KINETICS OF PLACENTAL ALKALINE PHOSPHATASE

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SUMMARY

The alkaline phosphatase of human placenta, prepared and purified as described in previous papers[1,2] hydrolyses β-glycerophosphate and phenyl phosphate at nearly the same rate. The alkaline phosphatases of the kidney and the intestinal mucosa, on the other hand, hydrolyze phenyl phosphate much more rapidly than β-glycerophosphate. The Michaelis-Menton constant, K_m, for placental alkaline phosphatase is also different.

INTRODUCTION

In a previous paper[3] we described the preparation and purification of the alkaline phosphatase of the human placenta. By extraction with butanol, fractionation with acetone and with ammonium sulphate, electrophoresis on paper and elution, a highly potent phosphatase preparation was obtained, whose activity appeared to be comparable with the most highly purified phosphatases from other tissues. Selective staining of electrophoretic filter-paper strips with lissamine green for total protein and *p*-nitrophenol (from *p*-nitrophenyl phosphate) for phosphatase seemed to indicate that about one quarter of the total protein was enzymically active. By repeating the electrophoresis on filter paper, elution with distilled water at +4° and dialysis in small cellophane bags, by the technique previously described[3], we have obtained an enzyme which now runs electrophoretically in a single band (Fig. 1). We have not

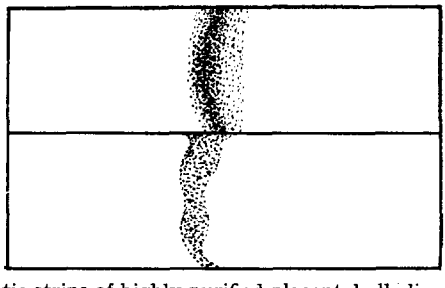

Fig. 1. Paper electrophoretic strips of highly purified placental alkaline phosphatase, prepared by butanol extraction, fractionation, electrophoresis, elution, dialysis and repeated electrophoresis; 1,750 KING-ARMSTRONG units/mg N. Top strip stained with lissamine green, bottom strip with *p*-nitrophenyl phosphate incubated at 37° and exposed to ammonia vapour.

Abbreviation: ATA, Aurine tricarboxylic acid.

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been able to raise its activity above the 1,750 King-Armstrong units previously obtained[3]. This communication describes studies of the chemical composition and kinetic behaviour of this preparation.

The absorption spectra, total and non-protein nitrogen and amino acid content have been measured. Substrate specificity, pH optima, activation and inhibition have been measured and the Michaelis constant determined with several substrates. The kinetic behaviour of the purified placental phosphatase has been compared with those of the kidney and intestinal phosphatases.

MATERIALS AND METHODS

Buffers

Carbonate–bicarbonate buffer: (see ref. 4), veronal buffer: (see ref. 5), glycine buffer: (SÖRENSEN), borate buffer: (PALITSCH). Ethanolamine buffer: ethanolamine was distilled at about 100° *in vacuo*. Buffers of different pH were prepared by the addition of *N* HCl to a 4 *M* solution of the ethanolamine.

Substrates

The following esters were used:

Monoesters: α- and β-glycerophosphate, phenyl phosphate, *o*-cresyl phosphate, bromophenyl phosphate, 2,4-dibromophenyl phosphate, ethyl phosphate and glucose-6-phosphate.

Diesters: Diphenyl and diethyl phosphate.

Polyphosphates: Meta- and pyrophosphates.

Enzymes: Purified placental, kidney and intestinal phosphatases were prepared as described in previous papers[2,3].

Estimation of phosphatase activity

Method of KING AND ARMSTRONG[6]; as modified by KING, ABUL FADL AND WALKER[7]. Hydrolysis of phenyl phosphate at pH 10 in Na_2CO_3 buffer.

Method of MORTON[8]: 0.5 ml sodium β-glycerophosphate solution (to give a final concentration of 0.02 *M*) + 2 ml ethanolamine buffer (pH 9.5 at 38°) (to give a final concentration of 0.04 *M*) + 0.1 ml magnesium sulphate solution (to give a final concentration of 0.01 *M*) + 2.3 ml distilled water in a test tube, were heated in a water bath for 3 min. Phosphatase solution (0.1 ml) of a suitable dilution to give not more than 5 % hydrolysis of the substrate was added. Reaction was stopped after exactly 5 min by adding 2.5 ml 25 % trichloroaceticacid. 1 ml of the mixture was taken + 2.3 ml distilled water + 0.5 ml ammonium molybdate (5 % in 5 *N* H_2SO_4) + 0.2 ml aminonaphthol sulphonic acid solution. After 10 min the blue colour was compared in a photoelectric colorimeter with a suitable phosphate standard treated in the same manner.

MORTON's *units*: The amount of enzyme which liberates 1 μg of inorganic P/min at 38° from sodium β-glycerophosphate.

Nitrogen: Micro-Kjeldahl digestion followed by Nesslerization[9].

Hydrolysis of placental enzyme

The highly purified enzyme, dialyzed in distilled water overnight at 4°, was hydrolyzed in 4 *N* HCl in a sealed tube at 120°, for 16 h. The tube was opened, the

excess HCl removed by evaporation under reduced pressure, and the residue dissolved in 1 ml of distilled water. A two dimentional chromatogram was run, using No. 1 Whatman filter paper (20 × 20 cm) with phenol and then with collidine. After having been dried the chromatogram was sprayed with 0.1 % ninhydrin solution in butanol–water.

Test for histidine: The two dimensional chromatogram was washed by elution with ethyl acetate, the paper left to dry and sprayed with a mixture of sulphanilic acid and sodium nitrite. After having been left to dry again, it was sprayed with 20 % sodium carbonate solution or was exposed to ammonia vapour. A red colour appeared if histidine was present.

pH measurements: These were made at 37°.

RESULTS

Absorption spectra were measured in the u.v. in a Beckman DU spectophotometer. The enzyme solutions were colourless. Fig. 2 shows the absorption spectra of four preparations, two prepared by autolysis and two by butanol extraction[2]. All curves are similar with a maximum absorption at about 278 mμ, probably due to cyclic amino acids. The low absorption at 260 mμ probably indicates an absence of nucleotide.

ROCHE AND BOUCHILLAUX[10] obtained a similar absorption spectrum for their highly purified intestinal phosphatase; and MORTON[8] for his purified intestinal, milk and kidney phosphatases; also ALVAREZ AND LORA-TAMAYO[11] for a purified kidney phosphatase.

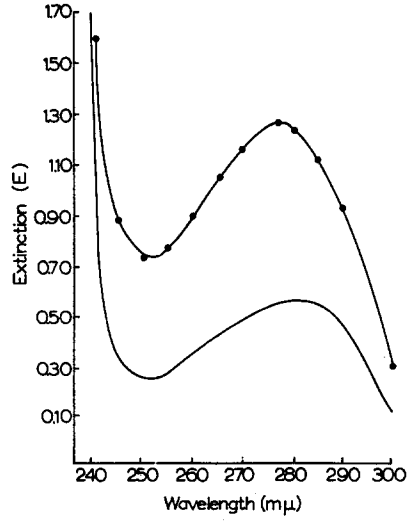

Fig. 2. Absorption spectra of placental alkaline phosphatases prepared from butanol extracts (top line) and from an autolysate of placental tissue in acetone, toluene and ethyl acetate (bottom line).

Chemical composition

Total nitrogen: For the electrophoretically isolated, eluted and dialyzed enzyme, 15.5 % N was obtained. ABUL-FADL AND KING[12] found 10–11 % N for purified

faecal phosphatase; MORTON[13] 16.2 % N for milk and 15.2 % N for intestinal phosphatases.

Carbohydrate: No carbohydrate could be detected with the Molisch reagent. SCHMIDT AND THANNHAUSER[14] reported as much as 21.2 % polysaccharide in purified intestinal phosphatase. ABUL-FADL AND KING[15] and ROCHE AND BOUCHILLAUX[16] reported that their intestinal phosphatase preparations were free of carbohydrate. The latter found 12–22 % carbohydrate in their initial intestinal phosphatase preparation, but only 1.5–4.4 % after purification, due they thought to the elimination of muco-protein. SCHRAMM AND ARMBRUSTER[17] found less than 5 % in intestinal phosphatase, MORTON[8] none by the Molisch reaction, but 2 % with an anthrone reaction[18].

Amino acids: The amino acids, determined by two dimensional chromatography, are compared with those found by ROCHE AND BOUCHILLAUX[10] for their intestinal phosphatase in Table I. Several amino acids are common to both preparations, but we were unable to show the presence of proline, arginine and histidine.

TABLE I

AMINO ACID CONTENT OF PLACENTAL AND INTESTINAL ALKALINE PHOSPHATASE

Amino acid	*Placental*	*Intestinal**
Valine	+	+
Lysine	+	
Serine	+	+
Glycine	+	
Glutamic	+	+
Threonine	+	+
Alanine	+	+
Leucine	+	+
Aspartic	+	+
Tyrosine	Traces	+
Methionine	?	
Phenylalanine	?	+
Proline	—	+
Arginine	—	+
Histidine	—	Absent or traces

* ROCHE AND BOUCHILLAUX[10].

Optimum substrate concentration

The optimum pH for hydrolysis varies with the nature of the substrate and with the substrate concentration[19,20]. Before determing the optimum pH, therefore, we determined the optimum substrate concentration at or near the optimum pH determined in a preliminary experiment. Phenyl phosphate and β-glycerophosphate were used with both carbonate and ethanolamine buffers. The duration of the hydrolysis at 37° was 5 min, accurately timed with the stop-watch. With phenyl phosphate and carbonate buffer the optimum substrate concentration was 0.0025 *M*, and with ethanolamine 0.005 *M*. Similar results were obtained with β-glycerophosphate.

Optimum pH

The activity curves with the two substrates in carbonate and ethanolamine buffers and also with veronal and glycine were determined. The optima were with phenyl phosphate: carbonate pH 10.3, ethanolamine pH 10.2, veronal pH 9.9, and glycine pH 9.6 (Fig. 3). With β-glycerophosphate they were pH 9.7 in ethanolamine, pH 9.9 in veronal, and pH 9.6 in glycine.

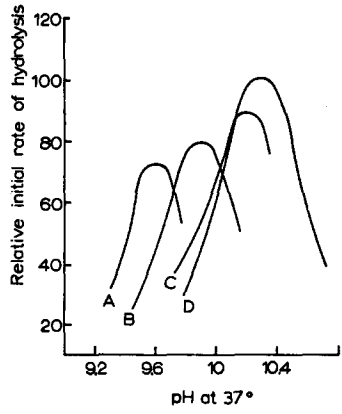

Fig. 3. pH activity curves for the hydrolysis of phenyl phosphate with different buffers. From left to right: glycine, veronal, ethanolamine, carbonate.

The rates of hydrolysis obtained at the optimum pH with the four buffers and both substrates are given in Table II. With phenyl phosphate the highest activity was obtained in carbonate buffer, with β-glycerophosphate in ethanolamine. The addition of magnesium increased the activity in carbonate, ethanolamine and glycine, but not in veronal (Table II). ZITTLE AND DELLA MONICA[21] found that carbonate ions were inhibitory to alkaline phosphatase. With the placental phosphatase and β-glycerophosphate the hydrolysis was a little less in carbonate than in ethanolamine, but in both was much higher than in veronal or glycine. Veronal gave similar results with milk- and intestinal phosphatase.

TABLE II

RATE OF HYDROLYSIS OF PHENYL PHOSPHATE AND β-GLYCEROPHOSPHATE IN DIFFERENT BUFFERS

Substrate	*mg P liberated/0.1 ml enzyme solution/min*							
	Carbonate		*Ethanolamine*		*Veronal*		*Glycine*	
	No Mg^{++}	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*
Phenyl phosphate	(pH 10.3)		(pH 10.2)		(pH 9.9)		(pH 9.6)	
	1.80	2.16	1.36	2.04	0.96	0.98	1.10	1.22
β-glycerophosphate	(pH 9.7)		(pH 9.7)		(pH 9.9)		(pH 9.6)	
	1.50	2.10	1.64	2.24	0.82	0.82	0.86	0.94

Substrate specificity

Like purified kidney and intestinal alkaline phosphatase, that of the placenta hydrolyzed all the monoesters of phosphoric acid tested, *i.e.* α- and β-glycerophosphate,

glucose-6-phosphate, ethyl phosphate, phenyl, bromophenyl, dibromophenyl and *o*-cresyl phosphates, but failed to attack diethyl and diphenyl phosphates and meta- and pyro-phosphates.

SCHMIDT AND THANNHAUSER[14] reported that their purified intestinal enzyme hydrolyzed both di- and polyphosphates as well as monoesters, but this is contrary to our experience. The highly purified placental phosphatase failed to show any activity against them at pH values from 7.5–10, using large amounts of enzyme and incubation periods of up to 2 h.

Comparison of the MORTON *and* KING-ARMSTRONG *units*

The ratio differs with different phosphatases, being highest with the placental (Table III). The big differences led us to investigate the rate of hydrolysis of both α- and β-glycerophosphates in comparison with phenyl phosphate by placental, kidney and intestinal phosphatases (Table IV). Placental phosphatase hydrolyzed the

TABLE III

DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY BY THE MORTON AND KING-ARMSTRONG METHODS

Source of phosphatase solution	MORTON *units/ml (I)*	KING-ARMSTRONG *units/ml (II)*	*Ratios* $\frac{I}{II}$	*Average ratios*
Placental:				
Crude	34.7	0.6	57.8	
Purified	98	1.8	54.4	57.6
Purified	14.6	0.24	60.8	
Kidney:				
Crude	9.8	0.36	27.2	26.4
Purified	30.6	1.2	25.5	
Dog intestinal:				
Purified	12	0.7	17.1	17.4
Purified	6	0.34	17.6	
Calf intestinal:				
Purified	33	1.4	23.6	23.8
Purified	60	2.5	24.0	

* β-glycerophosphate as substrate, ethanolamine–HCl buffer pH 9.5; μg P/min at 38°.
** Sodium phenyl phosphate as substrate, Na_2CO_3:$NaHCO_3$ buffer pH 10; mg phenol/15 min at 37°.

TABLE IV

RATE OF HYDROLYSIS OF α- AND β-GLYCEROPHOSPHATE AND PHENYL PHOSPHATE BY PLACENTAL, KIDNEY AND INTESTINAL ALKALINE PHOSPHATASES

Carbonate and ethanolamine buffers were used.

Enzyme	*mg P liberated/1 ml enzyme solution/15 min*					
	α-Glycerophosphate		*β-Glycerophosphate*		*Phenyl phosphate*	
	Carbonate buffer	*Ethanolamine buffer*	*Carbonate buffer*	*Ethanolamine buffer*	*Carbonate buffer*	*Ethanolamine buffer*
Placental	18	22	21	22	25	22
Kidney	2	4.5	3	7.5	14	9
Intestinal	3.5	10	6.5	20	47	39

three substrates at almost the same rate. Kidney and intestinal phosphatase hydrolyzed phenyl- much more rapidly than β-glycerophosphate, and it more rapidly than α-glycerophosphate, as first shown by KAY[22,23]. The two buffers carbonate and ethanolamine again gave very similar results with placental phosphatase, but quite different results with the kidney and intestinal enzymes. Fresh aqueous extracts of the three tissues confirmed these differences.

Effect of temperature on rate of hydrolysis

Fig. 4 shows the rates of hydrolysis of phenyl phosphate in carbonate buffer by placental phosphatase from 25–70°, with and without added magnesium. The increase is linear up to 55° without added magnesium, and up to 60° in its presence; and is much steeper in the presence of magnesium, which seems to have a protective effect at the higher temperatures. Similar results were obtained with β-glycerophosphate.

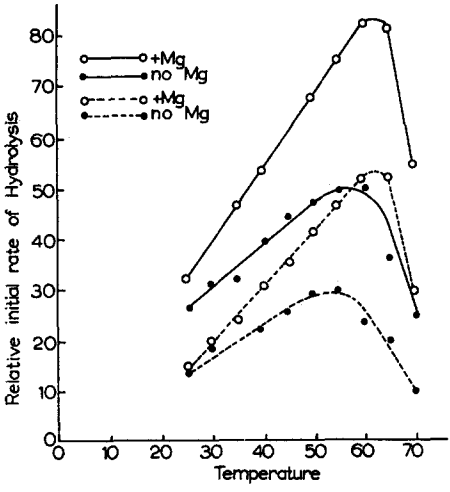

Fig. 4. Effects of temperature on the rate of hydrolysis of phenyl phosphate and β-glycerophosphate by placental alkaline phosphatase. Continuous lines, phenyl phosphate; broken lines, β-glycerophosphate.

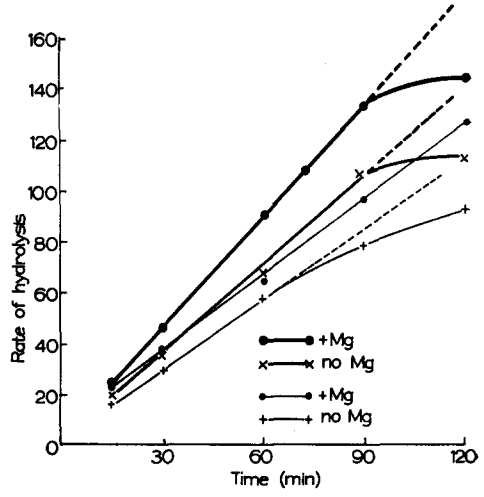

Fig. 5. Effects of time on rate of hydrolysis by placental alkaline phosphatase of phenyl phosphate in carbonate buffer (thick lines) and in ethanolamine buffer (thin lines).

Effect of time on rate of hydrolysis

The hydrolyses were at 37°. With phenyl phosphate in carbonate buffer the rate was linear up to 90 min with and without magnesium. In ethanolamine buffer the rate was linear only to 60 min in the absence of magnesium, while in its presence it remained linear to 120 min (Fig. 5). β-glycerophosphate in carbonate buffer was hydrolyzed in a linear manner for 60 min in the absence of magnesium, and for 90 min in its presence. In ethanolamine buffer the rate was linear for 90 min with and without added magnesium.

Activation and inhibition by cations

Fig. 6 shows the effects of several cations on the hydrolysis of phenyl phosphate in carbonate buffer of pH 10 by the highly purified placental alkaline phosphatase.

Magnesium showed the highest activation at 0.02 M concentration. The effect of Mg^{++} is less pronounced with placental phosphatase than with those from several other tissues, and provides further evidence for its difference from them. The smallness of magnesium activation with placental phosphatase was confirmed with other preparations, and was found whether the enzyme was purified or crude, dialysed or undialysed. Although several factors are known to influence the degree of activation of alkaline phosphatases by magnesium (*e.g.* concentration of Mg^{++}, nature of substrate and its concentration, degree of enzyme purity etc., (see ref. 23)) it nevertheless seems that some phosphatases are more activated by Mg^{++} than others.

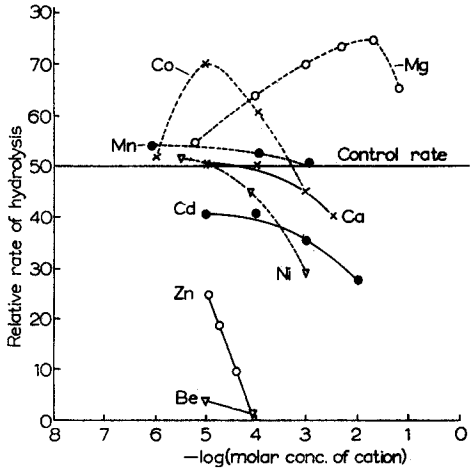

Fig. 6. Effects of cations on the hydrolysis of phenyl phosphate by placental alkaline phosphatase in carbonate buffer.

Co^{++} activated at 0.00001 M optimally; Mn was slightly activating; Ni^{++}, Ca^{++}, Cd^{++}, Zn^{++} and Be^{++} inhibiting. Zinc and beryllium were the strongest inhibitors. The effect of adding Mg^{++} to the enzyme solution in the presence of these cations is shown in Table V. With Co^{++} a slight activation is enhanced, and the powerful inhibition caused by Zn^{++} and Be^{++} disappeared. Where the activation was slight, *i.e.* with Ni^{++}, Ca^{++} and Mn^{++}, Mg^{++} had little or no effect.

TABLE V

ACTIVATION BY Mg^{++} IN THE PRESENCE OF OTHER CATIONS

Inhibitor	*Units/ml enzyme solution*	
	No Mg^{++}	*+ Mg^{++}*
Dialyzed enzyme	40	57
0.0001 M Co^{++}	52	57
0.0001 M Mn^{++}	47	47
0.00001 M Ni^{++}	46	48
0.0001 M Ca^{++}	41	46
0.0005 M Cd^{++}	32	32
0.00002 M Zn^{++}	8	57
0.00001 M Be^{++}	4	54

The experiment was repeated with ethanolamine buffer, with some differences: Mg^{++} was activating, Co^{++}, Mn^{++}, Ni^{++} and Ca^{++} were slightly activating, and Cd^{++}, Zn^{++} and Be^{++} were inhibiting. With Zn and Be the addition of Mg restored activity, although not fully. With the others nearly complete activity was restored.

The inhibition of alkaline phosphatase by Be^{++} has been extensively investigated by KLEMPERER, MILLER AND HILL[25], who claimed that the inhibition is independent of the nature of the substrate. DUBOIS, COCHRAN AND MAZUR[26] suggested that Be^{++} may interfere with the biological function of calcium and magnesium, for it occurs in the same group in the periodic table. ALDRIDGE[27] found that Mg^{++} can reverse the inhibition by Be^{++}. This agrees with our findings and shows that Be^{++} inhibition is largely due to competition with Mg^{++}. LINDENBAUM, WHITE AND SCHUBERT[28] found that ATA reversed the inhibition induced by Be^{++}. VEERKAMP AND SMITS[29] claimed that the inhibition *in vitro* bears no relation to the action of Be^{++} *in vivo* and that Be^{++} acts *in vitro* by precipitating the enzyme. SCHUBERT AND LINDENBAUM[30] suggested that the effectiveness of ATA can be accounted for by bringing about reversible chelation of Be^{++} with alkaline phosphatase.

Activation and inhibition by anions

Inhibition by anionic and related substances was studied at several concentrations. The results are set out in Table VI. Borate, fluoride, D.F.P., and iodo-acetate and thioglycolate were either uninhibiting or slightly inhibiting. Cyanide, iodine, formaldehyde and arsenate were strongly inhibiting, and could not be reversed by magnesium.

SIZER[31] showed that the inhibition of alkaline phosphatase by low concentrations of $KMnO_4$ and iodine was reversed by reducing agents (*e.g.* H_2S). He therefore suggested that the (OH) groupings of the cyclic amino acids, such as tyrosine, are essential to the catalytic action of the phosphatase. The inhibition of phosphatase by borate was studied by ZITTLE AND DELLA MONICA[21]. They concluded that it inhibits in a way similar to that of phosphate. JACOBSEN[32] showed phosphate inhibits both competitively and non-competitively, and from the general similarity in behaviour of arsenate to phosphate, the inhibition by arsenate is to be expected.

TABLE VI

EFFECT OF SOME ANIONS AND OTHER SUBSTANCES ON THE ACTIVITY OF PLACENTAL ALKALINE PHOSPHATASE

Inhibitor	*Units/ml enzyme solution*							
	None		*0.01 M*		*0.001 M*		*0.00001 M*	
	No Mg^{++}	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*	*No Mg^{++}*	*+ Mg^{++}*
Dialyzed enzyme	34	49						
+ Borate			25	33	33	49		
Cyanide			6	6	25	25	37	37
Fluoride			35	35	35	35		
DFP (diisopropylfluorophosphate)							34	49
Iodine					6.5	9.5		
Iodoacetate					35	45		
Thioglycolic acid					27	27		
Formaldehyde			3.5	3.5				
Phosphate					18	33		
Arsenate					2	2		

Macroionic inhibition

During our study of placental phosphatase we tried to concentrate an enzyme solution by placing it in a cellophane bag and then leaving it in a 15 % solution of polyvinyl pyrolidone (molecular weight 11,000). After a few hours the enzyme solution in the bag had undergone considerable concentration by volume, but upon testing proved to be without activity.

KATCHALSKI, BERGER AND NEUMANN[33] described the disappearance of pepsin activity when the enzyme was treated with polymers, and HAHN[34] the inhibition of hyaluronidase. DICZFALUSY *et al.*[35] described the inhibition of acid and alkaline phosphatase, among other enzymes, by phosphate polymers of various aromatic hydroxy and amino compounds.

Activation and inhibition by amino acids

All amino acids were used in a concentration of 0.001 *M*. Alanine alone produced slight activation, the rest either had no action or were slightly inhibitory (Table VII).

TABLE VII

EFFECT OF SOME AMINO ACIDS AND OF ASPARAGINE AND GLUTAMINE (ALL IN 0.001 *M* CONCENTRATION) ON THE ACTIVITY OF PLACENTAL ALKALINE PHOSPHATASE

	K-A. Units/ml enzyme solution		
Amino acid	$+Mg^{++}$	*Amino acid*	$+Mg^{++}$
None	53	Hydroxy proline	50
Alanine	59	Isoleucine	48
Arginine	45	Ketoglutaric	51
Asparagine	49	Lysine	47
Aspartic	50	Proline	49
Glutamic	52	Serine	54
Glutamine	44	Threonine	53
Histidine	45	Tyrosine	54

THOAI, ROCHE AND ROGERS[36] found that intestinal alkaline phosphatase lost most of its activity on prolonged dialysis, but pre-incubation with magnesium and addition of alanine restored its activity to a level higher than before dialysis. MORTON[13] found no activation of intestinal phosphatase by alanine in veronal buffer, but some in carbonate. The slight inhibition by histidine is in contrast to the activation of kidney phosphatase observed by ABUL-FADL AND KING[15] and AKAMATSU AND KOBAYASHI[37].

Michaelis constants

To determine K_m we used the following equation derived by HOFSTEE[38].

$$V_m = v + \frac{v}{S} K_m$$

Plotting v against v/S one gets a straight line cutting both axes. V_m is given by the intercept with the ordinate.

K_m was determined using phenyl phosphate and β-glycerophosphate as substrates and carbonate buffer (pH 10). Both substrates were used at the following

concentrations: 0.005, 0.0025, 0.001, 0.0005 M. The time of incubation was exactly 5 min. Fig. 7 shows the curves obtained using placental alkaline phosphatase. The following K_m values were obtained graphically from figures so drawn. The K_m values for purified kidney and intestinal alkaline phosphatases were similarly determined.

Substrate	*pH*	*Km values*		
		Placental	*Kidney*	*Intestinal*
Phenyl phosphate	10	0.0012	0.0057	0.0021
β-glycerophosphate	10	0.0018	0.025	0.012

These results show quite clearly that while the K_m or substrate affinity of kidney and intestinal phosphatase using phenyl phosphate and β-glycerophosphate are quite different, those obtained with the placental enzyme were nearly the same.

ROCHE AND SARLES[39] found different K_m values for liver, intestinal and bone phosphatase and concluded that such differences may be due to differences in the apo-enzyme. MORTON[8] found the K_m of intestinal phosphatase with phenyl phosphate to be 0.00086, while with β-glycerophosphate it was 0.017.

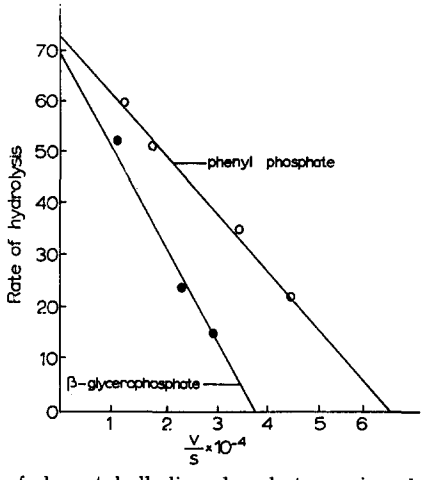

Fig. 7. Evaluation of K_m of placental alkaline phosphatase using phenyl phosphate and β-glycerophosphate as substrates.

On the basis of K_m values FOLLEY AND KAY[40] pointed out that the alkaline phosphomonoesterases seemed to have a much greater affinity for phenyl phosphate than for glycerophosphate. Their conclusions are supported by our findings for kidney and intestinal phosphatases, but cannot be extended to include placental phosphatase.

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