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Catalytic Properties of Alkaline Phosphatase from Pig Kidney

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The enzymic properties of alkaline phosphatase (EC 3.1.3.1) from pig kidney brush-border membranes were studied. 1. It hydrolyses ortho- and pyro-phosphate esters, the rate limiting step ($V_{max.}$) being independent of the substrate. It transphosphorylates to Tris at concentrations above 0.1M-Tris. 2. The pH optimum for hydrolysis was between 9.8 and 10. The pK of the enzyme–substrate complex is 8.7 for p-nitrophenyl phosphate and β-glycerophosphate. Excess of substrate inhibits the enzymic activity with decreasing pH. The pK of the substrate-inhibited enzyme–substrate complex, 8.7, is very similar to that for the enzyme–substrate complex. The pK values of the free enzyme appear to be 8.7 and 7.9. 3. Inactivation studies suggest that there is an essential tyrosine residue at the active centre of the enzyme. 4. The energy of activation (E) and the heat of activation (ΔH) at pH9.5 showed a transition at 24.8°C that was unaffected by Mg^{2+}. 5. Kinetic and atomic-absorption analysis indicated the essential role of two Zn^{2+} ions/tetrameric enzyme for an ordered association of the monomers. Zn^{2+} in excess and other bivalent ions compete for a second site with Mg^{2+}. Mg^{2+} enhances only the rate-limiting step of substrate hydrolysis. 6. Amino acid inhibition studies classified the pig kidney enzyme as an intermediate type of previously described alkaline phosphatases. It has more similarity with the enzyme from liver and bone than with that from placenta.

Mammalian alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) has been the object of many kinetic studies. However, most of the studies have been done with enzyme preparations of very low specific activity. The sources of the enzyme for kinetic studies were also very different, mainly human placenta (Ahmed & King, 1960; Harkness, 1968*b*; Ghosh & Fishman, 1968; Ghosh, 1969) and intestines of different species (Ghosh, 1969; Morton, 1954; Engström, 1961; Lazdunski & Ouellet, 1961; Fernley & Walker, 1965; Ghosh & Fishman, 1966; Fernley & Walker, 1967). The present paper describes catalytic properties of alkaline phosphatase from pig kidney (Wachsmuth & Hiwada, 1974), its pK values with special reference to the effect of Mg^{2+} and Zn^{2+} ions, substrate specificity and the inhibition by amino acids. The results indicate two different binding sites for Zn^{2+} and Mg^{2+}, which regulate the catalytic properties of the enzyme.

Experimental

Materials

All reagents were of analytical grade and several have been previously described (Wachsmuth & Hiwada, 1974). Sodium β-glycerophosphate, 1-amino-2-hydroxynaphthalene-4-sulphonic acid, L-tryptophan, D-tryptophan and L-histidine were purchased from Merck A.G., Darmstadt, West Germany; glycine, L-leucine, DL-serine, DL-threonine, the phosphorylated nucleotides, glucose 1-phosphate and glucose 6-phosphate were from Boehringer, Mannheim, West Germany. Tetranitromethane was from Fluka A.G., Buchs, Switzerland, and L-homoarginine hydrochloride from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Phosvitin and L-phenylalanine were obtained by the courtesy of Dr. M. Feurer, CIBA–GEIGY Ltd., Basel, Switzerland.

The alkaline phosphatase purified from pig kidney (Wachsmuth & Hiwada, 1974) had a specific activity of 1320 μmol/min per mg of protein at 37°C in 50mM-Tris–HCl–5mM-$MgCl_2$, pH9.5.

Methods

Enzyme assay. The hydrolysis rates of p-nitrophenyl phosphate were measured photometrically as described by Wachsmuth & Hiwada (1974) and recorded on a W+W 4201 recorder (W+W electronics Inc., Basel, Switzerland) fitted to an Eppendorf photometer. Corrections for the different molar extinction coefficients of p-nitrophenol below pH8.5 were made. For other substrates the amount of phosphate after enzymic hydrolysis was measured by the method of Fiske & SubbaRow (1925). The experi-

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Table 1. *K_m and $V_{max.}$ values for the hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase at pH9.5*

The rate of hydrolysis was measured at 37°C in 1ml of buffer containing 5mM-$MgCl_2$. For further details see the text.

		K_m (µM)			$V_{max.}$ (µmol/min per mg)		
Buffer system	Concn. of buffer ...	0.01M	0.05M	0.1M	0.01M	0.05M	0.1M
Tris–HCl		71	71	71	1200	1320	1442
+0.1M-NaCl		—	71	—	—	1409	—
+0.5M-NaCl		—	71	—	—	1496	—
Triethylamine–HCl		70	70	70	1165	1270	1355
Veronal–HCl		123	101	81	1166	954	797
Carbonate–bicarbonate		118	188	234	1170	1170	1170

mental procedure for both assay systems has been described (Wachsmuth & Hiwada, 1974).

For ATP or ADP hydrolysis 1mM-$MgCl_2$ was used for enzyme activation. For phosphate determination (Fiske & SubbaRow, 1925), the enzyme reaction was stopped by the addition of 0.5ml of 40% (w/v) trichloroacetic acid. Mixtures incubated simultaneously without enzyme but at corresponding substrate concentrations, and mixtures without substrate but with enzyme, served as controls. To the samples (2.5ml) were added 0.5ml of 2.5% (w/v) ammonium molybdate and 0.2ml of 0.25% aminonaphtholsulphonic acid in 15% (w/v) $NaHSO_3$ and, finally, water to give a total volume of 5ml. After 10min at 20°C the E_{691}^{1cm} of the phosphate complex was read in the Eppendorf photometer. Dilutions of 1mM-NaH_2PO_4 served as standards and all reactions were done at least in duplicate. The initial rates were linear up to 30% of substrate hydrolysed at pH7.5 and 9.5. The enzyme activity was expressed as µmol of product released/min per ml of enzyme at 37°C. The K_m and $V_{max.}$ values were measured by the method of Lineweaver & Burk (1934) at six substrate concentrations: for *p*-nitrophenyl phosphate at pH7.5 from 10µM to 0.1mM and at pH9.5 from 0.1 to 1mM; for ADP and ATP at pH7.5 from 0.1 to 0.25mM and at pH9.5 from 0.5 to 1.5mM: for all other substrates at pH7.5 from 0.2 to 0.4mM and at pH9.5 from 0.75 to 2.5mM. Tris–HCl buffer was used as described in the Results section. The pH was measured with an electrode before and after the enzyme reaction.

Zn^{2+} and Mg^{2+} were determined in an HGA-70 graphite cell of a Perkin–Elmer model 403 atomic-absorption spectrophotometer by Mr. O. Suter, CIBA–GEIGY Ltd., Basel, Switzerland.

Results

Conditions for analysis of the enzyme activity

When dialysed enzyme or enzyme that had been stored in 10mM-Tris–HCl–0.1% NaN_3, pH8.0, was used for hydrolysis in the presence of bivalent ions, the reaction rate increased rapidly within the first 2min and was then linear. Therefore all reactions were done by preincubating the enzyme in the reaction mixture in the absence of substrate for 5min. If not otherwise mentioned, this was followed by an addition of the substrate.

Tris buffer did not influence the K_m for *p*-nitrophenyl phosphate; altering the concentration, i.e. the ionic strength, had little effect on the maximum hydrolysis rate (Table 1). Carbonate–bicarbonate buffer, which is usually used for hydrolysis studies with alkaline phosphatase (Fishman & Ghosh, 1967*a*), inhibited the enzyme in a competitive manner. Triethylamine–HCl buffer showed the same

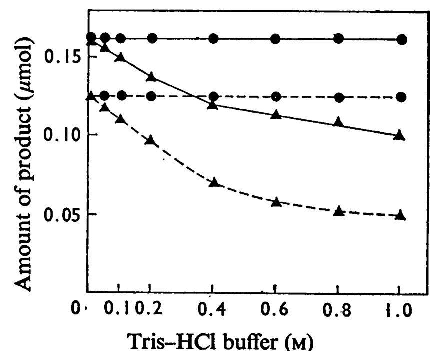

Fig. 1. *Transphosphorylation by alkaline phosphatase*

Enzyme (70 ng of protein at pH9.5 or 1.1µg at pH7.5) was incubated at 37°C in 3.0ml of Tris–HCl–5mM-$MgCl_2$ containing 3µmol of *p*-nitrophenyl phosphate at pH9.5 and 0.3µmol at pH7.5. The hydrolysis was followed in the photometer and stopped, by addition of 0.5ml of 40% (w/v) trichloroacetic acid at 0.162µmol of *p*-nitrophenol at pH9.5 and at 0.125µmol at pH7.5, when the hydrolysis rate was still linear. Each sample was estimated for both *p*-nitrophenol (●) and P_i (▲). ——, pH9.5; ----, pH7.5. The controls for enzyme and for substrate solutions remained constant at all Tris concentrations.

properties as Tris buffer. Veronal–HCl buffer was the most unsuitable buffer because of its influence on K_m and $V_{max.}$. Therefore all measurements were done in 50mM-Tris–HCl buffer.

Transphosphorylation in the Tris–HCl system

At concentrations higher than 50mM-Tris–HCl the amounts of *p*-nitrophenol and phosphate produced by enzymic hydrolysis of *p*-nitrophenyl phosphate became disproportionate. With increasing concentration of Tris at pH 7.5 or 9.5, the free phosphate decreased, indicating a phosphorylation to Tris by alkaline phosphatase (Fig. 1). With a 100-fold increase in Tris concentration the increase in *p*-nitrophenyl phosphate hydrolysis was only twofold at either pH.

pH optimum

The variation in $V_{max.}$ and K_m values as a function of pH was determined from Lineweaver–Burk plots. Because of substrate inhibition the substrate concentration had to be below 50 μM at pH 7.0, below 0.1 mM at pH 7.5 and pH 8.0 and below 0.5 mM at pH 8.5. The pH optimum for $V_{max.}$ was 9.8–10.0 and that for K_m was 7.5 (0.7 μM). Mg^{2+} increased $V_{max.}$ by up to five times relative to the value without Mg^{2+}; it had no effect on K_m.

pK values of ionization

With *p*-nitrophenyl phosphate and β-glycerophosphate as substrates the pK for the enzyme–substrate complex was 8.7 (Fig. 2b). The pK for the K_m value

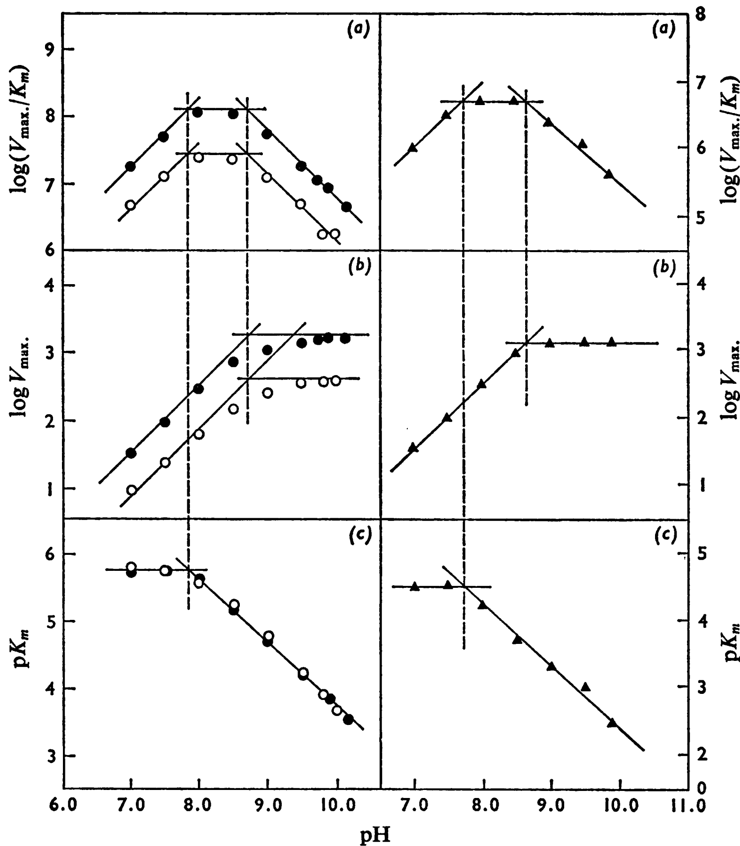

Fig. 2. *Effect of pH on the enzymic activity of alkaline phosphatase*

The enzymic hydrolyses of *p*-nitrophenyl phosphate in the absence of $MgCl_2$ (○) and in the presence of 5mM-$MgCl_2$ (●) and of β-glycerophosphate in the presence of 5mM-$MgCl_2$ (▲) were measured. (*a*) Variation of $\log(V_{max.}/K_m)$ with pH; (*b*) variation of $\log V_{max.}$ (μmol of substrate hydrolysed/min per mg of enzyme) with pH; (*c*) variation of pK_m (i.e. $\log 1/K_m$) with pH. For details see under 'Methods'.

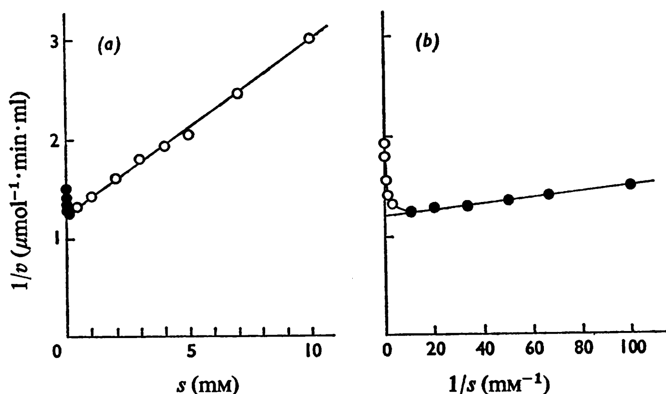

Fig. 3. *Inhibition by high substrate concentration* (*s*)

Initial hydrolysis rates of *p*-nitrophenyl phosphate were measured in 1 ml of 50mM-Tris–HCl–5mM-$MgCl_2$, pH8, at 37°C. (*a*) $1/v$ against s; (*b*) $1/v$ against $1/s$. Values at low (●) and inhibiting high substrate concentration (○).

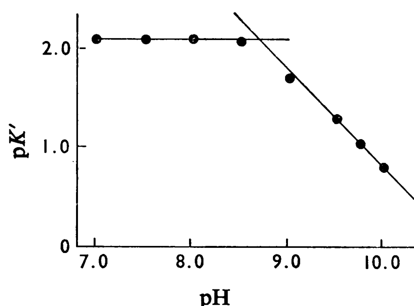

Fig. 4. *Effect of pH on the inhibition by high substrate concentrations*

pK'_s (i.e. $-\log K'_s$) was determined from the inhibition constants of *p*-nitrophenyl phosphate (M) (see Fig. 3) and plotted against pH.

was pH7.9 for *p*-nitrophenyl phosphate and about pH7.8 for *β*-glycerophosphate (Fig. 2*c*). From the quantity $V_{max.}/K_m$ the p*K* values of free enzyme and of the two substrates were estimated as pH8.7 and 7.9–7.8 (Fig. 2*a*).

Substrate inhibition

High concentrations of substrate inhibit enzymic hydrolysis by alkaline phosphatase (Fernley & Walker, 1965; Folley & Kay, 1935; Morton, 1957). The dissociation constant for inhibition by excess of substrate (K'_s) was estimated by plotting the reciprocal value of the hydrolysis rates against the concentration of *p*-nitrophenyl phosphate, thus at half-maximal velocity the value K'_s was derived (Fig. 3). Measurements at different pH values made it possible to estimate the p*K* for K'_s (Fig. 4), which was about 8.7, similar to that of the enzyme–substrate complex.

Effect of KCN, cystine and tetranitromethane

Incubation of the enzyme in 50mM-Tris–HCl buffer, pH8.0, at room temperature in the presence of 0.1mM-cysteine, 4mM-KCN or 6mM-tetranitromethane for 15min caused 50% inactivation. The inactivation by KCN and cysteine was completely reversible when the samples were dialysed for 24h against 5mM-$MgCl_2$ in 0.05M-Tris–HCl buffer, pH8.0. Inactivation by tetranitromethane was irreversible.

Temperature-dependence of the enzyme reaction

Alkaline phosphatase was inactivated above 50°C (Wachsmuth & Hiwada, 1974). The hydrolysis rate at pH9.5 increased with increasing temperature (Fig. 5), whereas substrate binding decreased (Fig. 6). Both graphs show two lines with two slopes crossing at a transition temperature of 24.8°C. In no case did Mg^{2+} affect the slope of either line. The heat of activation, ΔH, above the transition point was calculated to be −11kJ and below it −30kJ (Fig. 6). The energy of activation, *E*, was 12.7kJ above the transition point and 38.6kJ below it (Fig. 5). Ultracentrifugation studies in 50mM-Tris–HCl–10mM-$MgCl_2$–0.1% NaN_3 with a protein concentration of 1mg/ml, gave sedimentation coefficients $s_{20,w} = 6.2S$ at 36°C and $s_{20,w} = 6.87S$ at 9.2°C. Such changes in sedimentation coefficients and enthalpy may reflect a conformational change in the protein occurring around 25°C.

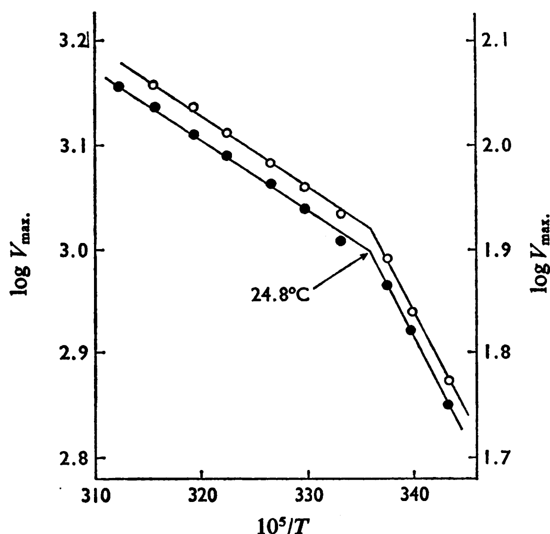

Fig. 5. *Arrhenius plot for p-nitrophenyl phosphate hydrolysis*

The hydrolysis rates ($V_{max.}$) are given in μmol/min per 1mg of enzyme in the absence (right scale; ○) and the presence (left scale; ●) of 5mM-$MgCl_2$ in 50mM-Tris-HCl buffer, pH9.5. Each point is the mean of three measurements.

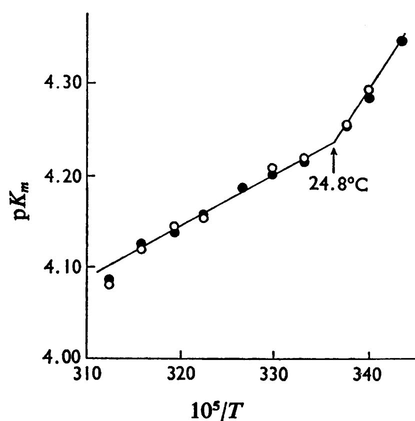

Fig. 6. *Effect of temperature on K_m for p-nitrophenyl phosphate*

The K_m values (M), plotted as pK_m (i.e. $\log 1/K_m$), were measured in 50mM-Tris-HCl buffer, pH9.5, with (●) and without (○) 5mM-$MgCl_2$. Each point is the mean of three measurements.

Effect of bivalent cations

Co^{2+} alone activates alkaline phosphatase about half as much as Mg^{2+} (Fig. 7). Only a little activation was found with Ca^{2+}, Mn^{2+} and Ni^{2+}. Zn^{2+} at 0.3μM inhibited enzymic activity by 50%. This inhibition was also seen with Mg^{2+} above 3mM and Co^{2+} above 1mM. Measurements of enzymic activity by using saturating concentrations of substrate gave with increasing Mg^{2+} concentration a non-competitive activation of the enzyme, with Zn^{2+} a non-competitive inhibition and with Co^{2+} a mixed-type between competitive and non-competitive activation with respect to the substrate.

On treatment with 0.3mM-EDTA at pH7–9.5, the enzyme lost all its activity. The enzymic activity could be restored in such a solution to about 40% by adding Mg^{2+}, and to 30% by adding Zn^{2+}. When Zn^{2+} in optimal concentration was added, Mg^{2+} then reactivated the enzyme to 100% of the original activity. The inhibitory effect of high concentrations of Zn^{2+} could be overcome by addition of relatively high concentrations of Mg^{2+}. This suggested an essential role of Zn^{2+} and Mg^{2+} for the hydrolytic activity of alkaline phosphatase. When alkaline phosphatase (0.93mg/ml) was dialysed against 4×2 litres of water for 48h to remove ions from the protein, the sample irreversibly lost 40% of its enzymic activity and still contained 500ng of Zn^{2+} and 200ng of Mg^{2+}/ml of enzyme, but only 4ng of Zn^{2+} and 3ng of Mg^{2+}/ml of dialysis fluid. This accounted for 1.3 atoms of Zn^{2+} and 1.3 atoms of Mg^{2+}/molecule of total protein or 2.2 atoms of Zn^{2+} and 2.2 of Mg^{2+}/molecule of hydrolytically active enzyme.

On prolonged dialysis for 72h no bivalent ion and no enzymic activity was detectable in the protein sample. The loss of enzymic activity might have been due to the acidity of distilled water (pH5–6) in promoting Zn^{2+} dissociation, because after dialysis at pH8.0 in Tris-HCl buffer no loss of enzymic activity was seen and 3Zn^{2+} atoms/molecule of enzyme were found (Table 2, Expt. 4). This high concentration of 3Zn^{2+} atoms was possibly due to unspecific adsorption to the protein and inefficient dialysis. This assumption was confirmed by inactivation studies in 0.01M-veronal-acetate buffer: at room temperature as little as 50μM-Zn^{2+} protected the enzyme completely from inactivation at pH5.0 and 4.5, whereas the control lost 60% of the hydrolytic activity within 15min at pH5.0 and 86% at pH4.5. This indicated that loss of enzymic activity was caused by the loss of Zn^{2+}.

EDTA also removed the Zn^{2+} from the enzyme (Table 2, Expt. 1). After dialysis only 40–50% of the original activity was reconstituted in the presence of Zn^{2+} and Mg^{2+} (Table 2, Expts. 1 and 2). Mg^{2+} alone was not bound by the enzyme (Table 2, Expt. 2), in contrast with Zn^{2+} (Table 2, Expt. 3). The data suggested that 2mol of Zn^{2+} was bound by 1mol of hydrolytically active enzyme.

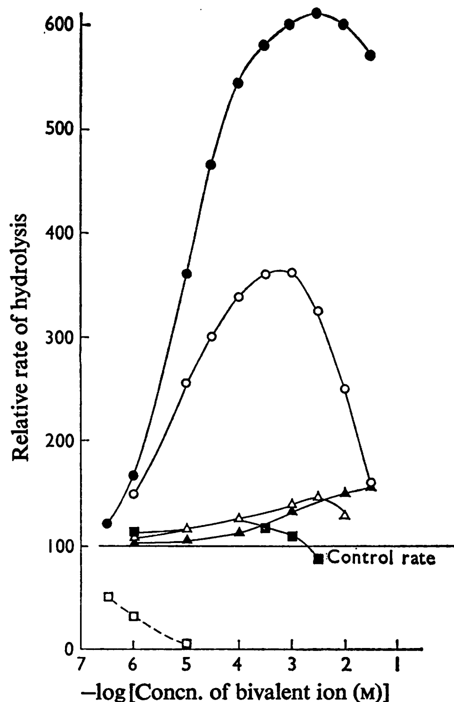

Fig. 7. *Effect of bivalent ions on alkaline phosphatase activity*

The hydrolysis rates for 1mM-*p*-nitrophenyl phosphate in 50mM-Tris–HCl buffer, pH9.5, by 27ng of alkaline phosphatase in 1ml at 37°C were measured in the presence of only one bivalent ion species. For these measurements the enzyme had been passed through a Sephadex G-200 column equilibrated with 10mM-Tris–HCl (pH8.0)–0.1% NaN_3 for removal of most bivalent ions. □, Zn^{2+}; ■, Ni^{2+}; △, Mn^{2+}; ▲, Ca^{2+}; ○, Co^{2+}; ●, Mg^{2+}. Control rate: hydrolysis rate at 1μM-$MgCl_2$ without any other bivalent ion. Other rates are relative to the control rate.

Substrate specificity

Mammalian alkaline phosphatase hydrolyses orthophosphate and pyrophosphate esters as substrates (Fernley & Walker, 1967; Morton, 1955*b*; Cox & Griffin, 1965; Eaton & Moss, 1967). This was now confirmed with the purified alkaline phosphatase from pig kidney (Table 3). All substrates tested were hydrolysed with very similar turnover numbers. The $V_{max.}$ increased in all cases from pH7.5 to 9.5 by a factor of 15–20. The ratio of the K_m at pH9.5 to that at pH7.5 for the mononucleotides was about half of that for the other orthophosphate esters, which might be due to the size of the substrates. The cyclic monophosphates and phosvitin, a macromolecule containing 10% phosphate at serine sites, were not hydrolysed, even after incubation for up to 3h at 37°C (Table 3). The hydrolysis of ATP and ADP was sequential with respect to the phosphate residues as assessed by chromatography of the intermediates during hydrolysis: ADP, AMP and adenine were intermediate products in the first case and AMP and adenine in the second case. In both cases P_i was released. Thus the K_m and $V_{max.}$ values determined (Table 3) are complicated by the hydrolysis rates of the intermediates.

Inhibition by amino acids

Double-reciprocal plots of velocity versus substrate concentration in the presence of different amounts of inhibitor gave straight lines in all cases. Aromatic amino acids inhibited the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase better than did hydrophobic ones (Table 4). Homoarginine was the best inhibitor. Hydrophilic amino acids did not show any effect. As judged from the K_i values, the amino acids would be rather poor inhibitors in terms of a physiological role.

Table 2. *Effect of Zn^{2+} and Mg^{2+} on enzymic activity of alkaline phosphatase*

In Expts. 1, 2 and 3 600μg of enzyme in 200μl was preincubated with 0.3mM-EDTA in the buffer system 50mM-Tris–HCl–0.1% NaN_3, pH8.0, and then dialysed for 48h at 4°C against 1 litre of the same buffer system containing in addition the given salts. In Expt. 4 the enzyme was not preincubated with EDTA but only dialysed against 2×1 litre of 50mM-Tris buffer–0.1% (w/v) NaN_3, pH8.0, for 48h at 4°C. The Zn^{2+} and Mg^{2+} contents were measured by atomic-absorption spectroscopy in the protein solution and the diffusate. From these values the contents (mol of metal/mol of enzyme) were calculated.

		Enzyme activity (test solution)			
Expt. no.	Diffusate	Bivalent ions in 50mM-Tris buffer, pH9.5	Recovery (%)	Zn^{2+} (mol/mol)	Mg^{2+} (mol/mol)
1	0.3mM-EDTA	None	0	0.17	0.023
		10mM-$MgCl_2$+0.3mM-$ZnCl_2$	47		
2	0.3mM-EDTA–1mM-$MgCl_2$	None	0.1	0.17	—
		10mM-$MgCl_2$+0.3mM-$ZnCl_2$	38		
3	0.5μM-EDTA–1μM-$ZnCl_2$	None	7.3	1.0	0.15
		10mM-$MgCl_2$	45		
4	Control	10mM-$MgCl_2$	100	3.2	—

Table 3. *Substrate specificity of alkaline phosphatase*

The rate of hydrolysis was measured at 37°C in 50mM-Tris–HCl buffer containing 5mM-$MgCl_2$ (for details see under 'Methods'). N.D., not detectable.

Substrate	K_m (μM)		$V_{max.}$ (μmol/min per mg of protein)	
	pH9.5	pH7.5	pH9.5	pH7.5
p-Nitrophenyl phosphate	71	0.7	1300	84
β-Glycerophosphate	1000	29	1300	96
Glucose 1-phosphate	1200	30	1200	60
Glucose 6-phosphate	1600	43	1000	61
5′-AMP	500	27	1100	65
5′-GMP	730	39	970	64
5′-CMP	980	45	1100	69
3′:5′-Cyclic AMP	N.D.	N.D.	N.D.	N.D.
3′:5′-Cyclic GMP	N.D.	N.D.	N.D.	N.D.
3′:5′-Cyclic CMP	N.D.	N.D.	N.D.	N.D.
5′-ADP	300	36	530	51
5′-ATP	630	41	360	41
Phosvitin	N.D.	N.D.	N.D.	N.D.

Table 4. *Inhibition of alkaline phosphatase by amino acids*

The rate of hydrolysis of *p*-nitrophenyl phosphate was measured in 1 ml of 50mM-Tris–HCl buffer containing 5mM-$MgCl_2$ (for details see the Results section).

Inhibitor	Concentration of inhibitor ...	Inhibition (%)		Type of inhibition	K_i value (mM)
		5mM	10mM		
L-Homoarginine		74	84	Uncompetitive	1.6
L-Histidine		46	64	Non-competitive	4.8
L-Tryptophan		47	61	Mixed-type	5.6
L-Phenylalanine		36	47	Uncompetitive	10.6
L-Leucine		39	48	Non-competitive	10.9
Glycine		6	9		
DL-Serine		0	0		
DL-Threonine		0	0		

Discussion

Alkaline phosphatases are classified by being inhibited either strongly and uncompetitively by L-phenylalanine, i.e. that from placenta (Ghosh & Fishman, 1968; Ghosh, 1969) or intestine (Ghosh, 1969; Ghosh & Fishman, 1966), or by L-homoarginine, i.e. that from liver and bone (Lin & Fishman, 1972). The pig kidney alkaline phosphatase was uncompetitively and strongly inhibited by homoarginine but also moderately by phenylalanine. Thus from the inhibition studies the kidney enzyme is an intermediate of the two extreme types, being more similar to the liver and bone enzymes.

The highly purified pig kidney alkaline phosphatase hydrolysed ortho- and pyro-phosphate esters. The K_m values were very similar for the different substrates, and for the mononucleotides were similar to the K_m of 5′-nucleotidase in the membrane (Center & Behal, 1966; Bosmann & Pike, 1971). Since both enzymes are membrane-bound, it may be that they are identical. The rate-limiting step for the hydrolysis by pig kidney alkaline phosphatase was of the same order of magnitude for all substrates used (Table 3). This suggests, by analogy with the *Escherichia coli* enzyme (Trentham & Gutfreund, 1968), that a conformational change of a phosphorylated enzyme intermediate may be the rate-limiting step. That such a phosphorylation occurs in the pig kidney enzyme is probable, since this alkaline phosphatase transphosphorylates to Tris at high Tris concentrations as shown above. This interpretation is supported by Morton's (1953) data, in which a transphosphorylation to glucose at high concentrations was shown.

The functional group of the enzyme, which becomes phosphorylated during the enzymic turnover, most likely has a p*K* of 8.7, because such a p*K* was found with two different substrates and because it is not seen in the K_m curve, i.e. its ionization is not affected by combination of enzyme with substrate. The

pK of 8.7 for substrate inhibition confirmed this estimate. Such a pK is, for alkaline phosphatase, probably not due to a thiol group, as suggested previously (Morton, 1954; Lazdunski & Ouellet, 1961; Ghosh & Fishman, 1966; Fishman & Ghosh, 1967*b*; Carey & Butterworth, 1969), because inactivation with CN^- or cysteine was completely reversible. The reported irreversible inactivation (Carey & Butterworth, 1969) may have been due to low pH of the dialysis water, which causes irreversible inactivation by the loss of Zn^{2+} and thus possibly dissociation and random association (Wachsmuth & Hiwada, 1974). Possibly a single tyrosine residue near the active centre is responsible both for the kinetic pK of 8.7 and for the sensitivity of the enzymic activity to tetranitromethane. Since in the plot of log $V_{max.}/K_m$ against pH the same pK values appear as in the plots for K_m and $V_{max.}$ against pH and since *p*-nitrophenyl phosphate has a pK of 7.04 (Kezdy & Bender, 1962) and *β*-glycerophosphate a pK of 6.34 (Delory & King, 1943), the free enzyme should have one pK of 8.7 and another of 7.9–7.8, which is similar to the pK of 7.6 found in intestinal alkaline phosphatase (Lazdunski & Ouellet, 1961).

The hydrolytic activity of alkaline phosphatase seems to depend on two different sites for bivalent-ion binding. The results of Harkness (1968*a*) on alkaline phosphatase from human placenta indicated 2–3 Zn^{2+} atoms/enzyme molecule of mol.wt. 125000, instead of 155000 (Wachsmuth & Hiwada, 1974), and a specific activity of 750 μmol/min per mg of protein at room temperature, which is about 25% less than we found. Recalculation of these values indicates about 4Zn^{2+} atoms/molecule of enzyme (Harkness, 1968*a*). Our own findings suggest that hydrolytically active alkaline phosphatase from pig kidney contains two Zn^{2+} atoms/tetrameric enzyme, which are firmly bound to the enzyme and are responsible for an ordered association of the monomers. They may be removed by EDTA or a slightly acid environment, which causes inactivation of the enzyme. The effect of Mg^{2+} was seen only on $V_{max.}$, i.e. the release of product, suggesting an enhancement effect on the dissociation of phosphate from the phosphorylated enzyme. Zn^{2+} competed for the Mg^{2+}-binding site, inhibiting the enzyme at higher concentrations. Other bivalent ions interfered with this site as well, an effect well known for alkaline phosphatases from different sources (Harkness, 1968*b*; Morton, 1955*a*; Clark & Porteous, 1965).

The interaction of substrate with the enzyme appears to be predominantly of hydrophobic nature because of the low degree of heat change, which is too low for ionic interaction. This also might be shown by the inhibition studies with amino acids, i.e. the more hydrophobic the amino acid the better it is as an inhibitor, the high K_i values in the mM range indicating an unspecific interaction.

The estimation of the energy of activation gave lower values than those for alkaline phosphatase from other sources (Harkness, 1968*b*; Bodansky, 1939; Ghosh & Fishman, 1966; Fernley & Walker, 1965). This discrepancy can be explained, because we found a transition for the energy of activation at 24°C, which was also seen in the heat change. This finding and the two differing sedimentation coefficients beyond the transition temperature suggest two different configurations of the enzyme. Since many membranes have phase transitions at around 25°C and since alkaline phosphatase is a membrane enzyme, a phase transition of the enzyme might reflect an adaptation of the protein for its function in the membrane, very similar to the dependence of *β*-glucoside transport on the membrane phase transition from more ordered to less ordered structure (Wilson & Fox, 1971).

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References

Ahmed, Z. & King, E. J. (1960) *Biochim. Biophys. Acta* **45**, 591–592

Bodansky, O. (1939) *J. Biol. Chem.* **129**, 197–206

Bosmann, H. B. & Pike, G. Z. (1971) *Biochim. Biophys. Acta* **277**, 402–412

Carey, M. J. & Butterworth, P. J. (1969) *Biochem. J.* **111**, 745–748

Center, M. S. & Behal, F. J. (1966) *Arch. Biochem. Biophys.* **114**, 414–421

Clark, B. & Porteous, J. W. (1965) *Biochem. J.* **95**, 475–482

Cox, R. P. & Griffin, M. J. (1965) *Lancet* **ii**, 1018–1019

Delory, G. E. & King, E. J. (1943) *Biochem. J.* **37**, 547–550

Eaton, R. H. & Moss, D. W. (1967) *Biochem. J.* **102**, 917–921

Engström, L. (1961) *Biochim. Biophys. Acta* **52**, 49–59

Fernley, H. N. & Walker, P. G. (1965) *Biochem. J.* **97**, 95–103

Fernley, H. N. & Walker, P. G. (1967) *Biochem. J.* **104**, 1011–1018

Fishman, W. H. & Ghosh, N. K. (1967*a*) *Advan. Clin. Chem.* **10**, 255–370

Fishman, W. H. & Ghosh, N. K. (1967*b*) *Biochem. J.* **105**, 1163–1170

Fiske, C. H. & SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400

Folley, S. J. & Kay, H. D. (1935) *Biochem. J.* **29**, 1837–1850

Ghosh, N. K. (1969) *Ann. N.Y. Acad. Sci.* **166**, 604–637

Ghosh, N. K. & Fishman, W. H. (1966) *J. Biol. Chem.* **241**, 2516–2522

Ghosh, N. K. & Fishman, W. H. (1968) *Biochem. J.* **108**, 779–792

Harkness, D. R. (1968*a*) *Arch. Biochem. Biophys.* **126**, 503–512

Harkness, D. R. (1968*b*) *Arch. Biochem. Biophys.* **126**, 513–523

Kezdy, F. J. & Bender, M. L. (1962) *Biochemistry* **1**, 1097–1106

Lazdunski, M. & Ouellet, L. (1961) *Can. J. Chem.* **37**, 1298–1309
Lin, C. W. & Fishman, W. H. (1972) *J. Biol. Chem.* **247**, 3082–3087
Lineweaver, H. & Burk, D. (1934) *J. Amer. Chem. Soc.* **56**, 658–666
Morton, R. K. (1953) *Nature (London)* **172**, 65–68
Morton, R. K. (1954) *Biochem. J.* **57**, 595–603
Morton, R. K. (1955*a*) *Biochem. J.* **60**, 573–582
Morton, R. K. (1955*b*) *Biochem. J.* **61**, 232–240
Morton, R. K. (1957) *Biochem. J.* **65**, 674–682
Trentham, D. R. & Gutfreund, H. (1968) *Biochem. J.* **106**, 455–460
Wachsmuth, E. D. & Hiwada, K. (1974) *Biochem. J.* **141**, 273-282
Wilson, G. & Fox, C. F. J. (1971) *J. Mol. Biol.* **55**, 49–60