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STUDIES ON ACID HYDROLASES
II. ISOLATION AND PROPERTIES OF SPLEEN ACID PHOSPHOMONOESTERASE

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SUMMARY

A procedure for the isolation of spleen acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) is reported.

Several of the physical and catalytic properties of the enzyme have been studied and shown to be extremely similar to those of prostatic acid phosphomonoesterase. The sedimentation coefficient of spleen acid phosphomonoesterase is close to 5.6 S. The pH–activity curve shows a broad maximum between pH 3.0 and 4.8.

INTRODUCTION

"Non-specific" acid phosphomonoesterase activity is very widely distributed in living organisms (see ref. 1 for a review). Only the acid phosphomonoesterase from human prostate has been obtained in a highly purified state[2–4], and no extensive purification has been reported so far for acid phosphomonoesterases from other sources.

A method developed in this laboratory for the preparation of spleen acid deoxyribonuclease[5], as modified for the large-scale preparation of this enzyme[6], lends itself, with only minor changes, to the isolation and purification of other acid hydrolases as well. These enzymes are also considered to be of lysosomal origin. We report in the present article the isolation and an investigation on the properties of one such enzyme, acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2). This enzyme appears to be very similar to the human prostatic enzyme.

EXPERIMENTAL PROCEDURE

Materials

Disodium *p*-nitrophenyl phosphate was a commercial product (Calbiochem, Los Angeles, Calif.; British Drug Houses, Poole, Dorset, England; Serva, Heidel-

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berg, Germany). Calcium [bis(*p*-nitrophenyl)phosphate]$_2$ was prepared according to PRIVAT DE GARILHE AND LASKOWSKI[7]; alternatively, a commercial sodium salt (British Drug Houses) was used. 2',3'-AMP, 5'-AMP, ATP, riboflavin 5'-phosphate, thiamine monophosphate, phosphoserine and phosphothreonine were purchased from Calbiochem. Glucose 1-phosphate was purchased from Merck, Darmstadt, Germany. All reagents used were of analytical grade.

Column chromatography and sucrose-gradient centrifugation

These were performed as already described[5,6,8].

Assay of enzymatic activity

The phosphomonoesterase activity was routinely assayed by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate. The reaction mixture (total volume 1.1 ml) contained: (a) 1 μmole of *p*-nitrophenyl phosphate (disodium salt), 150 μmoles of acetate buffer (pH 5.0) and 10 μmoles of EDTA; (b) enzyme; this was diluted, if necessary, with 0.15 M acetate buffer–0.01 M EDTA (pH 5.0) containing 0.05 % Armour bovine serum albumin.

After a 10-min incubation at 37°, the reaction was stopped by adding 0.2 ml of 2 M $(NH_4)OH$. The absorption at 400 mμ was measured and a suitable blank was subtracted from the reading. The concentration of liberated *p*-nitrophenol was calculated from the corrected reading taking an $A_{400m\mu}$ value of 12 000 for *p*-nitrophenol[9]. One activity unit is defined as the amount of enzyme which catalyses the liberation of 1 μmole of *p*-nitrophenol per min under the above conditions. Assays were performed using enzyme concentrations that would give $A_{400m\mu}$ readings not higher than 5.0. Under these conditions, a linear relationship was obtained between enzyme concentration and *p*-nitrophenol liberation. The specific activity was calculated by dividing the activity by the $A_{280m\mu}$ value for the enzyme solution. When using substrates other than *p*-nitrophenyl phosphate, the liberation of inorganic phosphate was determined according to MARTIN AND DOTY[10]. Other enzymatic activities (acid deoxyribonuclease, acid ribonuclease, phosphodiesterase and exonuclease) were assayed as already indicated[6].

RESULTS

Isolation of the enzyme

The starting material was crude spleen nuclease II, a preparation already described[8]. Acid phosphomonoesterase activity was determined at the successive steps leading to crude spleen nuclease II, and the results are given in Table I. For the sake of comparison, activity was also determined in two modifications of the extraction procedure, in which 0.15 M NaCl or 0.1 M HCl replaced 0.05 M H_2SO_4. On the average, crude spleen nuclease II contains 110–120 units of acid phosphomonoesterase activity per kg of ground spleen; the specific activity is 0.2–0.3.

TABLE I

PREPARATION OF SPLEEN ACID PHOSPHOMONOESTERASE

Values show total acid phosphomonoesterase activities per kg of trimmed spleen as determined on aliquots taken from a preparation at the successive steps indicated in the first column. The supernatants obtained by centrifuging products 1 and 2 at 8000 × *g* for 1 h, and the aqueous solution of precipitate 4, were dialyzed against 0.15 M NaCl and assayed. The dry weights of undialyzable material per kg of trimmed spleen as determined at the successive steps were reported in Table I of ref. 6.

Preparation step	*Extraction procedure*		
	0.15 *M NaCl*	0.1 *M HCl*	0.05 *M* H_2SO_4
1. Extraction	1460	2250	1420
2. Acidification (pH 2.5)*	350	405	318
3. 0.4 $(NH_4)_2SO_4$ saturation	—	—	—
4. 0.8 $(NH_4)_2SO_4$ saturation	160	221	162

* This was done with 0.2 M HCl for the extracts obtained with 0.15 M NaCl and 0.1 M HCl.

Chromatographic purification

The procedure used was patterned on Procedure C, already described for acid deoxyribonuclease. A summary of a typical purification is given in Table II.

Step I. DEAE-Sephadex A-50 (*Fig.* 1). This step was identical with Step I of Procedure C. Acid phosphomonoesterase activity was not retained by the column equilibrated with 0.05 M phosphate buffer (pH 6.8). Other enzymatic activities found in the same fraction were phosphodiesterase (Fig. 1), acid deoxyribonuclease and cytochrome *c* (Fig. 1, ref. 6) and two different ribonucleases (Fig. 1, ref. 11). A second, very small acid phosphomonoesterase activity peak was eluted by 0.5 M phosphate buffer (pH 6.8). This fraction is under investigation in this laboratory.

TABLE II

CHROMATOGRAPHIC PURIFICATION OF SPLEEN ACID PHOSPHOMONOESTERASE

All values reported refer to the fractions which were processed further or to the final product. The side fractions of the activity peaks were processed separately.

Fraction	*Volume (ml)*	*Total* $A_{280m\mu}$	*Total units*	*Specific activity*
Crude spleen nuclease II				0.25*
I. DEAE-Sephadex**	160	956	1570	1.65
II. Hydroxyapatite	120	170	1200	7.05
III. Sephadex G-100	130	10	784	78.4
IV. CM-Sephadex	65	1	368	368
CM-Sephadex***	12	0.45	184	408

* Average value found for several preparations.

** This product was formed by the phosphomonoesterase-rich fractions obtained from the first peak of DEAE-Sephadex and from the subsequent hydroxyapatite chromatography (Steps I and II of Procedure C in the acid deoxyribonuclease purification).

*** Rechromatography experiment.

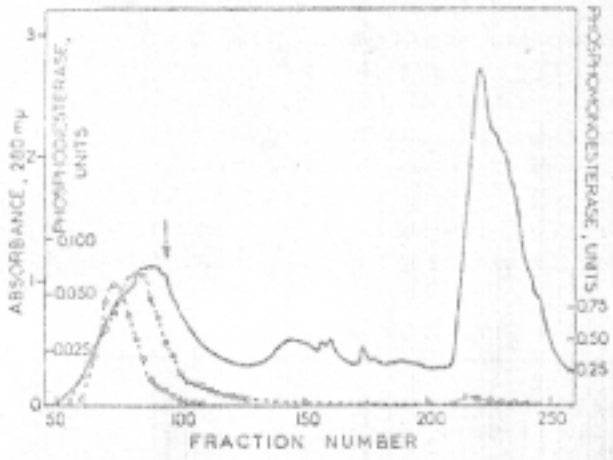

Fig. 1. Chromatography on DEAE-Sephadex A-50 of crude spleen nuclease II (see also ref. 6) (Step I). 330 ml of preparation HS 9 ($A_{280m\mu}$ = 10.3; $A_{260m\mu}$ = 6.9) were loaded on a 8 cm × 80 cm column of DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer (pH 6.8). This buffer was also used to elute the first protein peak. 0.5 M phosphate buffer (pH 6.8) was applied at the fraction indicated by the arrow. 24 ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Squares indicate the acid phosphomonoesterase activity (right-hand scale); circles, the phosphodiesterase activity, which is due to nucleoside polyphosphatase (left-hand inner scale). Acid deoxyribonuclease, cytochrome *c*, acid and basic ribonuclease were also assayed; the results are shown elsewhere[4,12].

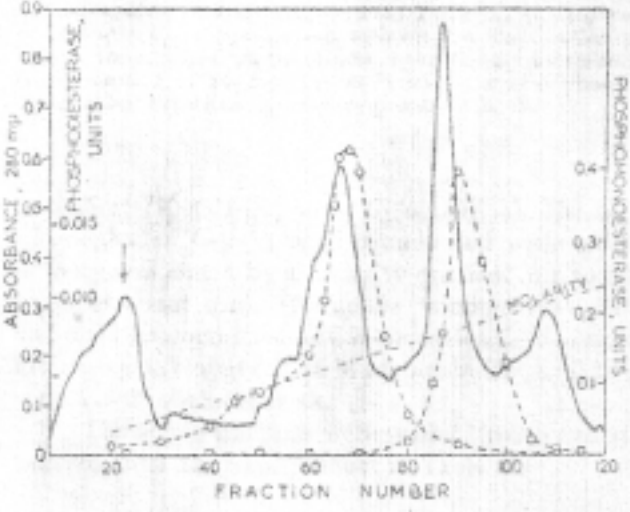

Fig. 2. Chromatography on hydroxyapatite of fractions 50–65 from Step I. 370 ml ($A_{280m\mu}$ = 1.48) were loaded on a 2 cm × 40 cm column of hydroxyapatite equilibrated with 0.05 M phosphate buffer (pH 6.8). A molarity gradient (0.05 to 0.5 M) was started at the fraction indicated by the arrow (at fraction 120, M = 0.35). 24 ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Squares indicate the acid phosphomonoesterase activity (right-hand scale); circles the phosphodiesterase activity, which is due to nucleoside polyphosphatase (left-hand inner scale).

Step II. Hydroxyapatite (*Fig.* 2). This step was identical with Step II of Procedure C. Acid phosphomonoesterase was eluted by the 0.05–0.5 M gradient of phosphate buffer (pH 6.8) at a molarity of about 0.20. Phosphodiesterase was eluted at about 0.12 M (Fig. 2). The elution pattern of other enzymatic activities is given elsewhere (see Fig. 2 of ref. 6 for acid deoxyribonuclease, cytochrome *c* and ribonuclease activities and Fig. 2 of ref. 11 for acid ribonuclease).

Step III. Sephadex G-100 (*Fig.* 3). The acid phosphomonoesterase activity from the previous step was loaded on a Sephadex G-100 column equilibrated with 0.1 M acetate buffer (pH 5.6). The activity was eluted before the main protein peak.

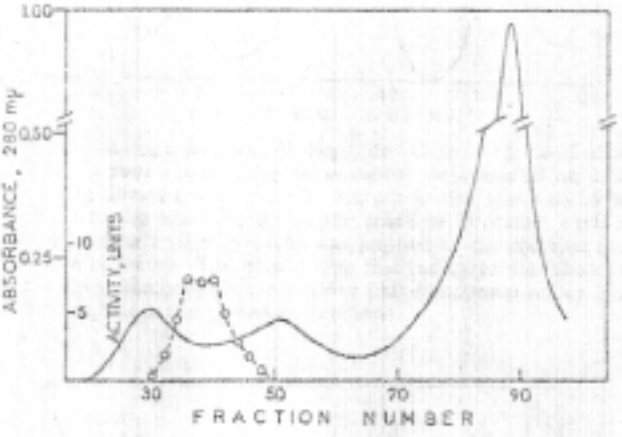

Fig. 3. Gel filtration on Sephadex G-100 of acid-phosphomonoesterase-rich fractions from a hydroxyapatite chromatography. 120 ml ($A_{280\,m\mu}$ = 1.40) were loaded on a 4 cm × 95 cm column of Sephadex G-100 equilibrated with 0.1 M acetate buffer (pH 5.6); the same solvent was used to elute the protein from the column. 13-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid phosphomonoesterase activity (left-hand inner scale). Fractions 34–42 were processed further.

Step IV. CM-Sephadex C-50 (*Fig.* 4). The active fractions were loaded on a CM-Sephadex C-50 column equilibrated with 0.1 M acetate buffer (pH 5.6). The activity was eluted by a molarity gradient, 0.1 to 0.3 M, of acetate buffer at a molarity of about 0.26. The active fractions were diluted with one volume of water and rechromatographed on CM-Sephadex C-50 as above (Fig. 5). Phosphomonoesterase was again eluted at 0.26 M acetate buffer, and the specific activity was practically constant through the peak.

The active fractions were pooled, frozen and stored at −60°. This material is referred to as the final product in Table II.

Properties of the enzyme

Physical properties. Owing to the very limited amount of enzyme which was available, the sedimentation constant was determined by centrifuging acid phosphomonoesterase in a sucrose-density gradient according to MARTIN AND

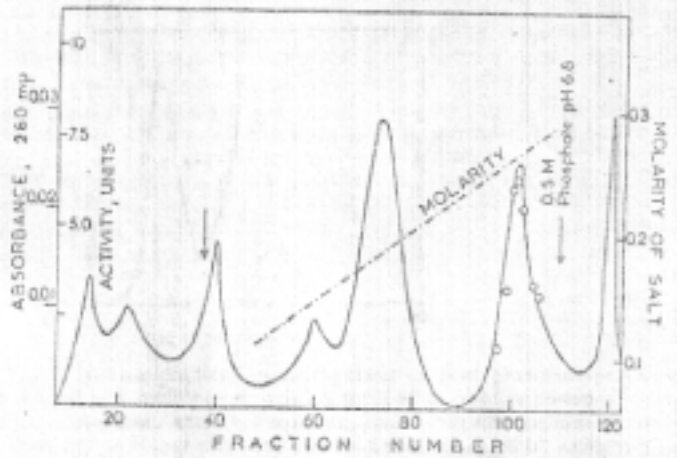

Fig. 4. Chromatography on CM-Sephadex C-50 of the acid phosphomonoesterase fraction from Sephadex G-100. 130 ml ($A_{280m\mu}$ = 0.080) were loaded on a 2 cm × 38 cm CM-Sephadex C-50 column equilibrated with a 0.1 M acetate buffer (pH 5.6). A molarity gradient (0.1 to 0.3 M) of acetate buffer was started at the fraction indicated with an arrow (right-hand ordinate). 0.5 M phosphate buffer (pH 6.8) was applied at the fraction indicated by the arrow. 5-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid phosphomonoesterase activity (left-hand inner scale). Fractions 97–109 were diluted with 1 volume of water and processed further.

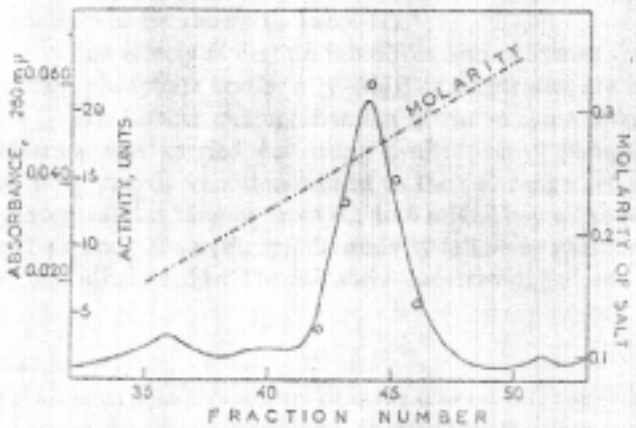

Fig. 5. Rechromatography on CM-Sephadex C-50 of the acid phosphomonoesterase fraction. 130 ml were loaded on a 0.9 cm × 16 cm column of CM-Sephadex C-50 equilibrated with 0.125 M acetate buffer (pH 5.6). A molarity gradient (0.125 to 0.5 M) of acetate buffer was used to elute the enzyme (right-hand scale). 3.2-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the acid phosphomonoesterase activity (left-hand inner scale).

AMES[12]. Cytochrome *c* and acid deoxyribonuclease were used as reference proteins. The results are shown in Fig. 6; a sedimentation constant equal to 5.6 S was calculated.

Purity. No acid deoxyribonuclease, acid ribonuclease, exonuclease and phosphodiesterase were detected after 2 h of digestion at 37° using 0.1 ml of the final

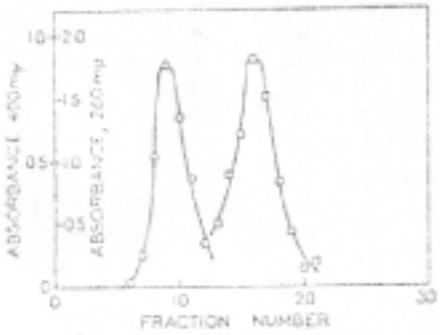

Fig. 6. Sucrose-gradient centrifugation of acid phosphomonoesterase; acid deoxyribonuclease was used as a reference protein. A total of 32 fractions were collected. Circles indicate acid phosphomonoesterase activity; squares, acid deoxyribonuclease activity. The bottom of the cell is at the left. A linear molarity gradient was obtained by using 5 % and 20 % sucrose solution in acetate buffer (pH 5.0) $\mu = 0.15$, containing 0.01 M EDTA. Centrifugation was carried out for 16 h at 38 000 rev./min using a SW-39 rotor and a Spinco Model-L ultracentrifuge.

product. When using 0.1 ml of a 1:100 dilution of the same enzyme solution, the liberation of 0.058 μmoles of *p*-nitrophenol from *p*-nitrophenyl phosphate was obtained in 10 min.

Enzymological properties. The initial rates of hydrolysis of several phosphomonoesters are shown in Table III.

The effects of certain inhibitors and activators on the enzyme, using *p*-nitrophenyl phosphate and/or 2',3'-AMP as substrates, are indicated in Table IV.

The further characterization of the enzyme properties of acid phosphomonoesterase was carried out using *p*-nitrophenyl phosphate as substrate. The pH–activity curves were determined in the pH range 2–6 using five different substrate concentrations ranging from 0.1 to 6 mM. They all show a broad maximum between pH 3.0 and pH 4.8 (Fig. 7), the activity fall being much more abrupt on the acidic than on the alkaline side. The Michaelis constant at 37° and pH 5.0, $\mu = 0.05$, was found

TABLE III

RELATIVE RATE OF HYDROLYSIS OF SEVERAL SUBSTRATES BY SPLEEN ACID PHOSPHOMONOESTERASE

The substrate concentration was 1.5 mM. The liberation of inorganic phosphate at 37° was determined at different incubation times. The initial rates were used.

Substrates	*Relative rate of hydrolysis* (%)
p-Nitrophenyl phosphate	100
2'-3'-AMP	96
5'-AMP	63
β-Glycerophosphate	60
Glucose 1-phosphate	9
Riboflavin phosphate	29
Thiamin phosphate	21
Phosphothreonine	6
Phosphoserine	4
ATP	0
Bis(*p*-nitrophenyl)phosphate	0

TABLE IV

EFFECTS OF SOME ACTIVATORS AND INHIBITORS ON SPLEEN ACID PHOSPHOMONOESTERASE

Assays were performed using the following incubation mixture: 2 μmoles of substrate in 2 ml of 0.05 M acetate (pH 5.0); activator or inhibitor in 0.2 ml; enzyme, diluted with bovine serum albumin, in 0.2 ml. The concentrations of activator or inhibitors given in this table refer to the solutions before adding the enzyme.

Activators or inhibitors	*2',3'-AMP* (%)	*p-Nitrophenyl phosphate* (%)
0.01 M cysteine	—	100
0.001 M cysteine	100	100
0.01 M EDTA	104	102
0.001 M Mg^{2+}	94	89
0.001 M D(−)-tartaric acid	—	90
0.001 M L(+)-tartaric acid	—	8.3
0.01 M Cu^{2+}	8.6	—
0.001 M Cu^{2+}	38	—
0.01 M F^-	2	—
0.001 M F^-	9	7.8
0.001 M Mo^{6+}	—	0.5

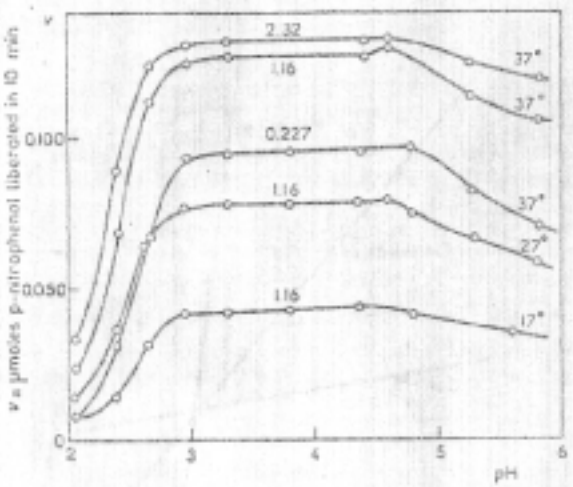

Fig. 7. pH–activity curves for acid phosphomonoesterase. The figure shows enzymatic activity as determined at three different substrate concentrations and three different temperatures. *p*-Nitrophenyl phosphate was used as the substrate; the solvent was 0.05 M acetate buffer.

to be equal to $7.25 \cdot 10^{-5}$ M (Fig. 8). Phosphate ions act as competitive inhibitors (Fig. 9), the inhibition constant being equal to about $3.0 \cdot 10^{-4}$ M. Cl^- also acts as a competitive inhibitor, the inhibition constant being equal to 0.2 M.

DISCUSSION

The results in Table I show that a striking decrease in the extracted acid phosphomonoesterase activity takes place upon acidification to pH 2.5; only about a quarter of the activity survives the acidification step. The enzymatic activity which

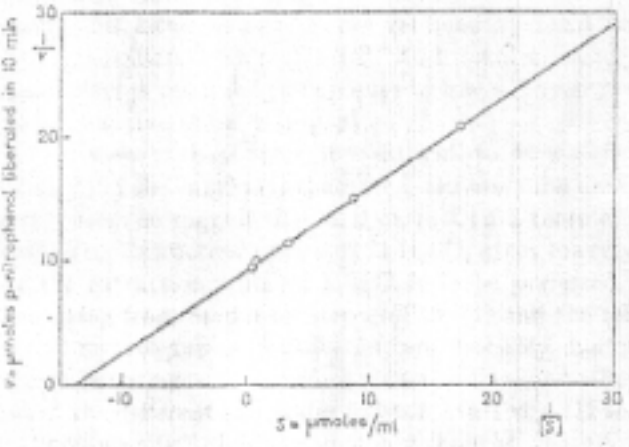

Fig. 8. Lineweaver–Burk plot for acid phosphomonoesterase; *p*-nitrophenylphosphate was used as the substrate; digestions were carried out at 37° in 0.05 M acetate buffer (pH 5.0).

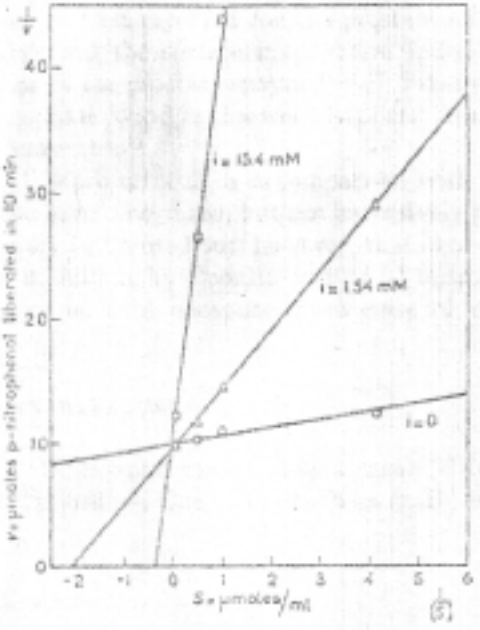

Fig. 9. Competitive inhibition of acid phosphomonoesterase by orthophosphate. Inhibitor concentrations are given in parentheses. Digestions were performed at 37° in 0.05 M acetate buffer (pH 5.0) using *p*-nitrophenyl phosphate as the substrate.

is destroyed by the pH 2.5 step is, however, different from that studied in this work. In fact, if the extract (Step 1 of Table I) is chromatographed on hydroxyapatite, about three-quarters of the total acid phosphomonoesterase activity loaded is not retained at 0.05 M phosphate (pH 6.8), whereas this fraction disappears after acidifi-

cation. This latter activity is not retained by DEAE-Sephadex equilibrated with 0.05 M phosphate buffer (pH 6.8). Additional evidence indicating that the enzyme studied here is resistant to exposure to low pH values is given by the fact that it is still fairly active at pH 2 (Fig. 7).

In view of these facts, it is difficult to draw definite conclusions as far as the "latency" of the enzyme is concerned, although the data obtained with the 0.1 M HCl extract seem to suggest that acid extraction is more effective than the 0.15 M NaCl extraction. Extraction with 0.05 M H_2SO_4 gives lower yields of enzyme, and therefore the extraction with 0.1 M HCl is to be preferred. The activity loss occurring when going from Step 2 to Step 4 (Table I) and the relatively low yield (15–20 %) upon chromatographic purification are probably due in part to the lability of the enzyme with regard to surface denaturation and dialysis. The relative activities toward the different substrates indicated in Table III show that the enzyme is much less "non-specific" than is commonly thought, the phosphomonoesters of threonine and serine being particularly resistant to it. The properties shown in Tables III and IV are very similar to those reported for prostatic phosphomonoesterase[13–18]. Other striking similarities between the spleen enzyme and the prostatic acid phosphomonoesterase are the following. The sedimentation constant found by us (5.6 S) is very close to that reported for the prostate enzyme (5.7 S; see ref. 4). The pH–activity curve and the surface inactivation found for the spleen enzyme closely resemble those of the prostate enzyme[14,16,17]. Finally, the competitive inhibition by inorganic phosphate found in this work has been recently reported for prostatic phosphomonoesterase, too[19].

More difficult is a comparison with other acid phosphomonoesterases which have been recognized, but not extensively purified, in different subcellular fractions. It may be pointed out, however, that in several of its properties, and particularly in its inhibition by fluoride and L(+)-tartrate, the spleen enzyme is similar to the lysosomal acid phosphomonoesterase of rat liver[18].

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