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## STUDIES ON ACID HYDROLASES

### IV. ISOLATION AND CHARACTERIZATION OF SPLEEN EXONUCLEASE

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#### SUMMARY

1. A new procedure for the preparation of spleen exonuclease is described. The enzyme preparation obtained is free from phosphomonoesterase, deoxyribonuclease, ribonuclease and adenosine deaminase activities.

2. Native DNA is degraded at a rate 25 times lower than alkali-denatured DNA. Poly (A), poly (U) and poly (I) are all degraded by the enzyme at comparable rates, whereas poly (C) is resistant. Bis-(*p*-nitrophenyl) phosphate is a very poor substrate. Using acid deoxyribonuclease digest as the substrate, the pH optimum of the enzyme is close to 5.5 in succinate buffer, whereas a higher value is found in acetate buffer; pH-activity curves are barely affected by the presence of  $Mg^{2+}$ .

3. The enzyme has a sedimentation constant of 4.6 S. Heating at 56° destroys 50 % of the enzyme activity.

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#### INTRODUCTION

Spleen exonuclease is an enzyme particularly useful in sequence studies of oligonucleotides derived from RNA and DNA, since it splits off, in a sequential way, 3'-phosphomononucleotides starting from the 5'-hydroxy end (see refs. 1 and 2 for a review of the literature). The enzyme has also been called spleen phosphodiesterase or spleen phosphodiesterase II, but this terminology will not be used here for reasons given in DISCUSSION.

A method for the partial purification of spleen exonuclease was described by HEPPEL AND HILMOE<sup>3</sup> in 1955 and by HILMOE<sup>4</sup> in 1960; this was later improved by RAZZELL AND KHORANA<sup>5</sup> and RICHARDSON AND KORNBERG<sup>6</sup>.

In a previous publication<sup>7</sup>, we have described a novel purification procedure

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leading to an enzyme preparation with a specific activity comparable to that of the best preparation of RAZZELL AND KHORANA<sup>5</sup>. Difficulties were encountered, however, during the purification of spleen exonuclease; these were mainly due to the instability and consequent loss of activity of the enzyme during the manipulations used, and led to low yields of the enzyme.

We have now improved this method and overcome these difficulties. The present procedure leads to the preparation, in excellent yield, of a stable spleen exonuclease free from phosphomonoesterase, adenosine deaminase, deoxyribonuclease and ribonuclease activities.

## EXPERIMENTAL PROCEDURE

### Materials

3'-Phosphodeoxyribooligonucleotides were obtained by digestion of calf thymus DNA in 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA by spleen acid deoxyribonuclease. The average size of the oligonucleotides present in these digests is 10–12 and the smallest fragments are trinucleotides<sup>8</sup>.

The *p*-nitrophenyl ester of thymidine 3'-phosphate, bis-(*p*-nitrophenyl) phosphate, *p*-nitrophenyl phosphate, *Escherichia coli* tRNA, and RNA core were preparations already described elsewhere<sup>9–12</sup>.

### Methods

Column chromatography and sucrose-gradient centrifugation were performed as already described<sup>9,10,13</sup>.

### Assay of enzymatic activity

The exonuclease activity was assayed by measuring the liberation of mononucleotides from 3'-phosphodeoxyribooligonucleotides. The reaction mixture (total vol. 1.25 ml) contained: (a) 1.2  $\mu$ moles of deoxyribooligonucleotide phosphate, 12.5  $\mu$ moles of EDTA, 187.5  $\mu$ moles of acetate buffer (pH 5.0); (b) enzyme; this was diluted, if necessary, with 0.15 M acetate buffer–0.01 EDTA, (pH 5.0), containing 0.05 % beef-heart cytochrome *c* (Sigma Chemical Company, Type V). After 10 min of incubation at 37°, the reaction was stopped by adding 1.25 ml of 2.5 % perchloric acid containing 0.25 % uranyl acetate. The mixture was chilled for 10 min in an ice-bath and clarified by centrifugation at 4°. The absorbance at 260  $m\mu$  of the supernatant fluid was measured and a suitable blank was subtracted. 1 activity unit is defined as the amount of enzyme that liberates in 1 min mononucleotides having an absorbance at 260  $m\mu$  equal to 1, under the conditions given above. No correction was made for dilution with perchloric acid.

The specific activity was calculated by dividing the activity by the absorbance at 260  $m\mu$  of the enzyme solution. Assays were performed using enzyme concentrations to obtain  $A_{260\text{ m}\mu}$  readings, corrected for blank, not higher than 2.5. Under these conditions, a linear relationship was obtained between enzyme concentration and mononucleotide liberation.

A comparison was made between the activities as measured by the present method and those measured by the procedure of RAZZELL AND KHORANA<sup>5</sup>. The initial hydrolysis rates were compared by using an enzyme preparation described elsewhere<sup>7</sup>. 1 activity unit, as defined above, was found to liberate 1.8  $\mu$ moles of *p*-nitrophenol per h from the *p*-nitrophenyl ester of thymidine 3'-phosphate. Contaminating enzymatic activities (acid deoxyribonuclease, acid and basic ribonuclease, acid phosphomonoesterase, phosphodiesterase) were assayed as described elsewhere<sup>10,12</sup>. Adenosine deaminase was assayed as described by KAPLAN<sup>14</sup>.

## RESULTS

### *Isolation of the enzyme*

This involved: (a) the preparation of a crude enzyme; (b) its chromatographic purification.

#### *Preparation of crude spleen nuclease III*

The following method is derived from the procedure used to prepare crude spleen nuclease II (ref. 10); the basic differences with that procedure are that acidification to pH 2.5 is avoided,  $(\text{NH}_4)_2\text{SO}_4$  precipitations are performed at different saturation levels, and the treatment of the  $(\text{NH}_4)_2\text{SO}_4$  precipitate at 60% satn. has been changed. All operations were carried out at room temperature, except where otherwise stated. Twice distilled water was used to prepare all buffers and solvents. Hog spleens were trimmed, ground with an electrical meat grinder, and homogenized with 0.05 M  $(\text{H}_2\text{SO}_4)$  (1 l per kg of ground spleen; 1 ml of iso-octanol was added to each batch). The final pH was about 4.2. The suspension was centrifuged for 1 h at  $8000 \times g$  at 4°. Solid  $(\text{NH}_4)_2\text{SO}_4$  (209 g/l; 35% satn. at 20°) and EDTA (3.72 g/l) were added to the clear supernatant fluid (clarified extract). The precipitate that formed was removed by filtration on a Seitz filter<sup>10</sup>. Solid  $(\text{NH}_4)_2\text{SO}_4$  (164 g/l; 60% satn. at 20°) and EDTA (3.72 g/l) were added to the clear filtrate. The precipitate that formed was collected by centrifugation (30 min at  $8000 \times g$ , at 4°), dispersed in 60% satd.  $(\text{NH}_4)_2\text{SO}_4$ , put in dialysis bags (Visking casing, boiled for 15 min in 1 mM EDTA-5% sodium bicarbonate and thoroughly washed with twice-distilled water), and dialyzed without stirring at 4° for 48-72 h against many changes of 0.05 M phosphate buffer (pH 6.3). A slight opalescence formed upon dialysis and the enzyme solution was clarified by centrifugation. This product will be called crude spleen nuclease III, to distinguish it from the previously described crude spleen nucleases I and II (refs. 9, 10). Alternatively, it will be called crude exonuclease. The supernatant obtained after the 60%  $(\text{NH}_4)_2\text{SO}_4$  satn. step was brought to 80% satn. and the precipitate which formed was used for preparing acid deoxyribonuclease.

#### *Properties of the clarified extract and the crude spleen nuclease III*

The clarified extract had a total activity of 13 000 units/kg of trimmed spleen, a specific activity of 0.4 and an  $A_{280 \text{ m}\mu}/A_{260 \text{ m}\mu}$  ratio of 1.55. The crude spleen exonuclease, as prepared by the present method, had a total activity of about 9000 units/kg of spleen, a specific activity of about 4, and an  $A_{280 \text{ m}\mu}/A_{260 \text{ m}\mu}$  ratio of 1.70. Slightly less than 1000 units/kg of spleen were found in the precipitate obtained at 35% satn.

of  $(\text{NH}_4)_2\text{SO}_4$  and slightly more than 1000 units/kg were present in the supernatant obtained at 60 % satn. of  $(\text{NH}_4)_2\text{SO}_4$ . During the dialysis of the crude spleen exonuclease preparation about 30 % of the total  $A_{280 \text{ m}\mu}$  was removed.

Some other enzymatic activities measured for crude spleen exonuclease are summarized in Table I.

TABLE I

## ENZYMATIC ACTIVITIES OF CRUDE SPLEEN NUCLEASE III

These data were obtained from preparation SE 5, obtained from 8 kg of trimmed spleen. Compare these results with those of Table I, ref. 7.

<i>Enzyme</i>	<i>Activity/kg of spleen</i>	<i>Specific activity</i>
Exonuclease	8 800	4.0
Acid ribonuclease	32 100	14.5
Acid deoxyribonuclease	550	0.25
Acid phosphomonoesterase	880	0.4

*Chromatographic purification*

The chromatographic purification of spleen exonuclease is derived from the procedures developed in this laboratory to prepare spleen acid deoxyribonuclease<sup>9,10</sup>. A summary of the purification is given in Table II. The following description refers, like Tables I and II, to preparation SE 5.

TABLE II

## CHROMATOGRAPHIC PURIFICATION OF SPLEEN EXONUCLEASE

The reported data refer to preparation SE 5, obtained from 8 kg of trimmed spleen. All values quoted refer to the fractions which were processed further, or to the final product; the sides of the activity peaks were processed separately.

<i>Fraction</i>	<i>Volume (ml)</i>	<i>Total <math>A_{280 \text{ m}\mu}</math></i>	<i>Total units</i>	<i>Specific activity</i>
Crude spleen exonuclease	475	17 800	70 000	4
Step I (CM-Sephadex)	570	910	44 000	48
Step II (Hydroxyapatite)	7.5	20	4 120*	206
Step III (Sephadex G-75)	225	6.7	2 580**	385
Step IV (Hydroxyapatite)	45	4.9	2 670	545

\* This fraction represents only 23 % of concentrated fractions 69-75 (Sec Fig. 2).

\*\* This value is probably underestimated.

*Step I.* CM-Sephadex C-50 (Fig. 1). Crude spleen nuclease III, dialyzed against 0.05 M potassium phosphate buffer (pH 6.3), was loaded on a CM-Sephadex C-50 column equilibrated with the same buffer. A linear gradient (0 to 0.4 M) of KCl in 0.05 M potassium phosphate buffer (pH 6.3) was used to elute the loaded proteins. 2 acid phosphomonoesterase activity peaks were eluted at the beginning of the gra-

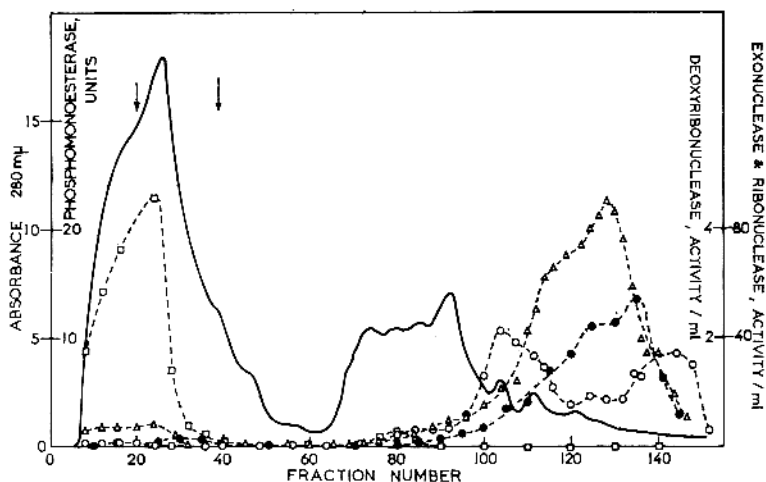


Fig. 1. Chromatography of crude spleen exonuclease on CM-Sephadex C-50 (Step I). 475 ml of preparation SE 5 ( $A_{280\text{m}\mu} = 37.4$ ;  $A_{260\text{m}\mu} = 22$ ) were loaded on a 3 cm  $\times$  90 cm column of CM-Sephadex C-50 equilibrated with 0.05 M potassium phosphate buffer (pH 6.3; at 22°). A molarity gradient (0 to 0.4 M) of KCl in 0.05 M potassium phosphate was used for elution. 25-ml fractions were collected. The enzyme solution was loaded at fraction number 0; at the fraction indicated by the first arrow the column was washed with the equilibrating buffer; the second arrow indicates the start of the linear molarity gradient. The continuous line indicates the absorption at 280 m $\mu$  (left-hand-outer scale). Squares indicate the acid phosphomonoesterase activity (left-hand-inner scale); circles the acid ribonuclease activity (right-hand-outer scale); filled circles the acid deoxyribonuclease activity (right-hand-inner scale); triangles the exonuclease activity (right-hand-outer scale).

dent and at 0.1 M KCl, respectively. These two activities have been identified as spleen acid phosphomonoesterase II (ref. 15) and I (ref. 11), respectively. The exonuclease activity was centered at 0.2 M KCl between 2 ribonuclease activity peaks and just before a deoxyribonuclease activity peak. The total yield of the exonuclease activity after this step was 92 %.

Extreme care should be used in checking the pH of the enzyme solution and of the buffer used in this chromatographic step; the value indicated above was measured at 22°. Using sodium instead of potassium salts in the elution system modifies considerably the elution pattern and the specific activity of the exonuclease peak is much lower.

*Step II.* Hydroxyapatite (Fig. 2). The exonuclease-rich fractions were pooled, adjusted to pH 6.7 with 0.05 M  $\text{K}_2\text{HPO}_4$  and loaded on a hydroxyapatite column. Elution was carried out with a linear gradient (0.05 to 0.4 M) of potassium phosphate (pH 6.8). Exonuclease activity very closely followed a ribonuclease activity peak eluted at about 0.12 M potassium phosphate buffer. A very weak deoxyribonuclease activity peak was exactly superimposed on the exonuclease peak. This seems to be due to the activity of exonuclease on DNA.

*Step III.* Sephadex G-75 (Fig. 3). The exonuclease-rich fractions were concentrated 10 times by freeze-drying and loaded on a Sephadex G-75 column equi-

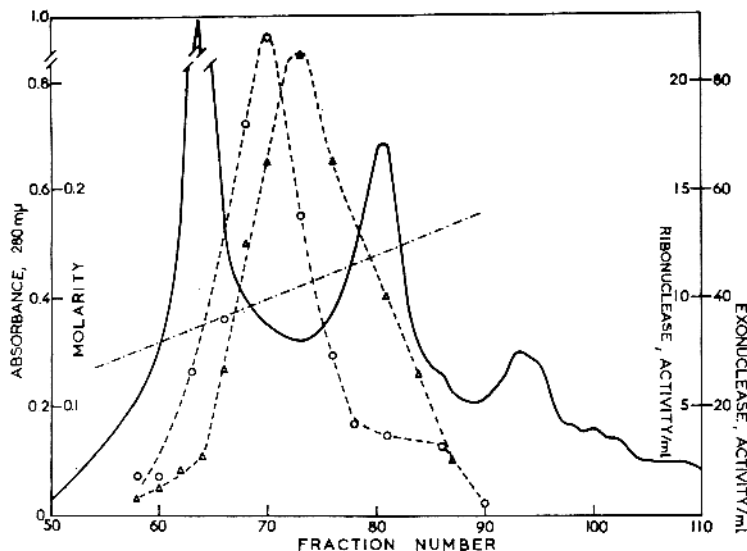


Fig. 2. Chromatography on hydroxyapatite of fractions 112-135 from Step 1 (Step 2). 570 ml ( $A_{280 \text{ m}\mu} = 1.65$ ) were loaded on a 3 cm  $\times$  60 cm column of hydroxyapatite equilibrated with 0.05 M phosphate buffer (pH 6.8). A molarity gradient (0.05 to 0.4) of phosphate buffer was used for the elution (left-hand-inner scale). 25-ml fractions were collected. The continuous line indicates the absorption at 280  $\text{m}\mu$  (left-hand-outer scale). Circles indicate the acid ribonuclease activity (left-hand-inner scale); triangles the exonuclease activity (right-hand-inner scale).

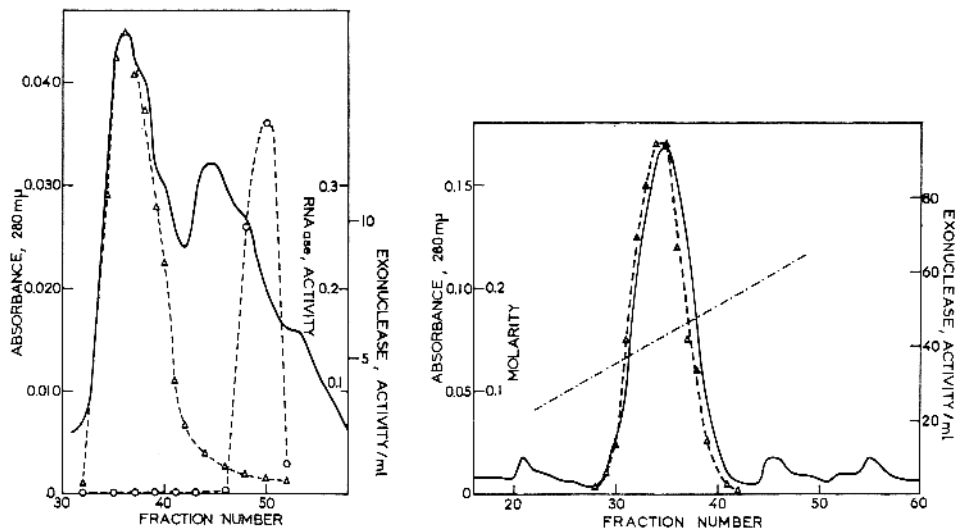


Fig. 3. Chromatography on Sephadex G-75 of an aliquot (23%) from concentrated fractions 69-75 from the previous step (Step 3). 7.5 ml ( $A_{280 \text{ m}\mu} = 2.675$ ) were loaded on a 6 cm  $\times$  90 cm column equilibrated with 0.05 M phosphate buffer (pH 6.8). Elution was carried out with the same buffer. 25-ml fractions were collected. The continuous line indicates the absorption at 280  $\text{m}\mu$ , (right-hand-outer scale). Circles indicate the acid ribonuclease activity (right-hand-inner scale); triangles indicate the exonuclease activity (right-hand-outer scale).

Fig. 4. Chromatography on hydroxyapatite of fractions 32-40 from Step 3 (Step 4). 225 ml ( $A_{280 \text{ m}\mu} = 0.030$ ) were loaded on a 1 cm  $\times$  10 cm column equilibrated with 0.05 M phosphate buffer (pH 6.8). Elution was carried out with a linear gradient (0.05 to 0.4 M) of phosphate buffer. 5-ml fractions were collected. The continuous line indicates the absorption at 280  $\text{m}\mu$  (left-hand-outer scale); triangles the exonuclease activity (right-hand-outer scale).

brated with 0.05 M potassium phosphate buffer (pH 6.8). A complete separation of the ribonuclease activity, which is retarded by the column, from the exonuclease activity, which is immediately eluted, was obtained.

*Step IV.* Hydroxyapatite (Fig. 4). The exonuclease activity peak was loaded on a hydroxyapatite column and eluted as in Step II. The exonuclease activity was eluted at a fairly constant specific activity. The preparation may be freeze-dried with only a small loss in activity.

### *Properties of the enzyme*

#### *Purity*

Spleen exonuclease, as obtained by the present procedure is completely free of the following enzymatic activities: acid phosphomonoesterase, acid deoxyribonuclease, adenosine deaminase. The contamination with ribonuclease seems to be ruled out by results obtained on seryl-tRNA.

It is evident from the last chromatogram shown that the enzyme, as obtained by the present procedure, does not yet exhibit constant specific activity. While efforts are being made towards this goal, we feel that the enzyme preparation is completely satisfactory for any analytical use, especially since it is remarkably stable.

#### *Enzymological properties*

Spleen exonuclease attacks RNA 'core'<sup>4</sup> and acid deoxyribonuclease digests very well. The pH-activity curve, using acid deoxyribonuclease digest as substrate, is shown in Fig. 5. Using succinate and phosphate buffers the pH optimum is found to be close to 5.5; a higher value is found in acetate buffer. The presence of 0.02 M Mg<sup>2+</sup> does not affect sensibly the pH activity curves.

Bis-(*p*-nitrophenyl) phosphate is a rather poor substrate for the enzyme. The pH activity curve obtained is different from that obtained with acid deoxyribonu-

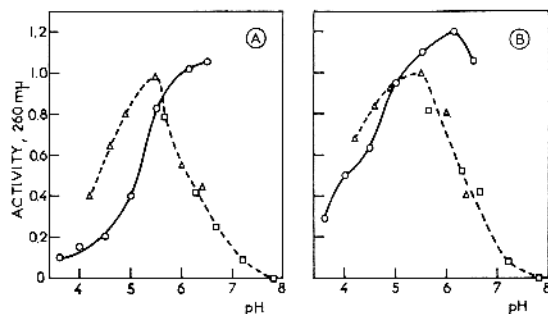


Fig. 5. Exonuclease activity on acid deoxyribonuclease digests; pH-activity curves at 37°. Solvents were: 0.1 M acetate (circles), 0.1 M succinate (triangles), 0.1 M phosphate (squares). Enzyme was in 0.1 M NaCl. The results shown in A and B were obtained in the absence and in the presence of MgCl<sub>2</sub> (0.02 M), respectively.

clease digest (Fig. 6), and resembles that obtained with acid deoxyribonuclease on this substrate (see the following article<sup>20</sup> for a comparison of the phosphodiesterase activities exhibited by exonuclease, acid deoxyribonuclease and nucleoside polyphosphatase).

That both activities are carried out by the same enzyme molecules is indicated by the finding that the ratio of the two activities is constant for different enzyme prep-

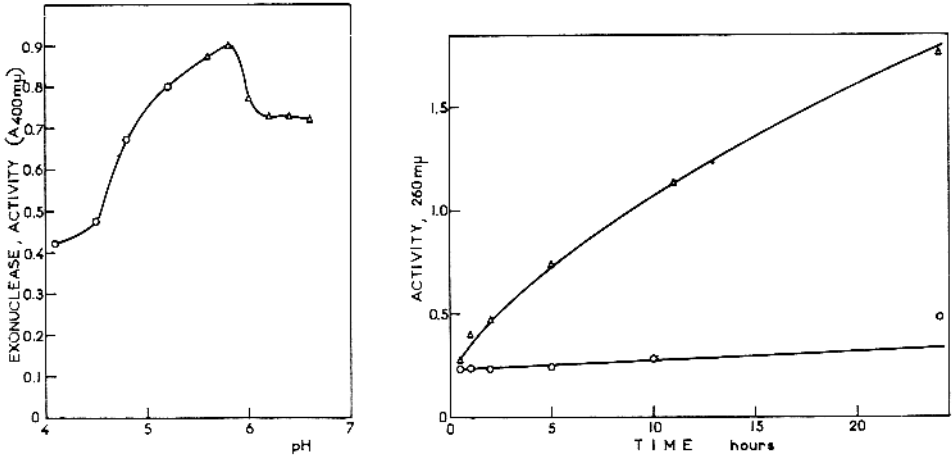


Fig. 6. Exonuclease activity on bis-(*p*-nitrophenyl) phosphate; pH-activity curves at 37°. Solvents were 0.1 M acetate (circles); 0.1 M succinate (triangles).

Fig. 7. Exonuclease activity on native (circles) and alkali-denatured (triangles) calf-thymus DNA. Experiments were carried out in 0.15 M acetate buffer (pH 5.0) + 0.01 M EDTA + 0.04 M Na<sub>2</sub>SO<sub>4</sub>. This was added because it very strongly inhibits acid deoxyribonuclease activity.

arations which have been obtained by various procedures and by the fact that the sedimentation constants of the two activities, as determined by sucrose-gradient centrifugation, are identical (see below). Native DNA is very resistant to enzymatic attack by spleen exonuclease, being split at less than 4 % the rate at which alkali-denatured DNA is degraded (Fig. 7). Interestingly enough, a small percentage of degradation is obtained at a very fast rate in both cases (Fig. 7); the products obtained have not yet been investigated in detail.

Among synthetic polyribonucleotides, poly (A), poly (U) and poly (I) are all degraded at comparable rates, which were lower than that obtained with acid deoxyribonuclease digest, whereas poly (C) was very highly resistant (Fig. 8). All these experiments were carried out at pH 5.0, in the presence of 0.01 M EDTA.

### Physical properties

The sedimentation constant was measured by centrifuging spleen exonuclease in a sucrose density gradient according to MARTIN AND AMES<sup>16</sup>; cytochrome *c* was used as the reference protein. The results of a typical experiment are shown in Fig. 9; a sedimentation constant equal to 4.6 S could be calculated. Identical results were obtained when using acid deoxyribonuclease digest or bis-(*p*-nitrophenyl) phosphate



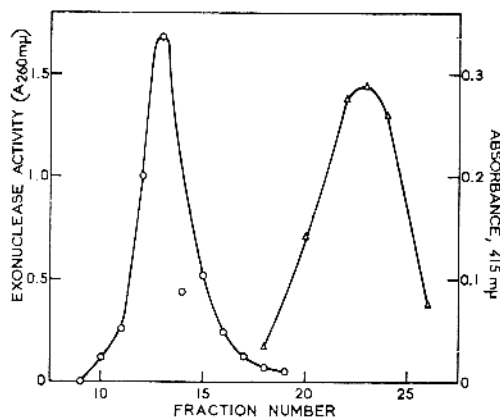
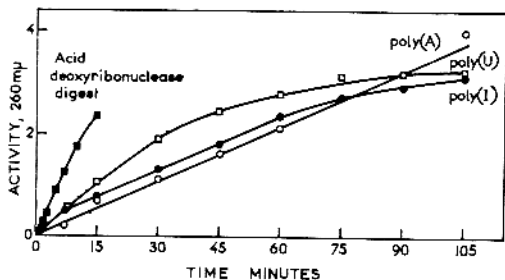


Fig. 8. Time course of the degradation of polyribonucleotides at 37° by exonuclease. The solvent was 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA. The absorbances at 260  $\mu$  of the polyribonucleotide solution before dilution with perchloric were equal to 8.0. The time course of the degradation of acid deoxyribonuclease digest is shown for comparison. Under the same experimental conditions no significant degradation of poly (C) could be detected.

Fig. 9. Sucrose-gradient centrifugation of a preparation of exonuclease; cytochrome *c* was used as a reference protein. A total of 28 fractions was collected. Circles indicate the exonuclease activity, triangles the absorbance of cytochrome *c* at 415  $\mu$ . The bottom of the cell is at the left. A linear density gradient was obtained by using 51 % and 20 % sucrose solutions in 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA. Centrifugation was carried out for 15 h at 4° at 38 000 rev./min using a SW-39 rotor in a Spinco Model L ultracentrifuge.

as the substrates. When using an enzyme preparation which still contained ribonuclease activity it was found that the latter had a sedimentation constant equal to that previously found<sup>12</sup> for acid ribonuclease ( $s = 2.3$  S).

The thermal inactivation curve of the enzyme in 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA is shown in Fig. 10; 50 % inactivation was obtained by heating at about 56°.

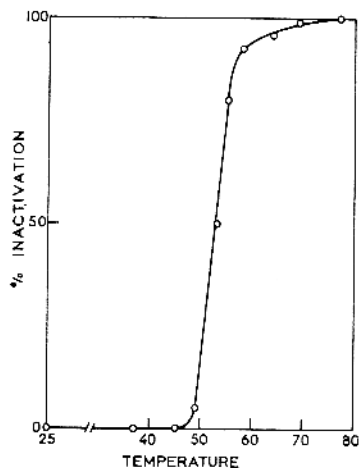


Fig. 10. Thermal inactivation curve. Exonuclease samples in 0.15 M acetate buffer (pH 5.0)–0.01 M EDTA were kept 20 min at the temperatures indicated on the abscissa; they were then chilled in an ice-bath and assayed at 37°.

## DISCUSSION

Compared to the previous method<sup>7</sup>, the yield of crude enzyme is more than doubled in the present procedure (*cf.* Table I of this article and Table I of ref. 7), mainly owing to the different treatment of the  $(\text{NH}_4)_2\text{SO}_4$  precipitate, which is dialyzed against 0.05 M phosphate buffer instead of being dialyzed against distilled water and freeze-dried.

A point of capital importance for the successful purification of spleen exonuclease, as described in the present article, was the choice of the oligonucleotides obtained by digestion of DNA by spleen acid deoxyribonuclease as the substrate. As already mentioned, these oligonucleotides have an average size of 10–12 and the smallest fragments are trinucleotides<sup>8</sup>. They have already been used for detecting exonuclease activity by DOSKOCIL AND SORM<sup>17</sup>. All other substrates used so far have serious drawbacks: (a) RNA core (refs. 3, 4), the water undialyzable 3'-phosphoribooligonucleotides obtained by exhaustive digestion of RNA with pancreatic ribonuclease, is also hydrolyzed by spleen ribonucleases<sup>12,18</sup>; (b) the *p*-nitrophenyl ester of thymidine 3'-phosphate<sup>5</sup> is also slowly hydrolyzed by spleen acid deoxyribonuclease<sup>9</sup> and by nucleoside polyphosphatase<sup>20</sup>; (c) bis-(*p*-nitrophenyl) phosphate is a very poor substrate for spleen exonuclease; furthermore, it is also hydrolyzed by spleen acid deoxyribonuclease<sup>9</sup> and spleen nucleoside polyphosphatase, the enzyme described by LASKOWSKI AND FILIPOWICZ<sup>19</sup>, and further investigated in the following paper<sup>20</sup>. It is clear that the enzyme studied in the present article should be called spleen exonuclease since its substrates are ribo- and deoxyribo-3'-phosphooligonucleotides, whereas non-specific phosphodiesterase substrates like bis-(*p*-nitrophenyl) phosphate are only slowly degraded. We agree with the proposal<sup>21</sup> to restore the original meaning of the term phosphodiesterase to a group name for all enzymes hydrolyzing phosphodiester bonds.

Among the properties exhibited by the enzyme two seem to be particularly valuable from a practical standpoint: (a) the very marked preference for denatured DNA compared to native DNA; this property, unknown so far, may make spleen exonuclease a substitute for exonuclease I from *E. coli*<sup>22</sup>; (b) the absence of ribonuclease contamination, which makes it a very interesting tool for RNA sequence work.

The results obtained with biosynthetic polyribonucleotides do not show any simple relationship between secondary structure and susceptibility to enzymatic hydrolysis; for instance, poly (A) and poly (C) have similar secondary structures at pH 5.0<sup>23</sup>, yet the first one is hydrolyzed at about the same rate as poly (U) which is devoid of secondary structure under the experimental conditions used<sup>23</sup> and the second one is very resistant to enzymatic attack. Clearly, more work needs to be done to elucidate this problem.

The intracellular localization of spleen exonuclease is not yet established. Although enzyme is present in the mitochondrial-lysosomal fraction, considerable activity is found in the soluble fractions<sup>24</sup>.

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