Isolation of Deoxyribonuclease II of Rat Liver Lysosomes

(Received for publication, October 12, 1971)

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SUMMARY

Although deoxyribonuclease II (acid DNase) is a widely distributed lysosomal enzyme, the liver enzyme has not been highly purified or well characterized. A procedure is presented for purifying the rat liver lysosomal enzyme 43,000-fold, employing chromatographic fractionations of an extract made from lysosomes. The purified enzyme is free of all other lysosomal enzymes assayed, including phosphatases, other nucleases, and, especially, phosphodiesterase, which has frequently been reported in DNase preparations.

Deoxyribonuclease II (acid DNase) is a lysosomal enzyme (1, 2) frequently used as a marker enzyme in the subcellular fractionation of cellular homogenates, and its participation in the lysosomal degradation of DNA seems evident from recent studies (3). Nonetheless, the only mammalian DNase II claimed to have been isolated in a highly purified state and extensively characterized is the spleen enzyme, work done largely by Bernardi and his collaborators (4). Little has been done to characterize the liver enzyme. Hodes et al. (5) and Lesca (6) have partially purified mouse liver DNase II, and Cordonnier and Bernardi (7) have separated chromatographically two forms of the enzyme from hog liver. In general, acid DNases from various tissues are similar, although a number of differences have been found (for example, see Reference 7). As pointed out under “Discussion,” even with the spleen enzyme there are conflicting reports as to whether the purified enzyme possesses phosphodiesterase activity towards low molecular weight synthetic substrates.

The isolation and characterization of acid DNase are important to understanding its role and regulation. In view of (a) the function of DNase in the lysosomal digestion of nucleic acids (3), (b) the lack of definitive information on a highly purified preparation of DNase obtained from liver lysosomes, (c) the recent interesting report on the presence in liver of an inhibitor of this enzyme (8), and (d) the suggestion (9) that the enzyme may be involved in genetic or mitotic events, it appeared desirable to purify and characterize the DNase of liver lysosomes. The rat liver enzyme was chosen for isolation because so many relevant studies are done with this species.

EXPERIMENTAL PROCEDURE

Materials

Male Wistar rats, 200 to 250 g, were obtained from Harlan Industries, Cumberland, Indiana. Chemicals were obtained from the following sources: Sephadex G-10 and G-100, Pharmacia; Whatman carboxymethylcellulose (CM-32), H. Reeve Angel Co., Clifton, N. J.; DEAE-cellulose, Sigma; bovine serum albumin, sperm whale myoglobin, and ovalbumin, Schwartz/Mann, Escherichia coli alkaline phosphatase (EC 3.1.3.1), E. coli β-galactosidase (EC 3.2.1.23), venom phosphodiesterase I (EC 3.1.4.1), and spleen phosphodiesterase II (EC 3.1.4.1), Worthington; cellulose (MN-300), Brinkmann Instruments, Inc., Westbury, N. Y. Other chemicals, except substrates, were reagent grade from various commercial sources.

Calf thymus DNA was purchased from Worthington. Sedimentation studies of this material, kindly performed by Mr. Sam Allen according to the method of Studier (10), gave $s_{20,w}$ of 20.2 S, corresponding to a molecular weight of $6.2 \times 10^6$. The $s_{20,w}$ of this preparation under alkaline conditions was 14.5 S, corresponding to a molecular weight of about $1.3 \times 10^6$. Under the conditions in which it was used as a substrate in this study, no nuclease, phosphodiesterase, DNase II, or RNase could be detected. The hypochromicity at 260 nm (in 0.1 N NaOH) was found to range, in different lots, from 25 to 37%. RNA (soluble, stripped, from E. coli B) was obtained from General Biochemicals, and polyadenylic acid and Micrococcus luteus DNA from Miles Labs, Kankakee, Illinois. The alternating copolymer poly[d(A-T) -d(A-T)] was a gift from Dr. Rose Litman. Other substrate sources were deoxyribonucleosides and deoxyribonucleoside 5'-phosphates, Sigma and P-L Biochemicals; deoxyguanosine 3'-phosphate, thymidine 3',5'-diphosphate, and p-nitrophenyl thymidine 5'-phosphate, Calbiochem; p-nitrophenyl thymidine 3'-phosphate, Raylo Chemicals, Ltd., Edmonton, Canada; bis[p-nitrophenyl] phosphate and phenolphthalein β-glucuronide, Sigma.

Methods

Acid DNase was routinely assayed, unless otherwise stated, in a 0.9 ml solution, 0.25 M in NaCl and 0.05 M in sodium acetate, pH 4.1, containing enzyme, 200 µg of calf thymus DNA (added...
from a stock solution of 2 mg per ml in 0.05 M sodium acetate, pH 5.0, and, when the enzyme was of specific activity greater than about 10,000, 10 μg of bovine serum albumin. This method was adapted from the methods of Bowers et al. (11) and Horvat and Touster (12), modified to the final form after the optimal conditions were determined (see under "Results"). All tissue fractions were treated, before assay, with Triton X-100 at a final concentration of 0.1% for at least 30 min in ice. Assays were performed at 37°C for 30 min and were stopped by addition of 1.0 ml of cold 10% perchloric acid. This mixture was allowed to stand in ice for 5 min and was then centrifuged in the cold room in a Sorvall SM 24 rotor at 15,000 rpm (37,000 × g) for 15 min. The supernatant solution containing acid-soluble oligonucleotides was decanted, and its absorbance read at 260 nm. The blank consisted of the assay mixture with enzyme present, except that the DNA was added after the perchloric acid. No breakdown of DNA to acid-soluble products occurred in the absence of enzyme. Data are expressed either directly as this absorbance value or corrected to standard units of activity as follows. The optical density obtained (after a blank correction) was multiplied by 4 to (a) correct for the dilution of the assay mixture by perchloric acid and to (b) adjust the value from 30 min to 1 hour. Thus 1 unit of DNase is the activity catalyzing a (corrected) optical density increase of 1.0 in an assay volume of 1.0 ml in 1 hour. In practice, sufficient DNase was usually used to yield an optical density increase of from 0.5 to 1.0 in the centrifuged supernatant solution, corrected for blank. One unit of activity corresponds to the production of 0.1 μmole of acid soluble oligonucleotide bases, assuming a molar extinction coefficient of 10,000 at 260 nm. Specific activity is expressed as units per mg of protein.

Acid DNase was assayed under the same conditions as given for DNase, except that E. coli-soluble RNA was substituted for DNA, at the same weight concentration. Phosphodiesterases activity against p-nitrophenyl thymidine 5'-phosphate and bis-(p-nitrophenyl) phosphate were assayed under the conditions described by Brightwell and Tappel (13), and the activity against p-nitrophenyl thymidine 3'-phosphate was assayed according to the method of Bernardi and Griffe (14). The nonspecific lysozymal acid nucleotidase was assayed according to the method of Aresenis and Touster (15), and acid phosphatase was assayed according to the method of Gianetto and de Duve (16). Three alkaline endonucleases catalyzing hydrolysis of DNA were assayed according to the methods of Beaufay et al. (2), O'Connor (17), and Curtis and Smellie (18).

Protein was measured by the method of Lowry et al. (19) as modified by Miller (20) and β-glucuronidase by the procedure of Stahl and Touster (21) as modified from Tulalay et al. (22). Cytochrome c and ovalbumin were determined by measuring absorbance at 410 nm and at 230 nm, respectively. E. coli alkaline phosphomonoesterase was assayed essentially according to the method of Garen and Levinthal (23), and β-galactosidase by the method of Craven et al. (24). Protein in the effluent from preparative column chromatography and gel filtration was monitored by the absorbance at 280 nm. Gel filtration was performed according to the procedure of Andrews (25).

Analytical disc gel electrophoresis was performed at pH 8.5 according to the method of Davis (26) after adding DNA to the gel as described by Boyd and Mitchell (27). Protein was visualized on gels with Coomassie brilliant blue (28); as little as 0.5 μg of ovalbumin (used as standard) could be detected. After a 30-min incubation in 0.25 M NaCl-0.05 M acetate, pH 4.1, DNA was detected by staining with methyl green according to the method of Boyd and Mitchell (27).

Oligonucleotides from the digestion of DNA by liver lysosomal DNase were separated according to size by the method of Tomlinson and Tener (29) and were desalted on Sephadex G-10. Specific details are given with the appropriate experiment under "Results." In the case of digestions which were not "sized," only the desalting step was employed to prepare the samples for subsequent work.

Thin layer chromatography was employed using cellulose (MN-300) spread as a 1:5 (w/w) suspension to a depth of 1.0 mm. The solvent systems of Felix et al. (30), as described for paper chromatography, were used; Rf values of standards were found to be similar to those of Felix et al. (30).

The method used for determination of terminal phosphate of oligonucleotides with E. coli phosphomonoesterase was similar to that of Koh, Waddell, and Aposthian (31), employing optimum conditions for the enzyme (Garen and Levinthal (23)). That is, 0.05 to 0.1 μmole of oligonucleotide phosphate was incubated in a final volume of 100 μl with 20 μmoles of Tris-Cl, pH 8.0, and 2 μl (20 μg) of E. coli phosphomonoesterase ( Worthington, Loto OCB, OEA, and 9GC) at 37°C for periods of 60 min up to 12 hours, and the liberated phosphate was determined by the method of Bartlett (32) for inorganic phosphate in the presence of phosphate esters, on one-tenth the scale used by this author. Total phosphate analysis was also performed according to Bartlett's procedure. Occasionally, two other methods were employed for confirmation (33, 34). The alkaline phosphatase concentration used was at least 10-fold the amount necessary to cleave the phosphate quantitatively from similar amounts of deoxyguanosine 3'– and 5'–phosphate standards under the same conditions.

Venom and spleen phosphodiesterases were employed in studies of the structures of the oligonucleotides after investigating the possible presence of significant amounts of contaminating enzymes. The conditions for utilizing venom phosphodiesterase were adapted from the method of Rassell and Khorana (35) and those for the spleen enzyme from the method of Hillmoe (36). p-Nitrophenol liberated by the phosphodiesterases was determined at 400 nm after stopping the reaction by adding 4 assay volumes of 0.1 N NaOH; the measurement of inorganic phosphate liberated by nucleotidases was performed as described above for alkaline phosphomonoesterase (32). The concentrations of the test substrates were identical (5 to 10 mg) to the concentrations of oligonucleotides exposed to the phosphodiesterases in the actual structural studies, as were all the other conditions (length of digestion, enzyme concentration, etc.). The amount of enzyme used was some 1000-fold in excess of that required to hydrolyze completely its typical substrate under the conditions (described below) which were used for the actual work on the oligonucleotides. Venom phosphodiesterase, which of course readily cleaved p-nitrophenyl thymidine 3'-phosphate, had no activity against 3'- or 5'-dGMP or thymidine 3',5'-di phosphate, or against p-nitrophenyl thymidine 3'-phosphate, the typical substrate for spleen phosphodiesterase. Spleen phosphodiesterase, which readily cleaved p-nitrophenyl thymidine 5'-phosphate, hydrolyzed, at most, 14% of the 3'-dGMP present in the assay, 10% of the 5'-dGMP, and 13% of the thymidine 3',5'-di phosphate, but had no activity against the substrate of venom enzyme, p-nitrophenyl thymidine 5'-phosphate. In view of the great excess of the enzymes employed in these tests, it may be con-
adenosine deaminase; all deoxyadenosine produced was converted to deoxyinosine during digestions with this phosphodiesterase. Digests by venom and spleen phosphodiesterases were carried out as follows. The optimum time and phosphodiesterase concentrations were established by examining the products of the digestion by thin layer chromatography (30). Digestion conditions were selected which resulted in a minimum amount of residual oligonucleotide remaining at the origin. The digestion mixtures consisted of 100 μl (final volume) of 0.1 M Tris-Cl, pH 9.0, for venom phosphodiesterase, and 100 μl of 0.1 M Tris-Cl, pH 7.0, for spleen phosphodiesterase, in the presence (in both cases) of 0.5 to 1.0 μmole of oligonucleotide phosphate. The optimum amount of venom phosphodiesterase ( Worthington, Lots OAA and OJB), for maximum hydrolysis in a digestion lasting 1 to 3 hours, was 10 to 20 μg (added from a stock solution in 0.05 M Tris-Cl, pH 9.0, stored frozen). The optimum amount of spleen phosphodiesterase ( Worthington, Lots 91A and 9GA) was found to be 100 to 200 μg (added as a stock solution in 0.05 M Tris-Cl, pH 7.0, stored frozen).

RESULTS

Isolation of DNase

Preparation of Extract of Lysosomes—In the procedure described below, “tissue volume” is defined as 1 ml of solution per g of original tissue. All operations were carried out in the cold room unless otherwise stated.

Rat livers (240 g) were passed through an Oster food grinder (model 430), suspended in 3 ml of 0.25 M sucrose per g of liver, and homogenized in a loose fitting (clearance 0.3 mm) motor-driven Potter-Elvehjem homogenizer, with 3 strokes at 1,000 rpm. The L fraction was isolated according to the method of Ragab et al. (37). The homogenate was first centrifuged in the Sorval SS-3 centrifuge equipped with the GSA rotor at 2,500 rpm for 10 min (10,200 × gmin; all values given here refer to forces at the bottom of the tube). The supernatant solutions were carefully decanted and the pellets were rehomogenized in 3 tissue volumes of 0.35 M sucrose and recentrifuged. This step was repeated once more, the pellets (nuclei and cell debris) were suspended in about 3 tissue volumes of 0.25 M sucrose for assay, and the combined supernatant solutions (cytoplasmic extract) were centrifuged (GSA rotor) at 4,500 rpm for 10 min (33,000 × gmin) to remove the bulk of the mitochondria, which were then suspended in about 2 tissue volumes of 0.25 M sucrose for assay. After removal of the mitochondria, the supernatant solution was centrifuged (GSA rotor) at 8,500 rpm for 35 min (409,500 × gmin) to sediment the lysosomes. The microsomes and soluble fraction were decanted. The lysosomal pellet was gently resuspended in one-half tissue volume of 0.25 M sucrose and recentrifuged. This step was repeated once more, the pellets (nuclei and cell debris) were suspended in about 3 tissue volumes of 0.25 M sucrose for assay, and the combined supernatant solutions (cytoplasmic extract) were centrifuged (GSA rotor) at 4,500 rpm for 10 min (33,000 × gmin) to remove the bulk of the mitochondria, which were then suspended in about 2 tissue volumes of 0.25 M sucrose for assay. After removal of the mitochondria, the supernatant solution was centrifuged (GSA rotor) at 8,500 rpm for 35 min (409,500 × gmin) to sediment the lysosomes. The microsomes and soluble fraction were decanted. The lysosomal pellet was gently resuspended in one-half tissue volume of 0.25 M sucrose and centrifuged (Spinco L, No. 30 rotor) at 25,000 rpm for 8 min and 20 s (609,000 × gmin). The supernatant solution and the loose fluffy layer (microsomes) on top of the lysosomal pellet were decanted, and the pellet (lysosomes) was suspended in about one-half tissue volume of 0.01 M acetate, pH 5.5. This suspension was dialyzed overnight against 2 liters of the same buffer to rupture the particles osmotically and was centrifuged as above in the Spinco No. 30 rotor for 30 min. The lysosomal-soluble fraction was discarded. To extract the DNase from the membranous pellet, the latter was suspended in one-quarter tissue volume of 0.4 M NaCl-0.01 M acetate, pH 5.5, stirred for 60 min, and recentrifuged as before. The extract contained most of the DNase.

Gel Filtration on Sephadex G-100—This step is especially important for the removal of RNase. The lysosomal membrane

Fig. 1. Gel filtration of lysosomal extract on Sephadex G-100.

The column (26 × 2.5 cm) was equilibrated against 0.05 M acetate, pH 5.5. After the sample was applied, 0.2 M NaCl and then 0.4 M NaCl, in the same acetate buffer, were used to elute DNase. Flow rate was 10 ml per hour and fraction size was 2.2 ml. Aliquots (20 μl) of Fractions 1 to 46 were used to assay for DNase (O——O) and 10 μl for RNase (O—-O). For other experimental details, see under “Results.”

Fig. 2. Chromatography of lysosomal DNase on CM-cellulose.

The column (26 × 2.5 cm) was equilibrated against 0.2 M NaCl-0.01 M acetate, pH 5.5. Flow rate was 6 ml per hour (using a pressure head of 5 cm). Fraction size was 3.3 ml. Protein was estimated by reading absorbance at 280 nm (——). Twenty microliters of each fraction were used in the standard assay for DNase (O——O) and 10 μl for RNase (O—-O). For other experimental details, see under “Results.”

Included that the two enzyme preparations were not appreciably contaminated with nucleotidases or phosphodiesterases which might make difficult the interpretation of the results of the structural studies of the oligonucleotides. It may be noted that, in confirmation of the report of Curtis and Smellie (18), it was found that spleen phosphodiesterase was contaminated with adenosine deaminase; all deoxyadenosine produced was converted to deoxyinosine during digestions with this phosphodiesterase.

The hydrolyses of the oligonucleotide products in DNase II digests by venom and spleen phosphodiesterases were carried

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Vol. 247, No. 5

Lysoosomal DNase II

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TABLE I

Purification of DNase II from rat liver lysosomes

For details, see under "Experimental Procedure" and "Results."

| Fraction                          | Total protein | Total activity | Yield of DNase | Specific activity | Purification ratioa |
|----------------------------------|---------------|---------------|----------------|------------------|---------------------|
| Nuclei                           | 17,400        | 33,200        | 100            | 2.62             | 1                   |
| Cytoplasmic extract              | 22,000        | 80,200        | 3.64           |                  |                     |
| Mitochondria                     | 5,150         | 19,200        | 3.74           |                  |                     |
| Membranes and soluble fraction   | 12,700        | 28,100        | 2.05           |                  |                     |
| Lysosomes                        | 960           | 38,300        | 39.9           | 12               |                     |
| Extract of lysosome membranes    | 205           | 30,900        | 154            | 47               |                     |
| Concentrate applied to Sephadex  | 67.5          | 28,700        | 426            | 130              |                     |
| Pooled Fractions 72-83 of Sephadex|              |               |                |                  |                     |
| G-100 column                     | 10.2          | 18,800        | 1,840          | 500              |                     |
| Pooled Fractions 81-83 of CM-cellulose column | 0.081        | 11,500        | 167,000        | 43,300           |                     |

a The purification ratio is based on the calculated specific activity (3.28) of the total liver homogenate (nuclei plus cytoplasmic extract).

The extract was concentrated with an Amicon ultrafiltration cell (UM-10 filter, 40 p.s.i. pressure) to 9.0 ml. This solution was dialyzed against 1 liter of 0.2 M NaCl-0.01 M acetate, pH 5.5, for 2 hours and then centrifuged for 10 min at 40,000 rpm (Spinco L-2, No. 40 rotor) to remove a considerable amount of insoluble protein formed during the concentration and dialysis. The supernatant solution (8.8 ml) was passed through Sephadex G-100 equilibrated in the same buffer. Fig. 1 illustrates the separation of DNase from most of the extracted protein and shows also the fairly complete removal of RNase.

Carboxymethylcellulose Chromatography—Fractions 72 to 83 from the Sephadex G-100 column (Fig. 1) were pooled and dialyzed overnight against 3 liters of 0.05 M acetate, pH 5.5. The dialyzed solution was applied to carboxymethylcellulose in the same buffer. After the sample was added, the column was washed with 0.05 M acetate, pH 5.5, and then eluted sequentially with 0.2 M NaCl-0.05 M acetate, pH 5.5, and 0.4 M NaCl-0.05 M acetate, pH 5.5. The elution patterns of proteins and of DNase are shown in Fig. 2. The small peak of enzyme activity eluted with 0.2 M NaCl (after the main protein peak) will be discussed later. The bulk of the enzyme, eluted with 0.4 M NaCl, was routinely stored in the eluting buffer.

The purification obtained during each step is shown in Table I. The final yield of enzyme was about 10% of that present in whole liver; a purification of 43,000-fold was achieved.

Properties of Purified Lysosomal DNase

Influence of pH, Salt Concentration, and DNA Concentration on DNase Activity—Fig. 3 illustrates the behavior of DNase at different pH values and salt concentrations in acetate buffer and NaCl. Maximum activity was observed at about pH 4.1 in 0.05 M acetate and 0.25 M NaCl. Fig. 4A illustrates the time course of the reaction over a period of 60 min. The short lag period observed in Fig. 4A is presumably due to the fact that formation of small products is being measured and is characteristic of DNases determined in this manner. A minor divergence from linearity is also seen in the study of enzyme concentration (Fig. 4B). The effect of calf thymus DNA concentration on the course of the reaction is shown in Fig. 4C. Since 200 μg of DNA per ml yielded the maximum rate, this substrate concentration was used in all routine assays.

Tests for Contaminating Enzymes—All DNase preparations of specific activity greater than 10,000 were found to be essentially free of RNase.
free of other enzyme activities possibly involved in oligonucleotide hydrolysis. The purified preparation described in Table I was assayed for contaminating activities at a DNase concentration sufficient to produce 17 μmoles (approximately 1.7 μeoles) of soluble oligonucleotide base from DNA in the standard assay. Assays were performed for the following enzymes (the substrates used and the activities found are listed): RNase (E. coli-soluble RNA), <0.005 μmole per hour; lysosomal acid nucleotidase (deoxyguanosine 3'- and 5'-phosphates), <0.002 μmole per hour; nonspecific acid phosphatase (β-glycerophosphate), <0.001 μmole per hour; three alkaline endonucleases (calf thymus DNA), <0.005 unit per hour (defined as above). The latter activities were either absent or negligible, whether the assays were performed directly to the assay mixture or at the pH and salt concentration of the standard assay for DNase.

### Table II

| Experiment | Test substance | Concentration | Activity |
|------------|----------------|---------------|----------|
| A          | Control        |               | 100      |
|            | 5'-dGMP        | 0.1           | 100      |
|            | 3'-dGMP        | 0.1           | 94       |
|            | 3',5'-TDP      | 0.1           | 98       |
|            | TpA            | 0.1           | 106      |
|            | Poly(A)        | 0.15          | 57       |
| B          | Control        |               | 100      |
|            | MgCl₂          | 5             | 105      |
|            | CaCl₂          | 20            | 92       |
|            | Na₂SO₄         | 20            | 32       |
|            | Zn(CH₂COO)₂    | 5             | 88       |
|            | Mn(CH₂COO)₂    | 5             | 99       |
|            | EDTA           | 5             | 99       |
|            | Mercaptoethanol| 5             | 108      |
|            | Dithiothreitol | 1             | 114      |
|            | N-Ethylethmaleimide | 1      | 101      |
|            | p-Hydroxymereuribenzoic acid | 1  | 97       |
|            | p-Hydroxymereurphenyl sulfonic acid | 1  | 73       |
|            | Iodoacetic acid| 1             | 52       |
|            | Iodoacetamide  | 1             | 5         |

### Table III

| Substrate                                                                 | Activity |
|--------------------------------------------------------------------------|----------|
| Native calf thymus DNA                                                  | 100      |
| Denatured calf thymus DNA                                               | 33       |
| Micrococcus luteus DNA                                                  | 71       |
| Poly[d(A-T).d(A-T)]                                                     | 143      |

**Effects of Cations, Nucleotides, and other Reagents**—The nucleotides tested had little effect on DNase activity (Table II). As observed with other acid DNases, sulfate is an inhibitor of the liver enzyme. Comparison with Fig. 3 makes it evident that the inhibition by Na₂SO₄ is mainly due to the sulfate ions, not the sodium ions. Of the metals tested, zinc had the most marked inhibitory effect. Neither EDTA nor the thiol compounds affected enzyme activity, and iodoacetic acid was the most potent inhibitor, as is the case also for the spleen acid DNase (4).

**Substrate Specificity**—DNase was active against native and denatured calf thymus DNA, *M. luteus* DNA, and poly[d(A-T).d(A-T)] (Table III). The denatured thymus DNA was found to retain one-third of its hyperchromicity after heat denaturation; its hydrolysis (about one-third of native DNA) may therefore reflect the activity of the enzyme on the residual double helical portions.

**Stability of DNase**—The purer the enzyme, the more stable it is towards storage. One preparation of specific activity about 160,000 retained one-third of its activity after 1 year's storage in the cold room (about 8° in the final eluting buffer (0.4 m NaCl-0.05 m acetate, pH 5.5), whereas a preparation with a specific activity of 79,000 retained one-third of its activity after only 24 months storage under these conditions. Less pure preparations, from preliminary purification steps, retain most of their activity for only several days.

The addition of 10 μg of bovine serum albumin per ml of assay volume is required to stabilize the purified enzyme during incubation at 37°; otherwise 50 to 75% of the activity is lost over the assay period of 30 min. Prior incubation of the enzyme with the above concentration of bovine serum albumin in the absence of substrate, followed by the standard assay for activity, did not disclose an activation effect by the bovine serum albumin.

**Analytical Disc Gel Electrophoresis**—A sample (10 μg) of the purified enzyme (specific activity 140,000) was electrophoresed as described under “Methods.” On staining for protein with Coomassie blue, a single band appeared with about one-third the mobility of the methylene blue tracking dye and about one-half the mobility of the two closely adjacent major bands of the ovalbumin used as staining standard in control gels. Since only the single band was detected by this procedure, the DNase preparation appeared to be reasonably pure, a result that was likely from the extensive purification achieved (Table I). It was ob-
viously desirable to determine whether an enzymatic test for DNase would give a band coincident with the protein band. When DNA-containing gel columns were used for the electrophoresis (see under “Experimental Procedure”), the column from the origin to the point at which the DNase protein band had been detected was found to give a negative response to the methyl green stain for DNA, a result suggesting that the DNA through which the DNase had passed had been cleaved into non-staining products. Control columns without added enzyme were stained throughout their length.

The stage in the procedure at which the degradation of the DNA in the gel occurred is not known. Traces of the DNase may have absorbed to the DNA, subsequent hydrolysis occurring during incubation of the gel. This explanation is in accord with the suggestion of Boyd and Mitchell (27), who also note that this event occurs with acid, but not alkaline, DNases. However, it is also conceivable that some hydrolysis occurred during the electrophoresis, since, although the liver DNase has no detectable activity at pH 8.5, a very large amount of DNase was used in this experiment.

**Molecular Weight**—The molecular weight of rat liver DNase II was estimated by Sephadex G-100 gel filtration (25) with the use of sperm whale myoglobin, ovalbumin, *E. coli* phosphomonoesterase, and *E. coli* β-galactosidase as molecular weight marker proteins. A value of 36,000 to 38,000 was obtained. A similar determination was made with Woodrington log spleen DNase, for which a similar value of 42,000 to 45,000 was found, not greatly different from 38,000 reported by Bernardi et al. (38) using a sedimentation technique.

**Investigation of Second, Minor Form of Acid DNase and Absence of Acid DNase in Nuclei**—The minor fraction of DNase which was eluted from CM-cellulose with 0.2 M NaCl-0.05 M acetate, pH 5.5 (see Fig. 2), was found to be eluted by the same salt concentration upon rechromatography. It comprises only 1 to 2% of the activity of the main DNase component eluted with 0.4 M NaCl-0.05 M acetate. It is most active at the pH and salt concentration optima of the major DNase. It has not been further characterized.

A report has appeared (39) citing cytochemical evidence suggesting the presence of an acid DNase in rat liver nuclei. Accordingly, nuclei were prepared according to Chauveau et al. (40) and were assayed for acid DNase and for two other lysosomal enzymes, aryl sulfatase, and β-glucuronidase. The following amounts of these enzymes were found, expressed as percentage of the activity found in the total liver homogenate: aryl sulfatase, 3.6%; β-glucuronidase, 3.9%; acid DNase, 2.1%. Also, the DNase extractable from nuclei by NaCl-acetate (as used for lysosomes) is eluted from CM-cellulose like the lysosomal enzymes, with 0.4 M NaCl-0.05 M acetate, pH 5.5. This experiment therefore suggests that rat liver nuclei do not contain an acid DNase active in our standard assay, other than amounts attributable to contamination by lysosomes or lysosomal enzymes.

**Nature of Products Formed**—It is obviously of interest to determine the mode of action of the lysosomal DNase on its substrates, for example, the points of cleavage of the DNA substrate in regard to the phosphodiester bond and the possible preference for certain bases. Only a brief account of this work will be presented because the matter is still under investigation.

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![Fig. 5. Fractionation of products produced from thymus DNA by the action of liver acid DNase. Calf thymus DNA (200 mg) was digested with 2000 units of DNase in 1 liter of 0.25 M NaCl-0.05 M acetate, pH 4.14. 50% bovine serum albumin at 37°C for 2 hours. The DNA was completely converted into acid-soluble products, and an increase in absorbance at 260 nm of 37% was observed. The digest, diluted 4 fold with H2O, was applied at 8° to a DEAE cellulose column (1.6 X 13 cm) equilibrated in 0.05 M acetate, pH 5.5. After the sample had been applied, smaller oligonucleotides were eluted with 0.25 M NaCl-0.05 M acetate, pH 5.5. This fraction, comprising about 15% of the material applied, was then diluted as above and applied to DEAE-cellulose (7 mm X 46 cm, equilibrated as above) and eluted with a linear gradient of 100 ml of 7 M urea-0.1 M acetate, pH 5.5, and 100 ml of 7 M urea-0.1 M acetate, pH 5.5, 0.2 M NaCl, at a rate of 10 to 12 ml per hour with a pressure head of 50 cm. Fractions of 1 ml were collected and the absorbance at 260 nm was read either without dilution (——) or after diluting the fraction with 9 volumes of water (—). The elution profile is shown in the figure. The peak fractions were pooled as shown (I to VI), applied to Sephadex G-10 (2.5 X 96 cm) in 0.01 M acetate, pH 4.5, and eluted in 2-ml fractions at a rate of 6 to 8 ml per hour. The desalted oligonucleotides were then lyophilized and taken up in small volumes for phosphate determinations and phoshoesterase studies.](http://www.jbc.org/)
Although there have been many reports of the purification of acid DNases from various tissues, the modest degree of purification usually reported, the presence of enzymatic contaminants, and the lack of adequate characterization all make it difficult to generalize regarding their comparative properties, mode of action, or composition. Among the tissues used to prepare partially purified DNase II have been mouse liver (3428-fold enrichment) (5), Holac cels (900 fold purification) (11), bovine spleen (1250-fold) (42), and sheep spleen (4360-fold) (43). Only the hog spleen enzyme has been stated to be homogeneous (4); it is not possible for us to calculate the fold purification of this preparation above the tissue level.

The procedure described herein for isolating the rat liver enzyme gives a preparation 43,000-fold enriched over the liver homogenate. As pointed out by Egor and Iodes (43), it is difficult to compare specific activities of different preparations of DNase reported in the literature, since assays vary so widely. However, it may be estimated that the preparation reported here may be 5 to 10 times as active as the best hog spleen preparation of Bernardi et al. (44). It is free of all lysosomal enzymes for which tests were performed, and it is devoid of detectable phosphodiesterase activity, reported by some investigators to be an intrinsic property of the spleen enzyme (14, 45).

Our procedure appears to be the first described which uses a lysosomal fraction as starting material for the extensive purification of DNase II. It is of considerable interest that essentially all the activity was present in the pellet obtained from centrifugation of osmotically disrupted lysosomes. This property should not be taken as strong evidence that the enzyme is localized in the lysosomal membrane, since some lysosomal enzymes, particularly N-acetylglycosaminidas (46), adsorb readily to the membranes.

The experiments on the distribution of DNase II in rat liver and on its purification indicated strongly that essentially all of the activity resides in lysosomes. A small amount of another form of the enzyme was detected by chromatography (Fig. 2). This second form is eluted by the same salt concentration upon rechromatography. Two acid DNases were detectable chromatographically in hog liver (7). Bernardi et al. (44) have suggested that the two forms isolated from hog spleen may be attributed to the acid treatment employed during the preparation, which perhaps caused deamination of glutamine or asparagine. It should be emphasized that the present study did not include the acid treatment usually employed by others in the purification of acid DNase.

The properties of the purified lysosomal enzyme resemble those found for other DNase II preparations. Thus, sulfate is an inhibitor (14, 47), as is iodoacetic acid (4), the pH optimum is dependent upon salt concentration (47, 48), and the molecular weight is in the range reported for the spleen enzyme (38).

The terms “DNase II” and “acid DNase” are generally used interchangeably, although, as pointed out by Bernardi (4), the nomenclature for this class of enzymes is quite unsatisfactory. Specifically, he states that the division of DNases into classes I and II does not strictly correspond to their ability to produce 5'-phosphorylated products and 3'-phosphorylated products, “even if it is frequently assumed to be so.” Most commonly, the designation “DNase II” is given to a DNase with an acid pH optimum, with a preference for native DNA over denatured DNA, and with certain responses to ions. Enzymes of this type are generally assumed to catalyze the production of 3'-phosphate termini in cleaving internal phosphodiester groups (4), a cleavage mechanism first reported by Koerner and Sinheimer (34). It is of interest that Bernardi and Bernardi (49),
employing very highly purified hog spleen DNase II, appear to have confirmed this evidence by demonstrating that their enzyme cleaves p-nitrophenyl thymidine 3'-phosphate but not the 5'-derivative. However, there are conflicting reports as to whether DNase intrinsically possesses such phosphodiesterase activity. Indeed, the situation may depend on the source of the enzyme. Swenson and Hodes (42) were able to remove from bovine spleen DNase the activity towards bis(p-nitrophenyl) phosphate and towards p-nitrophenyl thymidine 3’- and 5’-phosphates, and Stor (50) presented evidence that neither hog nor sheep spleen acid DNase possesses nonspecific phosphodiesterase activity. Sicard et al. (45), on the other hand, report that their homogeneous, electrophoretically pure preparation of hog spleenacid DNase does indeed possess nonspecific phosphodiesterase activity, and that the method used by Swenson and Hodes (42) to remove such activity from the enzyme from beef spleen and by Stor and Hodes (43) from sheep spleen is ineffective with the DNase obtained from hog spleen.

In the present work we have utilized the three simple phosphodiesterase substrates mentioned above and, to the extent permitted by the information available, the assay methods published in previous papers on this subject. Our results on the very highly purified rat liver enzyme are in accord with those reports which indicate that some acid DNases are devoid of phosphodiesterase activity. These findings raise questions regarding the purity of other acid DNase preparations, and perhaps about experiments on their base preferences in the DNA substrates.

A few comments are appropriate on the attempted characterization of the products of the action of the liver DNase on thymus DNA, although the problem must be studied further. To our knowledge, no similar attempt to characterize liver DNase has been published. A few studies of other acid DNases have been recorded, leading to the conclusion that the DNA products terminate in 3'-phosphate. In order to relate our own findings meaningfully to those of other workers, we should like to have employed identical phosphodiesterase digestion conditions and methods for the separation and identification of products. Unfortunately for the resolution of this point, it was not possible to be certain that we were duplicating earlier procedures. The work of Koerner and Sinsheimer (34) on calf spleen DNase is perhaps the most convincing, but details of chromatographic procedures were not given. Georgatos (31), studying mouse mammary tumors, briefly mentions that snake venom phosphodiesterase produced some nucleoside diphosphates from oligonucleotide fractions, but since the amounts were small due to the presumed contamination of the phosphodiesterase by 5’-nucleotidase, the fractions were dephosphorylated before the nature of the terminal bases was determined. In the reports of Rosenbluth and Sung (48) and of Ip and Sung (52) on the acid DNases of brain and hepatosomes, respectively, nucleoside diphosphate yields are given, but experimental details of the phosphodiesterase treatments are omitted. Finally, Laskowski (53) has only recently modified the description of the mode of action of snake venom phosphodiesterase and emphasized important methodological problems.

The size of the DNA used as substrate in our study and the absence of contamination of the DNA, of the DNase, or of the phosphodiesterases by known phosphatases suggest that the failure to obtain evidence for terminal phosphate groups on oligonucleotide products may be due to (a) the presence of oligonucleotide phosphatase activity or (b) some unexpected rearrangement involving the terminal phosphate. The possible possibility brings to mind the discovery of Becker and Hurwitz (54) of a phosphatase in E. coli able to cleave the 5'-phosphate group of polynucleotides, but not of 5' mononucleotides. The second may be relevant to the detection of the unknown product detected in thin layer chromatographic fractionation of the venom phosphodiesterase digest.

Finally, it should be mentioned that there is a considerable body of evidence suggesting that acid DNase may play a role in the replication of DNA (for review, see Reference 55). Slor and Lev (55) have very recently reported that calf thymus nuclei contain acid DNase. Our negative results with rat liver nuclei may reflect a not unexpected difference from thymus nuclei, although the presence of inhibitory material in the liver nuclei cannot be ruled out with certainty. However, it should be pointed out that no evidence for the presence of an inhibitor was observed in the examination of rat liver nuclei for acid DNase (see under "Results"), and appreciable amounts of inhibitory material appeared to be absent from the cytoplasmic extract as well (Table I). For additional information regarding the possible involvement of DNase in cell division, further work along this line is obviously required.

The method described herein for the extensive purification of rat liver DNase will hopefully soon permit its chemical characterization so that it may be related structurally to other lysozyme enzymes currently under study. Availability of the enzyme may also be of value in investigations of the fate of viruses and exogenous nucleic acids in cells.

Acknowledgment—We are grateful to Peter J. Oates for his participation in the early stages of this work and for useful conversations during the course of the investigation.

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*J. Biol. Chem.* 1972, 247:1424-1432.

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