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STUDIES ON ACID HYDROLASES.

V. ISOLATION AND CHARACTERIZATION OF SPLEEN NUCLEOSIDE POLYPHOSPHATASE.

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SUMMARY

1. A new procedure is described for the purification of spleen nucleoside polyphosphatase, an enzyme able to split both the terminal phosphates of nucleoside tri- and di-phosphates and bis-(*p*-nitrophenyl) phosphate. The enzyme was eluted from the final CM–Sephadex column at a fairly constant specific activity.

2. Using bis-(*p*-nitrophenyl) phosphate as the substrate, the enzyme showed a pH optimum close to 6.8, and a Michaelis constant equal to 0.95 mM. Phosphate ions are competitive inhibitors. The sedimentation constant of the enzyme was found to be equal to 3.2 S.

3. A comparison of the hydrolytic activities on bis-(*p*-nitrophenyl) phosphate of 3 enzymes, acid deoxyribonuclease, spleen exonuclease, and nucleoside polyphosphatase, is presented.

INTRODUCTION

KOERNER AND SINSHEIMER[1,2] and SHIMOMURA AND LASKOWSKI[3] observed that spleen acid deoxyribonuclease is accompanied by an enzyme capable of hydrolyzing bis-(*p*-nitrophenyl) phosphate. Both groups of workers referred to this enzyme as a phosphodiesterase. KOERNER AND SINSHEIMER[2] also observed that this enzyme has no exonucleolytic activity on deoxyribooligonucleotides terminated in 3'-phosphate. In an article published in 1958, which has been, so far, the only one devoted to this enzyme, LASKOWSKI AND FILIPOWICZ[4] described its partial purification; these authors noticed that the enzyme was able to split off the terminal phosphate of ribonucleoside tri- and diphosphates and called it nucleoside polyphosphatase.

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The present work will describe a new purification procedure of spleen nucleoside polyphosphatase, leading to a preparation which is chromatographically homogeneous on CM–Sephadex columns, and a characterization of the enzyme.

Since work from this laboratory has shown that both spleen acid deoxyribonuclease and spleen exonuclease are also able to split bis-(*p*-nitrophenyl) phosphate[5,6], we have compared the phosphodiesterase activities exhibited by these enzymes with that of nucleoside polyphosphatase. This comparison is important in view of the fact that bis-(*p*-nitrophenyl) phosphate has been very widely used to measure 'phosphodiesterase' activity, without knowing or realizing that this hydrolytic activity may be due to different enzymes which are very often present in the same extracts or partially purified preparations.

EXPERIMENTAL PROCEDURE

Materials and methods

Materials, column chromatography and sucrose-gradient centrifugation have already been described elsewhere[5,7,8]. The enzyme activity was routinely assayed by measuring the liberation of *p*-nitrophenol from bis-(*p*-nitrophenyl) phosphate. The reaction mixture (total vol. 1.1 ml) contained: (a) 1 μmole of bis-(*p*-nitrophenyl) phosphate and 250 μmoles of succinate buffer (pH 6.4); (b) enzyme; this was diluted, if necessary, with 0.25 M succinate buffer (pH 6.4) 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 $(NH_4)OH$ (containing 0.1 M EDTA, if the calcium salt of the substrate was used). 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 $A_{400\,m\mu}$ value of 12 000 for *p*-nitrophenol[9]. 1 activity unit is defined as the amount of enzyme which catalyzes the liberation of 1 μmole of *p*-nitrophenol per min under the above conditions. The specific activity was calculated by dividing the activity by the $A_{280\,m\mu}$ value for the enzyme solution. Activity on nucleoside triphosphates was measured according to the method of LASKOWSKI AND FILIPOWICZ[4].

RESULTS

Isolation of the enzyme

The starting material was crude spleen nuclease II, a preparation already described[10]. A much higher yield in phosphodiesterase activity was obtained if the acidification step to pH 2.5 was omitted, as for preparation of crude spleen nuclease III (ref. 6). Table I shows the results obtained by following the enzymatic activity during the $(NH_4)_2SO_4$ fractionation of a crude extract.

Two modifications of the procedure, in which 0.1 M HCl and 0.15 M NaCl, respectively, replaced 0.05 M H_2SO_4 in the extraction mixture were also studied and the enzymatic activities are reported in Table I.

TABLE I

PREPARATION OF SPLEEN NUCLEOSIDE POLYPHOSPHATASE

Values show total spleen nucleoside polyphosphatase 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, at 8000 × *g* for 1 h, products 1 and 2 and the aqueous solutions of precipitate 4, were dialyzed against 0.15 M NaCl and assayed. The dry wt. of undialyzable material per kg of trimmed spleen as determined at the successive steps was reported in Table I of ref. 9.

Preparation step	*Extraction procedure*		
	0.15 M NaCl	*0.1 M HCl*	*0.05 M H_2SO_4*
1. Extraction	0.1	12.6	14.6
2. Acidification to pH 2.5	2.95	6.65	1.29
3. $(NH_4)_2SO_4$, 40 % satn.	—	—	—
4. $(NH_4)_2SO_4$, 80 % satn.	3.04	7.75	1.10

Chromatographic purification

The procedure used was patterned on Procedure C, already described for acid deoxyribonuclease[10]. A summary of a typical purification is given in Table II.

TABLE II

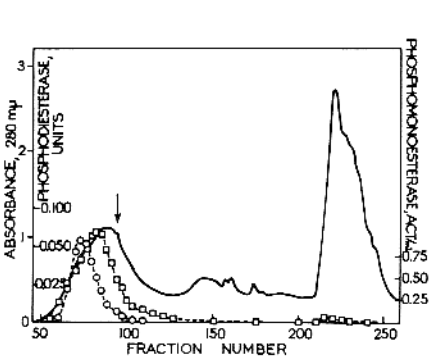
CHROMATOGRAPHIC PURIFICATION OF SPLEEN PHOSPHODIESTERASE

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. Steps I and II refer to preparation HS 9, already described elsewhere for other enzymatic activities[7,10]. Step III refers to preparation HS 11 also described elsewhere[10].

Fraction	*Volume (ml)*	*Total $A_{280\,m\mu}$*	*Total units*	*Specific activity*
Crude spleen nuclease II				
Step I (DEAE–Sephadex)	330	3450	528	0.155
Step II (Hydroxyapatite)	370	547	128	0.233
Step III (CM–Sephadex)	32	74	100	7.4

Step I. DEAE-Sephadex A-50 (Fig. 1). This step was identical with Step I of Procedure C. Nucleoside polyphosphatase 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 acid deoxyribonuclease and cytochrome *c* (Fig. 1, ref. 7) and 2 different ribonucleases (Fig. 1, ref. 10).

Step II. Hydroxyapatite (Fig. 2). This step was identical with Step II of Procedure C. Nucleoside polyphosphatase was eluted by the 0.05–0.5 molarity gradient of phosphate buffer (pH 6.8) at a molarity of about 0.12. Acid phosphomonoesterase was eluted at a molarity of about 0.20. The elution pattern of other enzymatic activities is given elsewhere (see Fig. 2 of ref. 9 for acid deoxyribonuclease, cytochrome *c* and ribonuclease activities, and Fig. 2 of ref. 10 for acid ribonuclease).



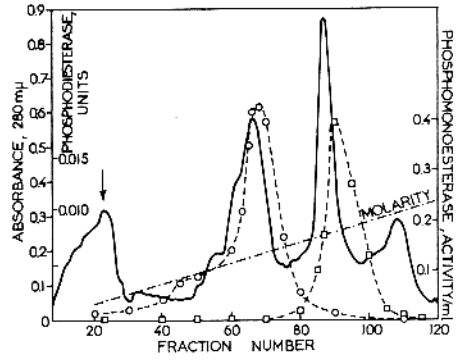


Fig. 1. Chromatography on DEAE–Sephadex A-50 of crude spleen nuclease II (see also ref. 7). (Step I). 330 ml of preparation HS 9 ($A_{280\,m\mu} = 10.3$; $A_{260\,m\mu} = 6.9$) were loaded on an 8 cm × 80 cm column of DEAE–Sephadex A-50 equilibrated with 0.05 M phosphate buffer (pH 6.8). This buffer was also used to elute the first protein peak. 0.5 M phosphate buffer (pH 6.8) was loaded at the fraction indicated by the arrow. 24-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the nucleoside polyphosphatase activity (left-hand inner scale); squares indicate the acid phosphomonoesterase activity (right-hand scale). Acid deoxyribonuclease, cytochrome *c*, acid and basic ribonuclease were also assayed; the results are shown elsewhere[9,10].

Fig. 2. Chromatography on hydroxyapatite of fractions 50–65 from Step I. 370 ml ($A_{280\,m\mu} = 1.48$) 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) was started at the fraction indicated by the arrow (at fraction 120, the molarity was 0.35). 24-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the nucleoside polyphosphatase activity (left-hand inner scale); squares indicate the acid phosphomonoesterase activity (right-hand outer scale). Acid deoxyribonuclease, cytochrome *c*, acid and basic ribonuclease were also assayed; the results are shown elsewhere[9].

Step III. CM-Sephadex C-50 (Fig. 3). The active fractions from the previous step were dialyzed against 0.01 M phosphate buffer (pH 5.7) and loaded on a CM-Sephadex column equilibrated with the same buffer. Some inactive material was removed from the column during the loading step and the washing of the column with the equilibrating buffer. The enzyme activity was eluted by a linear molarity gradient, 0.01–0.25 M, of phosphate buffer (pH 5.7) at a molarity of about 0.11. The specific activity was fairly constant across the peak.

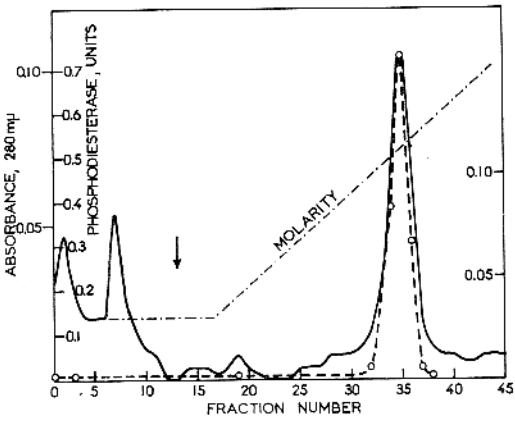
The active fractions were added to an equal volume of glycerol and stored at − 15°.

Properties of the enzyme

As already shown by LASKOWSKI AND FILIPOWICZ[4], the enzyme is able to hydrolyze both bis-(*p*-nitrophenyl) phosphate and the terminal phosphates of ATP and ADP. We have confirmed that it is not able to split deoxyribonucleoside 3′-phosphates.

The following enzymological properties were determined using bis-(*p*-nitrophenyl) phosphate as the substrate. The pH–activity curve was determined in the pH range 4–8 using a 1-mM-substrate concentration. A broad activity maximum centered at pH 6.8 was found (Fig. 4).

The Michaelis constant at 37° and pH 6.4 (0.25 M succinate buffer) was found



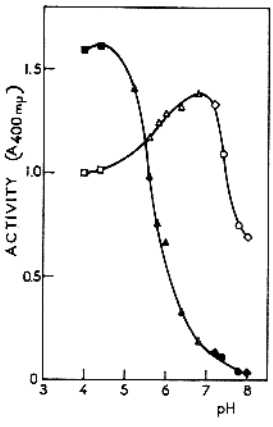


Fig. 3. Chromatography on CM-Sephadex C-50 of the nucleoside polyphosphatase-rich fractions from a hydroxyapatite chromatography. 24 ml ($A_{280\,m\mu} = 0.084$) were loaded on a 1 cm × 20 cm CM–Sephadex C-50 column equilibrated with 0.01 M phosphate buffer (pH 5.7). A molarity gradient (0.03 to 0.25 M) of phosphate buffer was started at the fraction indicated with an arrow (right-hand ordinate). 3.5-ml fractions were collected. The continuous line indicates the absorption at 280 mμ. Circles indicate the phosphodiesterase activity (left-hand inner scale).

Fig. 4. pH activity curve for nucleoside polyphosphatase using 1 mM bis-(*p*-nitrophenyl) phosphate as the substrate. Digestions were carried out at 37°. The solvents were 0.05 M succinate buffer (triangles), 0.05 M acetate (squares), 0.05 M veronal (circles), 0.05 M Tris (lozenges); in all solvents NaCl concentration was 0.1 M. Since the enzyme preparation used in this experiment contained acid phosphomonoesterase activity, this was also assayed in order to provide a correction for the further degradation of *p*-nitrophenyl phosphate (solid symbols).

to be equal to 0.95 mM. Phosphate ions act as competitive inhibitors, the inhibition constant being 1.2 mM. Sulfate ions did not inhibit the enzyme at concentrations as high as 80 mM.

The sedimentation constant was found to be equal to 3.2 S, using cytochrome *c* as a reference protein (Fig. 5).

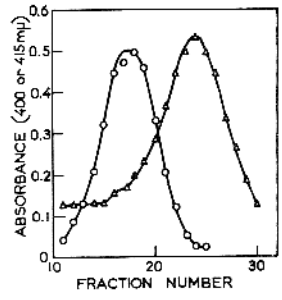


Fig. 5. Sucrose-gradient centrifugation of hog spleen nucleoside polyphosphatase; cytochrome *c* was used as a reference protein. A total of 30 fractions was collected. Circles indicate the nucleoside polyphosphatase activity, triangles the absorbance of cytochrome *c* at 415 mμ. Activity readings at 400 mμ were corrected for the absorption of cytochrome *c* in bis-(*p*-nitrophenyl) phosphate at alkaline pH. Bottom of the cell is at the left. A linear molarity gradient was obtained by using 5 % and 20 % sucrose solutions in acetate buffer (pH 5.0), $\mu = 0.15$, 0.01 M EDTA. Centrifugation was carried out for 16 h at 4° at 38 000 rev./min using a SW-39 rotor and a Spinco Model-L ultracentrifuge.

TABLE III

PROPERTIES OF ENZYMES ACTIVE ON BIS-(*p*-NITROPHENYL) PHOSPHATE

Properties	*Acid deoxyribonuclease*	*Spleen exonuclease*	*Nucleoside polyphosphatase*
1. Sedimentation constant*	3.4	4.6	3.2
2. pH optimum**	5.6–5.9	5.8	6.8
3. Substrates			
Bis-(*p*-nitrophenyl) phosphate	+	+	+
p-Nitrophenyl esters of:			
thymidine 5′-phosphate	−	−	−
thymidine 3′-phosphate	+	+	+
native DNA	+	−	−
ATP, ADP, etc.	−	−	+
3′-Phosphate oligonucleotides	−	+	−
4. Inhibitors**			
HPO_4^{2-}	+	+	+
SO_4^{2-}	+	−	−
Polyribonucleotides	+	+***	−
5. Thermal inactivation (50 %)	60°	57°	
6. Chromatographic properties:			
DEAE–Sephadex	pH 6.8; 0.05 M phosphate	pH 6.8; 0.05–0.1 M phosphate	pH 6.8; 0.05 M phosphate
Hydroxyapatite	pH 6.8; 0.3 M phosphate	pH 6.8; 0.12 M phosphate	pH 6.8; 0.12 M phosphate
CM–Sephadex	pH 6.8; 0.2 M phosphate	pH 6.3; 0.2 M KCl	pH 5.7; 0.11 M phosphate

* As determined by sedimentation in sucrose gradient, using cytochrome *c* as a reference protein; enzymatic assays were done on both bis-(*p*-nitrophenyl) phosphate and the natural substrates: the results were the same.

** Using bis-(*p*-nitrophenyl) phosphate in 0.25 M succinate buffer as the substrate.

*** The enzyme degrades polyribonucleotides to 3′-phosphomononucleosides; these are inhibitory.

A comparison of the hydrolytic activities on bis-(*p*-nitrophenyl) phosphate exhibited by 3 different enzymes from hog spleen, namely acid deoxyribonuclease, exonuclease and nucleoside polyphosphatase is given in Table III. The *minus* marks shown for several substrates and inhibitors indicate that they are completely resistant and completely ineffective, respectively, under the experimental conditions used.

DISCUSSION

The fact that nucleoside polyphosphatase was eluted from the final CM-Sephadex column at a fairly constant activity strongly suggests that our preparation has a higher degree of purity than that of LASKOWSKI AND FILIPOWICZ[4], who found the enzyme activity in the tail of a protein peak in their final CM-cellulose chromatography. This improvement in the enzyme purification seems basically to be due to the hydroxyapatite chromatography. A more precise comparison of the activities of the two preparations is impossible owing to the fact that LASKOWSKI AND FILIPOWICZ[4] did not publish the specific activity of their preparation.

The activity optimum found in the present work (6.8) is close to that (7.0) reported by LASKOWSKI AND FILIPOWICZ[4]. The Michaelis constant relative to the enzyme activity on bis-(*p*-nitrophenyl) phosphate could not be measured by LASKOWSKI AND FILIPOWICZ[4], apparently owing to the low solubility of the calcium salt of the substrate used by them; no difficulty was encountered by us with the sodium salt.

Nothing is known so far about the intracellular localization of the enzyme. Results reported in Table I suggest that nucleoside polyphosphatase is, like other hydrolases previously studied in this laboratory, a 'latent' enzyme. Extraction with 0.15 M NaCl is quite ineffective, whereas acid extraction of the tissue is a very efficient method of obtaining the enzyme. Activity losses are encountered after the acidification to pH 2.5, especially if this is done with H_2SO_4. Interestingly enough, acidification of the 0.15-M NaCl extract causes an increase in activity, suggesting that the small amount of enzyme extracted by 0.15 NaCl is in an inactive state. Preliminary results suggest that nucleoside polyphosphatase may be a lysosomal enzyme, like other hydrolases. If this should be confirmed, nucleoside polyphosphatase might have an important role in lysosomal activity.

A very important side issue of the present work is clarified by the results shown in Table III. They clearly indicate that the properties shown by 3 different enzymes using bis-(*p*-nitrophenyl) phosphate as a substrate are quite distinctive and cannot be due to any kind of cross-contamination.

A rather surprising result is the susceptibility of the *p*-nitrophenyl ester of thymidine 3'-phosphate and the resistance of the 5'-derivatives to all 3 enzymes. It appears that the activity on bis-(*p*-nitrophenyl) phosphate exhibited by these enzymes is to be interpreted as the activity on a very poor general substrate by enzymes whose natural substrates are different; the hydrolysis rate of bis-(*p*-nitrophenyl) substrate by these enzymes is one to two orders of magnitude lower than that of the natural substrates.

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