The Purification from Rat Liver of a Nuclease Hydrolysing Ribonucleic Acid and Deoxyribonucleic Acid

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1. The purification of a nuclease from rat-liver mitochondria is described. The mitochondria are rendered soluble by treatment with Triton X-100 and, after fractionation with ammonium sulphate and acetone, the active fraction is further purified by chromatography on DEAE-cellulose and Sephadex G-75 to give a purification of over 700-fold. 2. The purified enzyme was only very slightly contaminated with deoxyribonuclease II, phosphodiesterase and phosphomonoesterase. The individual activities of these enzymes did not exceed 0.1% of the activity of the liver nuclease. 3. The purified enzyme attacked RNA more rapidly than denatured DNA and hydrolysed native DNA more slowly than denatured DNA. 4. There is some evidence to suggest that the nucleolytic activity of the purified preparation towards native DNA, denatured DNA and RNA is associated with a single protein. 5. The enzyme is relatively labile but is stabilized in the presence of 20% (w/v) glycerol or 10mM-2-mercaptoethanol.

The presence of alkaline deoxyribonuclease in mammalian tissues has been demonstrated by Shack (1957), and Beaufay, Bendall, Baudhuin & de Duve (1959) showed that in rat liver this activity is largely localized in the mitochondria. The alkaline deoxyribonuclease of rat liver has been shown to hydrolyse denatured DNA preferentially (Burdon, Smellie & Davidson, 1964), suggesting that the enzyme may be similar to the lamb-brain endonuclease described by Healy, Stollar, Simon & Levine (1963) which has an absolute specificity towards denatured DNA. In the present studies the deoxyribonuclease from rat liver has been purified more than 700-fold without showing any increase in specificity towards denatured DNA.

EXPERIMENTAL

Materials. DNA from Landschutz ascites-tumour cells and calf thymus was used. DNA was prepared from calf thymus glands by the procedure of Kay, Simmons & Dounce (1952). This method was also employed for the isolation of DNA from the sediment obtained on centrifuging disrupted Landschutz ascites-tumour cells at 105000g for 1 hr. (Keir, Binnie & Smellie, 1962). The purified DNA was dissolved in water at a final concentration of 2mg./ml. for subsequent use. Thermally denatured DNA was prepared by heating a solution of DNA (2mg./ml. in water) in a boiling-water bath for 10 min. followed by rapid cooling in an ice bath.

Highly polymerized yeast RNA was purchased from the British Drug Houses Ltd., Poole, Dorset, and dissolved in water to give a solution containing 2mg./ml. Yeast s-RNA,*

prepared by the method of Bell, Tomlinson & Tener (1964), was kindly supplied by Dr D. Bell of this Department. The s-RNA, as judged by its behaviour in the ultracentrifuge and by the hyperchromicity observed on heating, was probably double-stranded. *p*-Nitrophenyl phosphate (disodium salt) was purchased from the British Drug Houses Ltd. and thymidine 5'-*p*-nitrophenyl phosphate (monosodium salt) from the California Corp. for Biochemical Research, Lucerne, Switzerland. Thymidine 3'-*p*-nitrophenyl phosphate was synthesized by the method of Turner & Khorana (1959). Triton X-100 was purchased from Rohm and Hass, Philadelphia, Pa., U.S.A., 2-mercaptoethanol from L. Light and Co. Ltd., Colnbrook, Bucks., and bovine serum albumin from the Armour Pharmaceutical Co., Eastbourne, Sussex. DEAE-cellulose (DE11 fibrous powder with a nominal capacity of 1m-equiv./g.) was purchased from Reeve Angel and Co. Ltd., London, E.C. 4, and was prepared according to the method of Peterson & Sober (1962). The DEAE-cellulose was equilibrated with buffer of 5 times the concentration of the eluting buffer and packed under pressure (approx. 15cm. Hg). The column was then washed with 10 times its bed volume of the eluting buffer. The pH of the effluent was checked to ensure that it was the same as that of the eluting buffer.

Sephadex G-75 (bead form) was purchased from Pharmacia, Uppsala, Sweden. The gel was pretreated and packed as described by Flodin (1962). The packed column (1.7cm. $\times$ 106cm.) was washed with 10mM-imidazole-HCl buffer, pH 6.8, made 5mM with respect to 2-mercaptoethanol, until a stable column had formed. Before use the void volume of the column was determined by measuring the volume of eluent required to move peak concentrations of Blue Dextran 2000 (mol. wt. 2×10^6 ; obtained from Pharmacia) from the top to the bottom of the column. Other reagents were A.R. grade except for tris and imidazole, which were laboratory reagents.

* Abbreviation: s-RNA, transfer or 'soluble' RNA.

Enzyme assays. One unit of activity is defined as the amount of enzyme which releases 1 μ mole of nucleotide or 1 μ g. atom of nucleotide phosphorus/hr. The specific activity is expressed as units/mg. of protein.

(a) Assay of liver nuclease. Activity was measured by following the ultraviolet absorption of the acid-soluble products as in the procedure of Lehman, Bessman, Simms & Kornberg (1958). The assay medium, containing 300 μ g. of denatured DNA, 25 μ moles of imidazole-HCl buffer, pH 6.8, 0.25 μ mole of $MgCl_2$ and 5 μ moles of 2-mercaptoethanol in a total volume of 0.4 ml., was incubated normally for 30 min. at 37° with 0.1 ml. of the enzyme solution containing approx. 0.6 unit. At the end of the incubation 0.3 ml. of bovine serum albumin solution (2 mg./ml.) was added as a carrier and, after mixing, 1.5 ml. of ice-cold $N-HClO_4$. After mixing again the tubes were allowed to stand for 10 min. at 0° before the addition of 0.9 ml. of ice-cold water. The precipitate was removed by centrifugation at 2° at 1000g for 15 min. and the extinction of the supernatant liquid at 260 $m\mu$ was measured. The amount of DNA rendered acid-soluble was calculated by assuming a molar extinction coefficient at 260 $m\mu$ in acid of 10200 (Keir, 1962).

The activity of the liver nuclease on native DNA was measured as above but with 300 μ g. of native DNA replacing denatured DNA. The assay medium for deoxyribonuclease II activity contained 300 μ g. of native DNA, 25 μ moles of acetate buffer, pH 4.7, and 100 μ moles of KCl in a total volume of 0.4 ml.; otherwise the procedure was as described above.

(b) Assay of ribonuclease. The activity of the liver nuclease towards RNA was measured as for deoxyribonuclease except that the denatured DNA was replaced by 300 μ g. of RNA. The corresponding molar extinction coefficient is 10600.

(c) Assay of phosphomonoesterase and phosphodiesterases I and II. The assay medium contained 2 μ moles of *p*-nitrophenyl phosphate, thymidine 5'-*p*-nitrophenyl phosphate or thymidine 3'-*p*-nitrophenyl phosphate respectively with 25 μ moles of imidazole-HCl buffer, pH 6.8, 0.25 μ mole of $MgCl_2$ and 5 μ moles of 2-mercaptoethanol in a volume of 0.4 ml. The enzyme solution was added in a volume of 0.1 ml. and the assay mixture was incubated at 37°. The reaction was stopped by the addition of 2.5 ml. of 0.1 *N*-NaOH and the extinction at 400 $m\mu$ was measured. The molar extinction coefficient under these conditions is 12000 (Razzell & Khorana, 1959).

Determination of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard. For effluents from columns, the extinction at 280 $m\mu$ gave an approximate indication of the distribution of protein.

Determination of ammonium salts. Ammonium salts were estimated with a commercial Nessler's reagent (British Drug Houses Ltd.). The solution was made up to a total volume of 7 ml. with water, 1 ml. of Nessler's reagent was added and the extinction at 490 $m\mu$ was measured. This procedure was satisfactory for samples containing 5–100 μ g. of nitrogen.

RESULTS

Purification of rat-liver deoxyribonuclease

Preparation of mitochondria. Ten male Porton strain rats from the Department colony weighing

180–220 g. were given water but no food overnight. The rats were anaesthetized with ether and killed by exsanguination. The livers were excised and immersed in crushed ice. Subsequent operations were carried out at 0–4° except where otherwise stated. The livers were weighed and cut into small pieces before homogenization in 8 vol. of 0.25 *M*-sucrose in a cooled Potter-type homogenizer. After the initial suspension of the tissue, about five passes of the pestle were found to give a homogenate relatively free of whole cells, as judged by microscopic examination of a smear stained with 1% crystal violet in 0.1 *M*-citric acid. The homogenate was centrifuged at 600g for 10 min. The supernatant liquid was decanted from the sediment and centrifuged at 14000g for 10 min. The supernatant liquid was discarded and the sediment, consisting of mitochondria and lysosomes, was suspended by gentle homogenization into a volume of 10 *mm*-tris-hydrochloric acid buffer, pH 7.5, made 0.1% with respect to Triton X-100, 1.25 times the volume of the original homogenate.

Ammonium sulphate fractionation. Saturated ammonium sulphate solution adjusted to pH 7.5 with aqueous ammonia was added slowly with stirring to the solubilized mitochondria to give 35% saturation. The suspension was kept for 20 min. and then centrifuged at 14000g for 20 min. The precipitate was occasionally found to form a pellet floating on the surface as well as at the bottom of the tube, and so the supernatant liquid was strained through two layers of muslin. To this solution a further volume of saturated ammonium sulphate was added slowly to bring the degree of saturation to 55%. Stirring was continued for 20 min. and the precipitate was collected by centrifugation at 14000g for 20 min. The precipitate was dissolved in the minimum volume of 10 *mm*-tris-hydrochloric acid buffer, pH 7.5, and the amount of ammonium sulphate present was estimated with Nessler's reagent. The concentration of ammonium sulphate was adjusted to 80 mg./ml.

Acetone fractionation. *m*-Tris-hydrochloric acid buffer, pH 6.5, was added to the dissolved ammonium sulphate fraction, 2 ml. being added for every 10 ml. of the solution. The solution was then immersed in an ethylene glycol bath at –15° and the measured volume of acetone, previously cooled to –15°, to bring the solution to 36.9% (v/v) acetone was added immediately with stirring. Stirring was continued for 5 min. and the precipitate was then removed by centrifugation at 700g for 15 min. at –15°. The supernatant liquid was decanted into a conical flask that had previously been immersed in the ethylene glycol bath at –15°. To it was added the required volume of precooled acetone to bring the concentration of acetone to 46.9% (v/v). The solution was stirred for 5 min.,

after which it was centrifuged at 700g for 15 min. at -15° . The precipitate formed a protein pellet situated between two liquid phases. Occasionally no such separation of the liquid phases occurred and it was invariably found that in these circumstances the enzyme preparation was relatively inactive. The liquid was carefully drained away, the pellets were dissolved in a minimum volume of 10mM-tris-hydrochloric acid buffer, pH 7.5, and the solution was dialysed against 7l. of 10mM-tris-hydrochloric acid buffer, pH 7.5, for about 5 hr. The solution was frozen and stored at -20° . No significant losses of activity occurred after storing for 1–2 months at this stage.

DEAE-cellulose. The bed volume of the column was such that the protein/DEAE-cellulose ratio did not exceed 1 mg. of protein/3 ml. packed bed volume. The column (4.7 cm. $\times$ 40 cm.) was equilibrated with 10mM-tris-hydrochloric acid buffer, pH 8.0. The acetone fraction was applied to the column and washed through with 10mM-tris-hydrochloric acid buffer, pH 8.0, contained in a reservoir held 1 m. above the surface of the cellulose. Under such conditions a flow rate of approx. 700 ml./hr. was obtained. Initially it was necessary to measure the protein content and deoxyribonuclease activity of those fractions containing ultraviolet-

absorbing material. The fractions containing activity were then combined. However, since the activity and protein were eluted together, it was found sufficient to combine those fractions with an extinction at 280m μ greater than 50% of the maximum extinction obtained (Fig. 1). Though no separation of activity from inactive protein is apparent in Fig. 1, more than 95% of the protein applied to the column was retained and about 30% of the enzymic activity was eluted (Table 1).

Sephadex G-75. The Sephadex G-75 column (1.7 cm. $\times$ 106 cm.) was equilibrated at 0–4 $^{\circ}$ with 10mM-imidazole-hydrochloric acid buffer, pH 6.8, made 5mM with respect to 2-mercaptoethanol. The void volume had previously been determined with Blue Dextran 2000 and found to be 72 ml. The enzyme was concentrated from the DEAE-cellulose effluent by precipitation in 70% saturated ammonium sulphate. The precipitate was dissolved in 1 ml. of 10mM-imidazole-hydrochloric acid buffer, pH 6.8, made 5mM with respect to 2-mercaptoethanol, and applied to the bed surface of the column. The column was connected to a reservoir containing the same buffer and the flow rate was adjusted to 8 ml./hr. by varying the height of the reservoir. Fractions (3 ml.) were collected and assayed for protein and deoxyribonuclease (Fig. 2). Those fractions containing the enzyme with a specific activity greater than 50% of the maximum

Fig. 1. Elution pattern of the liver nuclease from a column of DEAE-cellulose. The acetone fraction (19 ml.) contained 210 mg. of protein, 1880 deoxyribonuclease units and 2960 ribonuclease units. The column (4.7 cm. $\times$ 40 cm.) was equilibrated with 10mM-tris-HCl buffer, pH 8.0, and was eluted with the same buffer. The effluent was collected in 20 ml. fractions after the first 400 ml. The extinction at 280m μ of each fraction was measured, and each fraction was assayed for deoxyribonuclease and ribonuclease (as described in the Experimental section). The total protein recovered was 7 mg. ●, Protein; ○, deoxyribonuclease activity; △, ribonuclease activity.

Fig. 2. Fractionation of the liver nuclease on a column of Sephadex G-75. The fraction from DEAE-cellulose was precipitated with $(\text{NH}_4)_2\text{SO}_4$, dissolved in 1 ml. of 10mM-imidazole-HCl buffer, pH 6.8, made 5mM with respect to 2-mercaptoethanol, and contained 4.5 mg. of protein and 405 units of nuclease. The column (1.7 cm. $\times$ 106 cm.) was equilibrated and eluted with the same buffer. The flow rate was 8 ml./hr. and 3 ml. fractions were collected. The arrow (↑) indicates the void volume. The protein content of each fraction was determined and each fraction was assayed for activity towards denatured DNA, native DNA and RNA (as described in the Experimental section). ●, Protein; ○, activity towards denatured DNA; □, activity towards native DNA; △, activity towards RNA.

Table 1. *Purification of the rat-liver nuclease*

Experimental details are given in the text.

Fraction	Total volume (ml.)	Activity (units/ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. of protein)	Yield (%)	Purification
Homogenate	500	10	5000	7300	0.69	100	1
Triton X-100	650	9.6	6250	2200	2.85	120	4.1
(NH ₄) ₂ SO ₄ (35–55% satn.)	80	44.5	3560	720	4.78	71	7
Acetone (36.9–46.9%, v/v)	20	165	3300	210	15.7	66	23
DEAE-cellulose	80	11.7	940	7.0	134	19	195
(NH ₄) ₂ SO ₄ (0–70% satn.)	1	506	506	4.5	112.5	10	163
Sephadex G-75	12	17.1	206	0.44	492	4	715

value were pooled to give the final enzyme solution. The enzyme was in general used immediately, but could be stored at 0° in 20% (w/v) glycerol for up to a week. A summary of a purification procedure is given in Table 1.

Purity of the preparation. The purified enzyme was assayed for deoxyribonuclease II, phosphomonoesterase and phosphodiesterase activities. Deoxyribonuclease (1.7 units) was incubated with the various substrates for 4 hr. at 37°. Under these conditions deoxyribonuclease II, phosphomonoesterase and phosphodiesterase individually amounted to no more than 0.1% of the total deoxyribonuclease activity.

The preparation hydrolysed RNA and denatured DNA at comparable rates and native DNA less rapidly. The elution profile on Sephadex G-75 (Fig. 2) of the activity towards RNA and native DNA suggests that the activities belong to the same protein as that hydrolysing denatured DNA. This possibility draws support from the observation that the proportional activities towards RNA, native DNA and denatured DNA of the various fractions obtained during the purification procedure are relatively constant (Table 2), the main discrepancies being in the homogenate and the final fractions. The values of the proportional activities for the homogenate are probably not too meaningful since they represent net results of many concurrent reactions, whereas those shown for the final fractions are rather different from the proportions obtained at earlier stages in the purification and closer values have been obtained in other experiments.

The enzyme hydrolysed s-RNA at a rate such that the ratio of the rate of hydrolysis of denatured DNA to that of native DNA (calf-thymus DNA, purchased from the Sigma Chemical Co.) was approximately the same as the ratio of the rate of hydrolysis of single-stranded RNA to that of s-RNA.

Table 2. *Ratios of the activities of various enzyme fractions towards (i) denatured DNA and native DNA and (ii) RNA and denatured DNA*

Experimental details are given in the text.

Fraction	Activity with denatured DNA	Activity with RNA
	Activity with native DNA	Activity with denatured DNA
Homogenate	13.7	3.3
Triton X-100	2.3	2.8
(NH ₄) ₂ SO ₄ (35–55% satn.)	2.8	3.6
Acetone (36.9–46.9%, v/v)	3.2	2.3
DEAE-cellulose	2.2	3.4
(NH ₄) ₂ SO ₄ (0–70% satn.)	1.9	4.0
Sephadex G-75 (fractions 32–35)	7.0	1.5

Storage of the nuclease

Before the DEAE-cellulose step in the purification, the enzyme preparation could be stored at –20° for 1–2 months without any loss of activity. After passage through DEAE-cellulose the stability of the enzyme was considerably decreased, so that large losses of activity occurred on storage by freezing or freeze-drying. In the presence of 20% glycerol or 10 mM-2-mercaptoethanol the enzyme retained both deoxyribonuclease and ribonuclease activity when stored at 0° for 1 week (Table 3). The assays for deoxyribonuclease and ribonuclease showed no dependency on 2-mercaptoethanol when a freshly prepared DEAE-cellulose effluent was used. However, if this solution was stored in ice for more than 5 hr., both assays were stimulated by as much as 17% by 2-mercaptoethanol.

Table 3. *Effect of storage on the activity of the liver nuclease*

Samples (2 ml.) contained 20 mM-imidazole-HCl buffer, pH 6.8, and 83 μ g. of protein from the DEAE-cellulose fraction/ml. One sample was stored in ice without further additions, a second sample contained glycerol (final concn. 20%, w/v), and a third was made 10 mM with respect to 2-mercaptoethanol. The samples were assayed for deoxyribonuclease and ribonuclease (as described in the Experimental section) at zero time and after 3, 6 and 28 days. The results are expressed as percentages of the zero-time values.

Storage conditions	Activity measured	Activity (% of original activity)		
		3 days	6 days	28 days
Buffer only	Deoxyribonuclease	3.8	0	4
	Ribonuclease	50.5	35.6	24
20% Glycerol	Deoxyribonuclease	107	111	52
	Ribonuclease	111	111	79
10 mM-Mercaptoethanol	Deoxyribonuclease	49.5	50	65
	Ribonuclease	112	115	85

DISCUSSION

The procedure described for the purification of the liver nuclease provides a means of obtaining the enzyme relatively free of contaminating activities, in particular deoxyribonuclease II, phosphomonoesterase and phosphodiesterase, so that the characteristics and specificity of the enzyme may usefully be examined. The enzyme preparation is not homogeneous with respect to protein, as is clearly indicated by its behaviour on Sephadex G-75. Three pieces of evidence suggest that the nucleolytic activities towards RNA, native DNA and denatured DNA are associated with the same protein. First, the elution profiles of the three activities from a Sephadex G-75 column are exactly superimposed; secondly, the proportional activities of the preparation towards denatured DNA, native DNA and RNA are approximately constant for the fractions obtained during the purification procedures; thirdly, the ratio of the rate of hydrolysis of denatured DNA to that of native DNA is approximately equal to the ratio of the rate of hydrolysis of single-stranded RNA to that of s-RNA.

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