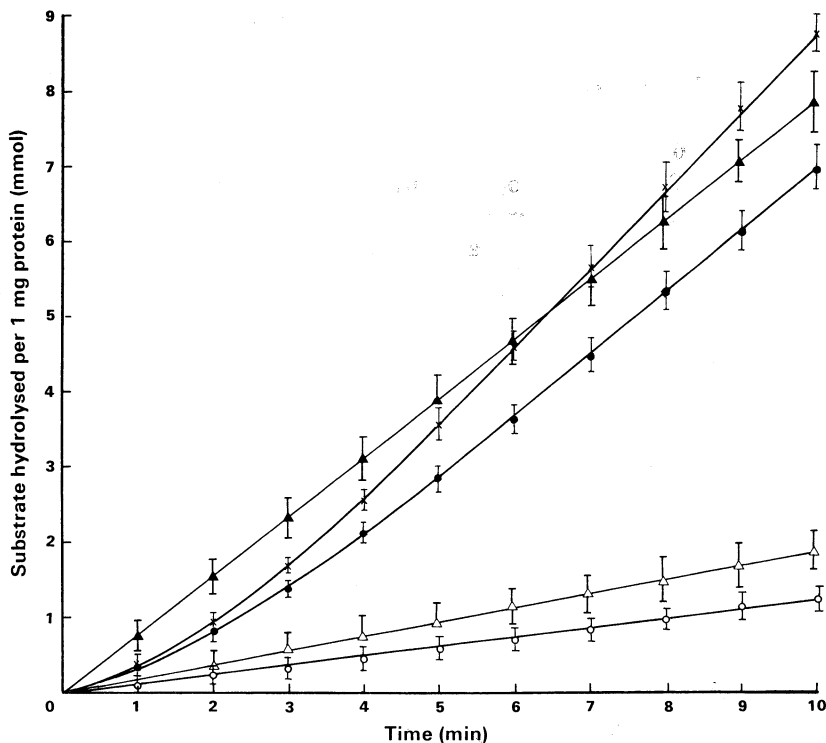


Fig. 5. Kinetics of reconstitution of the apoenzyme of mouse uterine alkaline phosphatase. Cations were each added at 1.0 mM final concentration while substrate (*p*-nitrophenyl phosphate) was added at 6.0 mM final concentration. ▲ Native enzyme in the absence of any added cations. ● Apoenzyme incubated with both Mg^{2+} and Zn^{2+} before adding substrate. × Apoenzyme incubated with Zn^{2+} alone before adding Mg^{2+} and substrate. ○ Apoenzyme incubated with Mg^{2+} alone before adding Zn^{2+} and substrate. △ Apoenzyme incubated with substrate before adding Mg^{2+} and Zn^{2+} .

Effects of Neuraminidase

Treatment of purified preparations of alkaline phosphatase with neuraminidase significantly ($P < 0.001$) decreased the anodal migration of the enzyme during electrophoresis on cellulose acetate membranes but did not significantly alter the activity of the phosphatase or its elution profile on Sephacryl S200 columns (results not shown). On electrophoresis the native and desialylated enzymes migrated as single, well-defined zones of activity.

Apparent K_m values for the native and desialylated enzymes hydrolysing *p*-nitrophenyl phosphate at pH 10.5 were 0.82 ± 0.05 and 0.80 ± 0.02 mM, respectively.

The native and neuraminidase-treated enzyme preparations displayed similar activity patterns after heating at temperatures between 35 and 50°C, and in both cases about 92% of the original activity remained after heating at 50°C for 15 min.

After 15 min at 55°C, however, only 51 and 42% of the original activity remained in the native and neuraminidase-treated samples, respectively. A Student's *t*-test showed that these activity values following heating at 55°C were significantly different ($P < 0.05$). When the preparations were heated at 60°C for 15 min, complete inactivation of the enzyme occurred in all cases.

Fig. 6 shows that the optimal pH for enzyme activity in the native and desialylated preparations decreased with decreasing substrate concentration. At a substrate concentration of 6.0 mM, the optimal pH for activity was 10.5; at a substrate concentration of 0.6 mM, the optimal pH for activity was 10.0. All enzyme preparations, whether treated or not treated with neuraminidase, displayed very similar responses to changes in pH and substrate concentration.

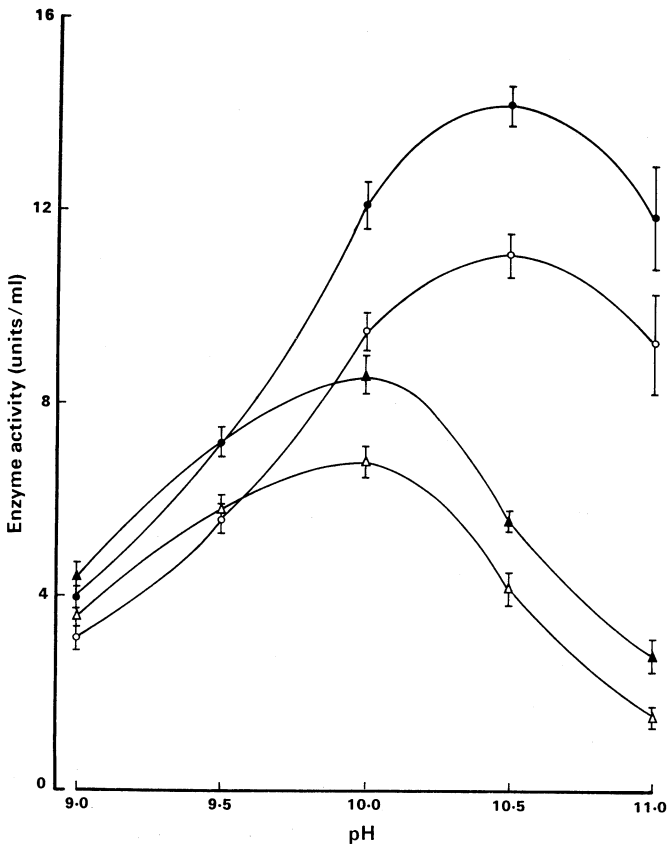


Fig. 6. Effects of neuraminidase treatment on the response of purified mouse uterine alkaline phosphatase to pH and substrate concentration. ● Control, 6.0 mM substrate. ○ Neuraminidase-treated, 6.0 mM substrate. ▲ Control, 0.6 mM substrate. △ Neuraminidase-treated, 0.6 mM substrate.

Effects of pH

The dependence of the optimum pH for enzyme activity on substrate concentration was further confirmed in an experiment employing a wide range of substrate concentrations and buffers of pH ranging from 8.5 to 11.0. Under these conditions, an optimal pH of 11.0 was found at substrate concentrations of 8 and 16 mM. As

substrate concentration decreased, the optimal pH also decreased until a value of 9.75 was recorded at substrate concentrations of 62.5 and 125 μM . Furthermore, enzyme activities became increasingly independent of substrate concentration as pH decreased and values of the apparent K_m could only be determined from Lineweaver-Burk plots when the pH exceeded 8.5.

Apparent K_m values decreased with decreasing pH. When a plot of $\text{p}K_m$ versus pH was constructed (Fig. 7) a $\text{p}K$ value of 9.45 was obtained. The data suggest that only one functional group with this $\text{p}K$ value may be involved at the active centre because the graph of the measured values misses the intersection point of the theoretical curves by a vertical distance of $\log 2$. If two groups with similar $\text{p}K$ values were involved, the vertical distance would be $\log 3$ (Dixon and Webb 1964).

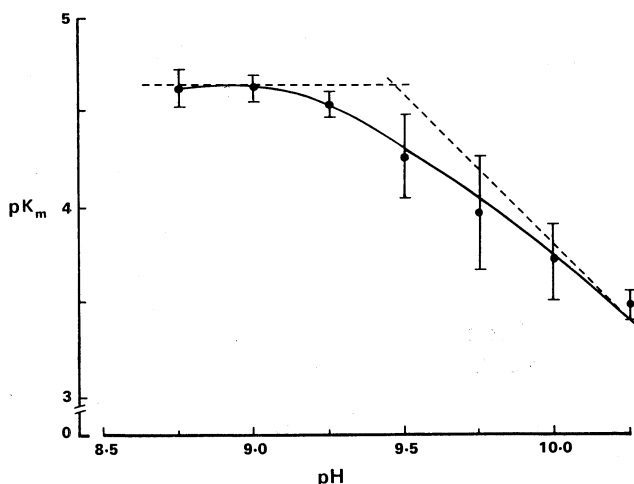


Fig. 7. Variation in the apparent K_m values of alkaline phosphatase as a function of pH. Solid lines are measured values. Dotted lines are theoretical curves drawn according to the theory described by Dixon and Webb (1964).

The effects of exposing the enzyme to buffers of various pH values on the subsequent activity at pH 10.5 were also studied. Incubation in buffer of pH 5.0 irreversibly inactivated the enzyme to the extent that only 77% of the original activity was retained after 5 min and 28% retained after 320 min. Incubation in buffer of pH 7.0 had no effect on the subsequent activity at pH 10.5 while exposure to buffer of pH 11.5 slightly increased ($P < 0.05$) the subsequent activity above that recorded prior to incubation. This effect was still apparent even after 320 min incubation at pH 11.5.

Specificity Studies

The ability of mouse uterine alkaline phosphatase to hydrolyse various phosphate esters was studied and the results are presented in Table 2 together with some values of the apparent K_m . All of the phosphate esters tested were hydrolysed by the enzyme and *p*-nitrophenyl phosphate was attacked at the most rapid rate. Apparent K_m values varied from 1.05 mM for *p*-nitrophenyl phosphate to 3.30 mM for β -naphthyl acid phosphate.

Arrhenius Plot and Activation Energy

The rate of enzyme-catalysed hydrolysis of *p*-nitrophenyl phosphate at pH 10.5 increased with increasing temperatures between 0.5 and 35.5°C. The activation energy, determined from a linear Arrhenius plot (Dixon and Webb 1964), was 50.1 kJ mol⁻¹.

Table 2. Specificity of alkaline phosphatase and the values of K_m for various substrates hydrolysed at pH 10.5

Substrate (4 mM concn)	Enzyme activity ^A	K_m (mM)	Substrate (4 mM concn)	Enzyme activity ^A	K_m (mM)
<i>p</i> -Nitrophenyl phosphate	230 ± 11.7	1.05 ± 0.26	Ribose 5-phosphate	104 ± 9.0	—
β-Naphthyl acid phosphate	212 ± 10.6	3.30 ± 0.96	Fructose 6-phosphate	87 ± 7.4	—
β-Glycerophosphate	143 ± 8.7	1.67 ± 0.45	Phosphoenol pyruvate	87 ± 8.5	—
<i>O</i> -phospho-D,L-serine	140 ± 9.2	1.70 ± 0.47	3-Phosphoglyceric acid	60 ± 6.4	—
Glucose 1-phosphate	140 ± 10.4	1.33 ± 0.30	α-Glycerophosphate	60 ± 5.8	—
5'-AMP	133 ± 11.0	—	Glucose 6-phosphate	60 ± 7.1	—
6-Phosphogluconic acid	124 ± 10.6	—	ATP	53 ± 5.1	—

^A Expressed as μmol Pi liberated min⁻¹ (mg protein)⁻¹.

Discussion

The present study describes an improved method for the purification of mouse uterine alkaline phosphatase. An essential step in the procedure following homogenization involved the solubilization of the enzyme with Triton X-100. Although the value of this non-ionic detergent in solubilizing alkaline phosphatase in mouse uterine homogenates was previously recognized (Murdoch *et al.* 1978, 1979), the present study indicates that care must be exercised to adjust the concentration of the detergent to suit the conditions of tissue homogenization if maximum solubilization of the enzyme is to be achieved.

The requirement for the presence of Triton X-100 in the purification process of mouse uterine alkaline phosphatase is not unique. Ikehara *et al.* (1978) indicated that while alkaline phosphatases from many sources can be solubilized without addition of any detergent once they are extracted from membranes with *n*-butanol, the enzymes from other sources, including rat ascites hepatoma cells, need Triton X-100 for solubilization. At present there is no satisfactory explanation for the requirement of the detergent in these instances. However, because an increase in specific activity with only 5% loss of total activity occurred in the present investigation following extraction with Triton X-100 and *n*-butanol, membrane lipid components may not be essential for the activity of alkaline phosphatase in the mouse uterus. This proposal supports the observations of Murdoch *et al.* (1979).

Most mammalian alkaline phosphatases are glycoproteins and several have been shown to contain carbohydrate residues which interact with concanavalin A bound to Sepharose, enabling purification of the enzymes by affinity chromatography (Komoda and Sakagishi 1976*a*, 1976*b*; Latner and Hodson 1976; Trépanier *et al.* 1976; Yokota 1978*b*; O'Keefe and Kinsella 1979). The affinity of mouse uterine alkaline phosphatase for the immobilized lectin in the present study clearly indicates that this enzyme is also a glycoprotein and suggests that the phosphatase may contain branched polysaccharides with α-D-glucopyranosyl or α-D-mannopyranosyl

residues (Goldstein *et al.* 1965a, 1965b). Yokota (1978a) separated two isozymes of alkaline phosphatase from cultured rat liver cells by concanavalin A-Sepharose affinity chromatography. Although both isozymes were assumed to be glycoproteins, only one contained sugar residues which were capable of reacting with the lectin. In the uterus of the mouse, however, alkaline phosphatase appears to exist as a single isozymic form because, unlike the results reported by Yokota (1978a), it remained bound to columns of immobilized concanavalin A in the absence of any added methyl- α -D-mannopyranoside and electrophoresis in polyacrylamide gels and on cellulose acetate membranes resolved only a single zone of enzyme activity.

Investigations of the subunit structure of alkaline phosphatases have clearly established the oligomeric nature of these enzymes and suggest that they generally exist either as dimers or tetramers (see Yokota 1978a, 1978b). The present estimate of the apparent molecular weight of $205\,000 \pm 10\,000$ for the enzyme in the mouse uterus is similar to that found for other phosphatases, but because of the small amount of protein available for study, we have not been able to confidently determine its subunit structure.

Cathala *et al.* (1975) have indicated that there are two classes of mammalian phosphatases, those which need only Zn^{2+} for activity and those which need both Zn^{2+} and Mg^{2+} . It is generally considered that with the phosphatases in the latter class, Zn^{2+} is required both for the preservation of the structure and activity of the enzyme while Mg^{2+} regulates the mode of binding of Zn^{2+} and stimulates the enzymatic activity over and above that attained in the presence of Zn^{2+} alone (Ahlers 1974, 1975; Hiwada and Wachsmuth 1974; Cathala *et al.* 1975; Linden *et al.* 1977; Petitclerc and Fecteau 1977). The present results place the mouse uterine enzyme into the class of phosphatases requiring both Zn^{2+} and Mg^{2+} and demonstrate that both cations must be available to the apoenzyme for the restoration of any significant level of activity. It is clearly apparent that a precisely ordered interaction occurs between these bivalent metal ions and substrate to influence the activity of the enzyme.

Many workers over the past decade have reported that neuraminidase treatment of various alkaline phosphatases liberates sialic acid from the carbohydrate component and subsequently decreases the anodal migration of the enzyme during electrophoresis (see Tarachand and Heald 1979). The reduced electrophoretic mobility of the mouse uterine enzyme in the present investigation following treatment with neuraminidase suggests that sialic acid is also a component of this glycoprotein. However, as has been found for other sialic acid-containing phosphatases (Saraswathi and Bachhawat 1968, 1970; Mulivor *et al.* 1978; Otani *et al.* 1978), desialylation failed to alter the activity or the catalytic properties of the uterine enzyme suggesting that the sialic acid residues do not constitute a part of the active centre of the enzyme. Attempts to elucidate the functional significance of sialic acid in alkaline phosphatase enzymes and other glycoproteins have shown that this component may nevertheless influence antigenic specificity (Badger and Sussman 1976) and the ability of the protein to inhibit the agglutination of viruses (Usategui-Gomez *et al.* 1969).

It is evident from the present results that mouse uterine alkaline phosphatase, either with or without sialic acid, does not possess the heat-stable structure of the human placental enzyme (Harkness 1968) but displays a response to heat similar to that of the enzyme from rat uterine deciduomata (Tarachand and Heald 1979). The dependence of optimum pH for activity on substrate concentration together

with decreases in the value of the apparent K_m with decreasing pH are findings which are also in agreement with reports on other alkaline phosphatases (Harkness 1968; Hiwada and Wachsmuth 1974; Lopez *et al.* 1976; Van Belle 1976; Adler 1978) and support the proposal that the enzyme may be catalytically active under physiological conditions of pH and substrate concentration (Morton 1957; Harkness 1968; Murdoch 1971). Furthermore, like other alkaline phosphatases (Fernley 1971) the enzyme in the present study was stable in neutral or mildly alkaline solution but was inactivated at pH 5.0.

The functional group involved at the active centre with a pK of 9.45 could either be an ϵ -amino group of lysine or a phenolic hydroxyl group of tyrosine. A number of other investigators (see Hiwada and Wachsmuth 1974; Ahlers 1975) have proposed that these groups may occur in various alkaline phosphatases from both prokaryotic and eukaryotic sources, but confirmation in the present case requires further study using group specific inhibitors.

The estimation of the energy of activation gave a value similar to that reported for alkaline phosphatases from other sources (Harkness 1968; Cathala *et al.* 1975; Hung and Melnykovich 1976) but greater than that reported by Hiwada and Wachsmuth (1974). The latter authors, however, found a transition for the energy of activation of pig kidney alkaline phosphatase at 24°C which possibly reflected a change in the configuration of the enzyme.

In conclusion, these studies describe a considerably improved method, compared with that reported previously (Murdoch *et al.* 1979), for the purification of mouse uterine alkaline phosphatase. The results also indicate that the enzyme is a sialoglycoprotein in nature and has the ability to hydrolyse a variety of compounds having either phosphomonoester or pyrophosphate linkages. Mg^{2+} and Zn^{2+} ions are necessary for hydrolytic activity and the response of the enzyme to changes in pH and temperature generally resembles that of other alkaline phosphatases from a variety of mammalian sources.

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