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Purification and Characterization of a Major Endonuclease from Rat Liver Nuclei†

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ABSTRACT: A major endonuclease has been purified approximately 800-fold from rat liver nuclei using poly(A) as substrate. The enzyme had a molecular weight of about 50,000, and active fractions were obtained which contained no nucleic acid. Enzymatic activity was optimal between pH 6 and 7 and was totally dependent on the presence of a divalent cation. The reaction was inhibited by high ionic strength, polydextran sulfate, heparin, and sodium pyrophosphate. The purified enzyme readily hydrolyzed poly(A), poly(U), poly(C), and denatured DNA, whereas poly(G) was not degraded, and transfer RNA, ribosomal RNA, and native DNA were hydrolyzed only at relatively slow rates. These data suggest that the enzyme may be specific for single-stranded polynucleotides. The purified enzyme was essentially devoid of exonuclease activity, and the products of exhaustive endonuclease digestion of poly(A) were small oligonucleotides terminated with a 5′-phosphoryl group. Evidence was obtained that this endonuclease is localized in the nucleoplasm. Possible functions for this activity are discussed.

It is now well established that the different polyribonucleotides of animal cells are synthesized in the nucleus as larger precursor molecules which are subsequently cleaved to generate the RNA molecules of the cytoplasm and fragments which are degraded in the nucleus (Weinberg, 1973; Darnell et al., 1973). However, very little is presently known about the nucleases which may be involved in the processing of these precursor molecules or in the breakdown of the discarded segments. Several reports have appeared previously describing the existence of nuclear endonucleases. Heppel described an endonuclease from pig liver nuclei which to date has been used mainly for the preparation of oligoribonucleotides (Heppel, 1966; Razzell, 1967). During purification of a nuclear exoribonuclease, Sporn also demonstrated the existence of a nuclear endonuclease which was not characterized further (Lazarus and Sporn, 1967; Sporn et al., 1969). More recently, nucleolar endonucleases from HeLa cells and L cells have been identified which cleave the precursor ribosomal RNA associated with preribosomal particles to the size of mature ribosomal RNA (Kwan et al., 1972; Mirault and Scherrer, 1972; Winicov and Perry, 1974). However, these nucleolar endonucleases from Novikoff hepatoma cells, HeLa cells, L cells, and rat liver show less specificity with naked 45S preribosomal RNA as substrate (Boctor et al., 1974; Kwan et al., 1972; Prestakyo et al., 1972; Winicov and Perry, 1974). The cleavage of heterogeneous nuclear RNA by an endonuclease present in 30S ribonucleoprotein particles of rat liver nuclei has also been reported (Niessing and Sekeris, 1970). Another endonuclease which may be specific for double-stranded regions of RNA has been identified in HeLa cell nuclei (Birge and Schlessinger, 1974). In order to gain further insight into the processing of RNA and the functions of nuclear nucleases, we have purified and characterized what appears to be a major endonuclease of rat liver nuclei.

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Experimental Procedure

Materials

Biochemicals. AG1-X2 anion exchange resin (200–400 mesh, chloride form) was purchased from Bio-Rad Laboratories. Bovine serum albumin (A grade), calf thymus DNA, and dithiothreitol were products of Calbiochem. Ovalbumin was obtained from Mann Research Laboratories. Poly(A) (potassium salt), poly(C) (potassium salt), poly(G) (sodium salt), and poly(U) (ammonium salt) were from Miles Laboratories, Inc. Aquasol from New England Nuclear was used as the scintillation fluid. Sodium dextran sulfate 500 and Sephadex G-100 were purchased from Pharmacia Fine Chemicals, Inc. Diethylaminoethylcellulose (Whatman microgranular DE 52, pre-swollen) and cellulose phosphate (Whatman P 11, powder) were obtained from Reeve Angel. Ammonium sulfate (special enzyme grade), [^{14}C]poly(A) (potassium salt), [^{3}H]poly(A) (potassium salt), and sucrose (RNase free) were from Schwarz/Mann. Heparin (sodium salt, 170 USP units per mg) was a product from Sigma Chemical Co. *Escherichia coli* alkaline phosphatase was obtained from Worthington Biochemical Corp.

Preparation of Substrates. Poly(dA) and [^{3}H]poly(dA) were prepared by the method of Riley et al. (1966). Rat liver rRNA was prepared by the procedure of Moldave (Moldave, 1967; Moldave and Skogerson, 1967). DNA was denatured by heating at 100° for 10 min and immediately cooling in ice.

Methods

Determination of Protein and Nucleic Acid Content. Protein concentration was determined by the method of Lowry et al. (1951) and by absorbance at 220 and 210 nm (Webster, 1970). Nucleic acid content was determined by the ratio of absorbance at 280 nm to that at 260 nm (Layne, 1957).

Light and Electron Microscopy. Unfixed nuclear pellets were stained with Toluidine Blue for light microscopy. For electron microscopy the pellets were fixed in 4% glutaraldehyde–0.1 *M* cacodylate buffer (pH 7.3) for 4 hr at 4°, followed by post-fixation in 2% osmium tetroxide–0.1 *M* cacodylate buffer (pH 7.3) for 2 hr at 4°. The nuclear pellets were then dehydrated, embedded, sectioned, and stained as described by Goldblatt et al. (1969). Thin sections were examined in a Philips EM 300 electron microscope at 60 kV.

Spectrophotometric Assay of Endonuclease Activity. Endonuclease activity was assayed by a modification of the procedure of Lazarus and Sporn (1967). The standard reaction mixture contained in 350 μl: 25 m*M* potassium phosphate (pH 7.0 at 37°); 2 m*M* $MgCl_2$; 0.2 m*M* dithiothreitol; 100 μg/ml of bovine serum albumin; and 1 m*M* poly(A) expressed as nucleotide. The molar extinction coefficient (supplied by the manufacturer) used for determining the concentration of poly(A) was $\epsilon_{260} = 9.8 \times 10^3$. The reaction was initiated by the addition of enzyme and terminated after 15-min incubation at 37° with 0.7 ml of 0.6 *N* perchloric acid. After 15 min in ice, the mixture was centrifuged at 13,300*g* for 10 min. The concentration of adenylate residues released was calculated utilizing $\epsilon_{257} = 15.1 \times 10^3$ (P-L Biochemicals, Inc., 1969). One unit of endonuclease was defined as that amount of enzyme which released one micromole of adenylate residues per hour. Where necessary, corrections were made for the nonlinearity of the assay or for the presence of inhibitors (EDTA, salt) in the enzyme solutions. In the early stages of purification interfering exonuclease activity was inhibited by 3 m*M* sodium fluoride (see Results section).

Thin-Layer Chromatography of Poly(A) Hydrolyzed by Nuclear Endonuclease or Exonuclease. Poly(A) (1 m*M*) was incubated at 37° with 0.22 unit of purified endonuclease, 0.18 unit of exonuclease, or without enzyme in the standard assay mixture for 50 hr and placed in ice. Fourteen microliters of the reaction mixture was applied to a thin-layer chromatography sheet (cellulose with fluorescent indicator) and developed in Eastman thin-layer sandwich chambers with 1 *M* ammonium acetate–95% ethanol (4:1, v/v) (Thach and Doty, 1965). Poly(A) and AMP were also applied to the sheet as markers.

Results

Purification of Nuclear Endonuclease. (1) ISOLATION OF NUCLEI. The entire procedure was carried out at 4°. Nuclei were prepared from the livers (about 660 g) of 150 fasted CF-N female rats (115–145 g) by the aqueous procedure of Chauveau et al. (1956) as modified by Busch and Smetana (1970). Animals were decapitated, the livers rapidly excised, rinsed in 0.25 *M* sucrose, and homogenized in 5 vol of 2.4 *M* sucrose–3 m*M* calcium acetate by three up-and-down strokes with a Teflon glass homogenizer (0.006–0.009 in. pestle clearance) at roughly 1350 rpm. After filtering through two layers of cheesecloth, the combined homogenate of 25 livers was brought up to volume with 2.4 *M* sucrose–3 m*M* calcium acetate (12 ml/g of wet liver), stirred, and centrifuged at 37,000*g* for 70 min. The supernatants were discarded, the tubes wiped, and the nuclear pellets rinsed with 0.88 *M* sucrose–1 m*M* calcium acetate. The nuclear pellets were then resuspended in the same medium (1 ml/g of wet liver) by gentle homogenization. The nuclear suspensions were stored at −73°. The combined nuclear preparations (695 ml) were thawed and centrifuged at 2300*g* for 10 min and the nuclear pellets saved for extraction. The nuclei isolated by this procedure were essentially free of cytoplasmic contamination as shown by both light and electron microscopy.

(2) pH 6.2 EXTRACTION. Steps 2 through 5 are a modification of the procedure of Lazarus and Sporn (1967). Wet nuclei, 45 g, were suspended in 708 ml of solution 1 (5 m*M* potassium phosphate (pH 6.2)–0.1 *M* NaCl–2 m*M* $MgCl_2$–1 m*M* dithiothreitol) by three up-and-down strokes first with a loosely fitting pestle in a Dounce homogenizer and then with a tightly fitting pestle. The suspension was stirred for 15 min and centrifuged at 20,100*g* for 8 min. The extraction was repeated with 354 and 177 ml of solution 1, and the supernatants were discarded. The pH 6.2 extraction served to remove a large amount of exonuclease activity. Electron microscopic examination of the pellets showed intact nuclei with aggregated chromatin.

(3) pH 8.0 EXTRACTION. The nuclear pellets were extracted further with four successive portions of solution 2 (20 m*M* Tris-Cl (pH 8.0)–80 m*M* NaCl–20 m*M* EDTA–1 m*M* dithiothreitol) of 623, 312, 156, and 156 ml, and the combined supernatants were saved. Approximately 80% of the nuclease activity extracted at pH 8.0 was due to endonuclease as determined by NaF inhibition and chromatographic analysis of the products. A small amount of endonuclease activity remained in the pellet even after four extractions. Electron microscopic inspection of the residue showed only chromatin and intact nucleoli.

(4) AMMONIUM SULFATE FRACTIONATION. Ammo-

Table I: Purification of Nuclear Endonuclease

Step	Total Act.[a] (Units)	Sp Act.[b] (units/mg)	Rel Purification (–Fold)	Yield (%)
1. Nuclei	5779	2.2	1	100
2. Combined pH 6.2 extract	Negligible			
3. Combined pH 8.0 extract	5418	6.6	3	94
4. Ammonium sulfate	2969	26	12	51
5. Combined DEAE-cellulose	1935	255	116	33
6. Combined phospho-cellulose	1118	1247	567	19
Peak tube	220	1802	819	

[a] Endonuclease activity in the first four steps was measured in the presence of 3 m*M* sodium fluoride to inhibit contaminating exonuclease. [b] Concentration of protein was determined by the Lowry method for steps 1, 3, and 4 and by absorbance at 210 nm for steps 5 and 6 as described under Methods.

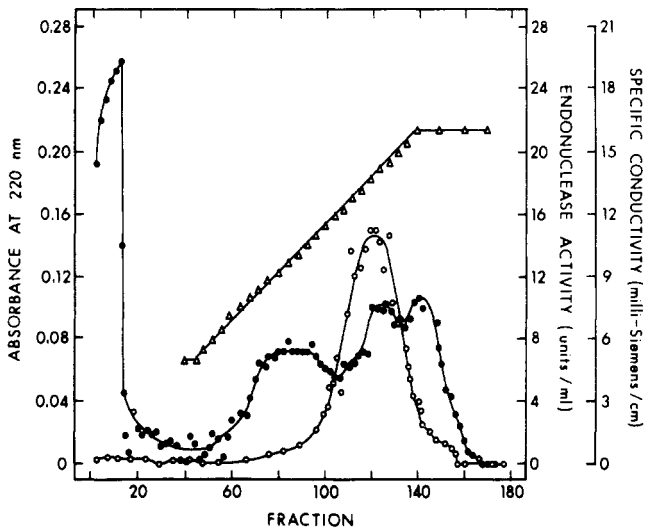

FIGURE 1: Phosphocellulose chromatography of the nuclear endonuclease. Enzyme purified through DEAE-cellulose (120 ml, 550 units) in solution 5 was added to a phosphocellulose column (0.9 × 9.1 cm) and washed with solution 6 until the A_{220} dropped below 0.02. The enzyme was eluted with a linear gradient (100 ml) from solution 6 to the same solution containing 0.2 *M* KCl. At fraction 142 the column was washed with solution 6 containing 0.2 *M* KCl. The 10-ml (1-16) and 1-ml fractions (17-177) were analyzed for absorbance at 220 nm (●), endonuclease activity (○), and specific conductivity (△).

nium sulfate (208 g) was added to 1245 ml of combined solution 2 extracts over 15 min, stirred 15 min more, and centrifuged, and the pellets were discarded. Ammonium sulfate (150 g) was added to the supernatant fraction with stirring and centrifugation as above. The ammonium sulfate pellets were extracted three times with 40 ml of solution 3 (10 m*M* Tris-Cl (pH 8.0)–0.14 *M* NaCl–0.1 m*M* dithiothreitol), stirred for 30 min, and centrifuged at 113,000*g* for 30 min. The combined supernatants, 120 ml, were dialyzed against solution 4 (10 m*M* Tris-Cl (pH 8.0)–0.1 m*M* dithiothreitol) overnight to remove the sodium chloride and were again centrifuged as above. The sodium chloride in solution 3 was necessary for the solubilization of the endonuclease

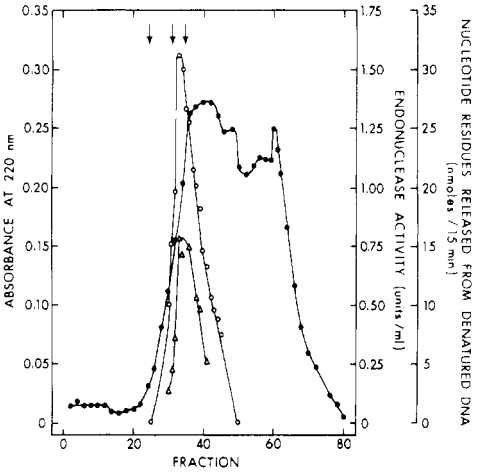

FIGURE 2: Molecular weight determination of the nuclear endonuclease. A column of Sephadex G-100 (0.9 × 27.5 cm) was equilibrated at 4° with 50 m*M* Tris-Cl (pH 8.0), 1 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.1 m*M* dithiothreitol, and 10% glycerol. Bovine serum albumin (mol wt 67,000) and ovalbumin (mol wt 45,000) were used as standards, and Blue Dextran (mol wt 2,000,000) was employed to determine the void volume as indicated by the arrows. Samples of 0.5 ml were applied and the column washed with the above buffer at 3.0 ml/hr. Fractions of 0.4 ml were collected. Endonuclease, purified through step 5, was concentrated approximately 30-fold in an Amicon Diaflo ultrafiltration apparatus using a UM-10 membrane. The concentrated enzyme was made 0.05 *M* with respect to Tris-Cl (pH 8.0) and 0.5 ml of the nuclease (10.9 units) was applied to the Sephadex G-100 column. The collected fractions were monitored for endonuclease activity (○), endonuclease hydrolysis of denatured DNA (△), and absorbance at 220 nm (●), and the molecular weight determined as described by Fischer (1969).

from the ammonium sulfate pellets, while the centrifugation removed precipitated material present after the dialysis.

(5) DEAE-CELLULOSE CHROMATOGRAPHY. The dialyzed ammonium sulfate fraction was added to a column of DEAE-cellulose (2.5 × 13.2 cm) equilibrated with solution 4, and the column was washed with the same solution until the A_{280} in the effluent was below 0.01. Endonuclease activity was eluted from the column under these conditions, whereas the exonuclease activity remained bound to the column and could be eluted only at higher salt concentrations. In addition, this procedure served to remove most of the nucleic acid from the preparation (see below).

(6) PHOSPHOCELLULOSE CHROMATOGRAPHY. The combined fractions after DEAE-cellulose chromatography were made 10 m*M* in potassium phosphate (pH 7.8) and 40 m*M* in KCl and added to a phosphocellulose column (2.5 × 3.8 cm) previously equilibrated with solution 5 (10 m*M* Tris-Cl (pH 8.0)–10 m*M* potassium phosphate (pH 7.8)–40 m*M* KCl–0.1 m*M* dithiothreitol). The column was washed with solution 6 (10 m*M* potassium phosphate (pH 7.8)–50 m*M* KCl–0.1 m*M* dithiothreitol) until the A_{220} fell below 0.03. The endonuclease was eluted with a linear gradient consisting of 150 ml of solution 6 and 150 ml of the same solution containing 1 *M* KCl. This purification procedure is summarized in Table I. In more recent experiments we have been able to achieve greater purification of the enzyme by employing a more shallow gradient for the phosphocellulose chromatography. An example of this improved procedure is shown in Figure 1 in which a specific activity of 3300 units/mg was attained in the peak tubes. Attempts to purify the endonuclease further have so far been unsuccessful, mainly due to the small amount of protein obtained and to the instability of the enzyme with further manipulation.

Table II: Requirements of the Standard Assay.[a]

Assay Conditions	Adenylate Residues Released (nmol/15 min)	Rel Release of Adenylate Residues
Complete	67.2	1.00
− Mg^{2+}	<1.0	<0.02
− Bovine serum albumin	32.1	0.48
− Dithiothreitol	60.1	0.89
− Enzyme	1.6	0.02

[a] Purified endonuclease (0.33 unit) was assayed under standard conditions except for deletion of one component of the standard assay as indicated.

Stability and Storage of the Endonuclease. The endonuclease was stable in isolated nuclei for at least 1.5 months at −73°. The purified enzyme from phosphocellulose, quickly frozen at −73°, was stable for at least 19 months. Storage of the same enzyme at −20° in 50% glycerol led to about a 90% decrease in activity after 4 months.

Determination of Nucleic Acid Content. The ratios of absorbance at 280 nm to that at 260 nm of individual fractions of endonuclease after DEAE-cellulose chromatography have been as high as 1.82 indicating the absence of any nucleotide material in active fractions. However, upon combination and concentration of fractions after DEAE-cellulose chromatography, ratios of about 1.40 were obtained. This lowered ratio was probably due to the leaching of some ultraviolet-absorbing material from the membranes used for concentrating. Nevertheless, the data obtained from fractions prior to concentrating indicate that the enzyme does not require the presence of nucleic acid for activity.

Molecular Weight of Endonuclease. The endonuclease after DEAE-cellulose chromatography had a molecular weight of approximately 50,000 as determined by gel filtration on a column of Sephadex G-100 (Figure 2).

Requirements of the Enzyme. The requirements of the purified enzyme are shown in Table II. Degradation of poly(A) was totally dependent on the presence of enzyme and a divalent cation. Bovine serum albumin stimulated the reaction about twofold presumably by stabilization of the diluted enzyme, whereas dithiothreitol was not required. The optimal concentration of Mg^{2+} was 0.5 to 2 m*M*, and higher concentrations inhibited. Mg^{2+} could be partially replaced by some other divalent cations. Thus, 2 m*M* Co^{2+} (using 25 m*M* Tris-Cl buffer (pH 7.2) and 2 m*M* Mn^{2+} were approximately 75 and 50% as effective as 2 m*M* Mg^{2+}, respectively. Other divalent cations (2 m*M* Ba^{2+}, 2 m*M* Ca^{2+}, and 2 m*M* Sr^{2+}) were ineffective. EDTA inhibited the enzyme, and this inhibition could be reversed by addition of Mg^{2+}. The optimal poly(A) concentration in the presence of 2 m*M* Mg^{2+} was 0.5 to 1 m*M*. At higher poly(A) concentrations endonuclease activity decreased, possibly due to limiting Mg^{2+} or to an increase in the average size of the product which would be expected in the presence of excess substrate. If a larger proportion of product was acid insoluble, it would appear that endonuclease activity had decreased. The pH optimum of the enzyme acting on poly(A) was between 6 and 7 (Figure 3).

Time Course of the Standard Assay. Due to the endonucleolytic nature of the enzyme's action, the early cleavage products were not acid soluble, and the release of products was not linear with time (Figure 4) or enzyme concentration. Whenever possible, this nonlinearity was minimized by adjusting the concentration of enzyme so that approximately the same amount of adenylate residues was released in each assay. In addition, a standard curve was constructed relating enzyme concentration to release of adenylate residues under the usual conditions of assay. This curve was then used to calculate the enzyme concentration in other experiments.

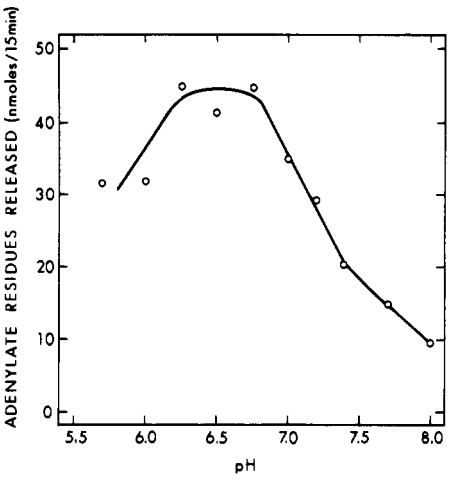

FIGURE 3: The effect of pH on the release of adenylate residues by the nuclear endonuclease. Purified enzyme (0.28 unit) was assayed under standard conditions except that the pH of the potassium phosphate buffer (37°) was varied as indicated.

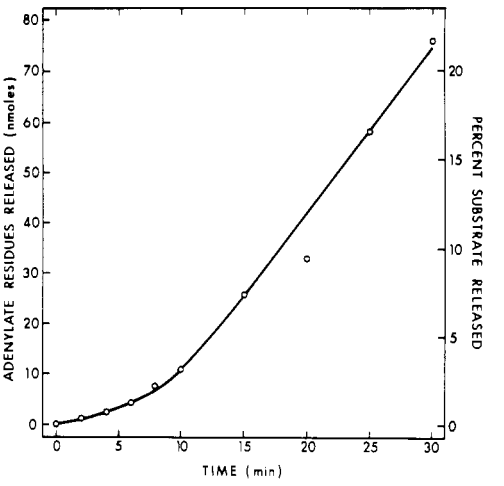

FIGURE 4: Time course of the standard assay. Purified enzyme (0.21 unit) was assayed under standard conditions except for the variation of time of incubation as indicated.

Temperature Sensitivity of the Endonuclease. The purified enzyme proved to be relatively heat labile, losing about half its activity in 5 min at 47°, and essentially all its activity in 5 min at 54° (Figure 5). However, the nuclease was stable at 37° for 5 min when compared to a control kept in ice for the same period of time. At most, 15% of the activity was lost in 30 min at 37°.

Inhibition of Endonuclease Activity by High Ionic Strength. The purified nuclear endonuclease was sensitive to increasing concentrations of various salts. Thus, 50% inhibition of endonuclease activity was obtained with 40 m*M* potassium phosphate and 55 m*M* potassium chloride. High concentrations of sodium chloride, ammonium sulfate, and Tris-Cl buffer also inhibited endonuclease activity.

Polyanion Inhibition of Endonuclease Activity. Polydextran sulfate was an extremely potent inhibitor of the purified nuclear endonuclease, inhibiting the enzyme nearly

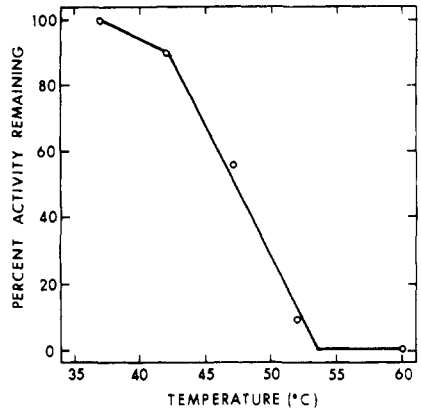

FIGURE 5: Temperature sensitivity of the nuclear endonuclease. Purified enzyme (0.26 unit) was incubated 5 min at various temperatures and cooled in ice before addition to the standard assay. These data are presented as the percentage of activity remaining compared to a control sample kept in ice for 5 min.

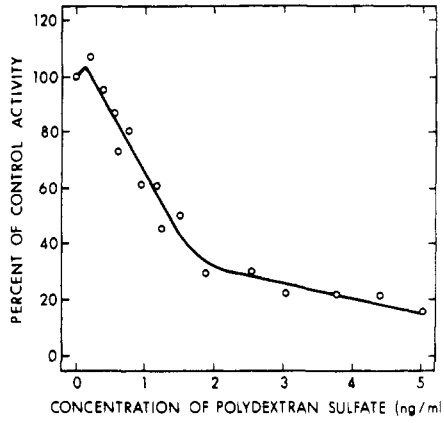

FIGURE 6: Polydextran sulfate inhibition of the nuclear endonuclease. Purified enzyme (0.23 unit) was assayed under standard conditions except for the addition of the indicated concentrations of polydextran sulfate.

50% at a concentration of 1.3 ng/ml (Figure 6). Higher concentrations of polydextran sulfate were needed to inhibit the endonuclease in less purified fractions. Thus, approximately 350 ng/ml of polydextran sulfate was needed to inhibit the endonuclease purified through DEAE-cellulose 50%. Apparently, other nuclear proteins which were removed upon further purification on phosphocellulose also bound to the polyanionic polydextran sulfate. Another polyanion, heparin, also proved to be a strong inhibitor of the purified endonuclease, inhibiting the enzyme approximately 50% at a concentration of 22 ng/ml.

Substrate Specificity of the Endonuclease. The substrate specificity of the enzyme is presented in Table III. Activities were determined with varying concentrations of each substrate, but only the activity at the optimal polymer concentration is shown and compared to that with poly(A). Poly(U) and poly(C) were degraded more rapidly than poly(A), whereas poly(G) was resistant to the enzyme. It is known that poly(U), poly(C), and poly(A) would be single stranded under these conditions (Fasman et al., 1964; Holcomb and Tinoco, 1965, Shin, 1973), whereas poly(G) would be a multi-stranded helix (Shin, 1973). The endonuclease was relatively inactive with either ribosomal RNA or transfer RNA, both of which contain an appreciable amount of double-stranded structure (Spencer, 1972). The purified nuclear endonuclease preparation also degraded

Table III: Substrate Specificity of the Endonuclease.[a]

Substrate	Optimal Concn (m*M* nucleotide)	Nucleotide Residues Released (nmol/15 min)	Rel Release of Nucleotide Residues
Poly(A)	1.0	50.0	1.00
Poly(U)	0.9	144.6	2.89
Poly(C)	0.5	86.6	1.73
Poly(G)	1.0	<1.0	<0.02
Rat liver tRNA	0.9	4.8	0.10
Rat liver rRNA	0.3	6.2	0.12
Calf thymus DNA	1.8	3.3	0.07
Calf thymus denatured DNA	0.2	24.2	0.48

[a] Purified enzyme (0.29 unit) was assayed under standard conditions except for the substitution of different substrates at the optimal concentration and the determination of acid-soluble material at the appropriate wavelength. The following molar extinction coefficients (supplied by the manufacturer) were used for the determination of the concentration of the substrate: poly(A) ($\epsilon_{260} = 9.8 \times 10^3$), poly(U) ($\epsilon_{260} = 8.8 \times 10^3$), poly(C) ($\epsilon_{260} = 5.6 \times 10^3$), and poly(G) ($\epsilon_{260} = 7.8 \times 10^3$); an $\epsilon_{260} = 8 \times 10^3$ was assumed for RNA and DNA. The following molar extinction coefficients were used for the calculation of nucleotide residues released: AMP ($\epsilon_{257} = 15.1 \times 10^3$), UMP ($\epsilon_{262} = 10.0 \times 10^3$), CMP ($\epsilon_{280} = 13.0 \times 10^3$), GMP ($\epsilon_{256} = 12.2 \times 10^3$) (P-L Biochemicals, Inc., 1969); an $\epsilon_{260} = 10 \times 10^3$ was assumed for the nucleotide residues released from RNA and DNA. Reactions with poly(U) were terminated with 0.7 ml of 50 m*M* sodium acetate (pH 5.5 at 4°)–10 m*M* magnesium acetate–0.8 m*M* lanthanum nitrate–50% ethanol, and kept at −20° for 20 min before centrifugation (Ambellan and Hollander, 1966).

denatured DNA at an appreciable rate, whereas it was relatively inactive against native DNA. The presence of a deoxyribonuclease activity in the preparation was also shown by its activity with poly(dA) (Table IV), although poly(A) was, by far, the preferred substrate. At the present time it is not known whether this deoxyribonuclease activity is due to the same protein or to a contaminant. However, the deoxyribonuclease activity copurified with the ribonuclease activity during chromatography on DEAE-cellulose, Sephadex G-100 (see Figure 2), and phosphocellulose, suggesting that only a single enzyme might be involved. The results presented here also indicate that the enzyme has a marked preference for single-stranded polynucleotides.

Effect of Other Synthetic Polynucleotides on the Hydrolysis of Poly(A). Although poly(G) was not a substrate for the nuclear endonuclease, it did inhibit the hydrolysis of [^{3}H]poly(A) suggesting that it does bind to the enzyme (Figure 7). As expected, poly(C) and poly(U) also inhibited since these polymers are alternative substrates. The poorer inhibition by poly(U) may have been due to the formation of double- and triple-stranded helices between poly(A) and poly(U) which had poor affinity for the enzyme.

Specificity of Chain Cleavage and Chain Length of Product. In order to investigate the specificity of chain cleavage and the chain length of the limit product, [^{14}C]poly(A) was digested for increasing periods of time with large amounts of enzyme, and the average size of the product was determined by measurement of nucleotide to nucleoside ratios as described in the legend to Figure 8. The progressive decrease in chain length with time indicated that the endonuclease generated oligonucleotides terminated with 3′-hydroxyl groups. The validity of this experiment was confirmed by the absence of phosphatase activity in the purified enzyme preparation. Hydrolysis of poly(A) reached a limit at a chain length of about four residues, suggesting

Table IV: Sugar Specificity of the Endonuclease.[a]

Polynucleotide	Nucleotide Residues Released (pmol/ 15 min)	Rel Release of Nucleotide Residues
[^{3}H]Poly(rA) (7500 cpm per nmol)	394	1.00
[^{3}H]Poly(dA) (2370 cpm per nmol)	33	0.08

[a] Purified enzyme (0.04 unit) was added to reaction mixtures containing in 100 μl: 25 mM potassium phosphate (pH 7.0 at 37°); 2 mM $MgCl_2$; 0.2 mM dithiothreitol; 100 μg/ml of bovine serum albumin; and 50 μM radioactive substrate, expressed as nucleotide. After 15-min incubation at 37°, the reaction was stopped by the addition of 0.3 ml of carrier poly(A) (1 mM) and 0.4 ml of 20% trichloroacetic acid. After 15 min in ice and 10 min centrifugation (13,000g), 0.4 ml of the supernatant was dissolved in 10 ml of scintillation fluid and the acid-soluble counts measured in a liquid scintillation counter.

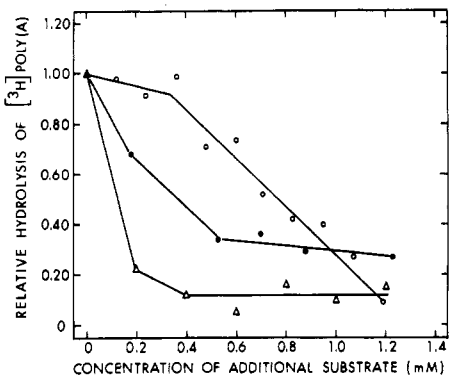

FIGURE 7: Inhibition of hydrolysis of [^{3}H]poly(A) by other synthetic polynucleotides. Purified enzyme (0.38 unit) was added to reaction mixtures containing 0.6 mM [^{3}H]poly(A) (700 cpm per nmol) as described under Table IV except for the addition of poly(U) (○), poly(C) (●), or poly(G) (△) as indicated.

that the enzyme does not hydrolyze very small oligonucleotides. This was confirmed by thin-layer chromatography which showed that the endonuclease generated small oligonucleotides and only a trace of AMP in contrast to the nuclear exonuclease which released only AMP.

We have also determined the products of the DNase activity associated with the nuclear endonuclease. Extensive hydrolysis of ^{3}H-labeled denatured DNA from *Bacillus megaterium* led to the production of oligonucleotides, but no mononucleotides as determined by thin-layer chromatography. The oligonucleotide products were completely converted to mononucleotides by the 3′ → 5′ exonuclease activity of *E. coli* DNA polymerase I indicating that they were terminated with a 3′-hydroxyl group. These data further suggest that the RNase and DNase activities are part of the same protein.

Differentiation of the Two Major Rat Liver Nuclear Nucleases. Rat liver nuclei also contain an active exonuclease which hydrolyzes poly(A) and interferes with determination of endonuclease activity in cruder fractions (Lazarus and Sporn, 1967). The two enzymes could be partially differentiated by their response to sodium fluoride or sodium pyrophosphate as shown in Table V. The sodium fluoride inhibition of the nuclear exonuclease was particularly useful for assaying the endonuclease in crude fractions and for the studies described below. The ratio of the relative hydrolysis in the presence of sodium fluoride to the relative hydrolysis

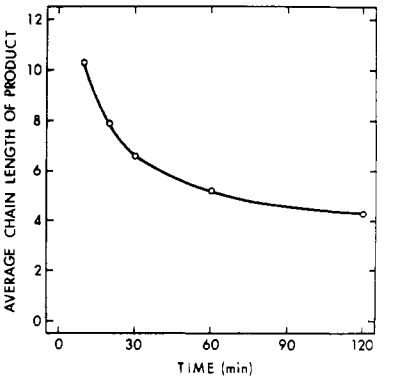

FIGURE 8: Specificity of chain cleavage and chain length of product. Purified endonuclease (0.17 unit) was incubated at 37° with 0.26 mM [^{14}C]poly(A) (560 cpm per nmol) in the standard reaction mixture (350 μl) for different periods of time. The products were made 0.1 N in sodium hydroxide, hydrolyzed at 60° for 2 hr, and put in ice. The pH was adjusted to between 5 and 6 with 1 N hydrochloric acid, and the sample was applied to an AG1-X2 column (0.6 × 1 cm). Nucleosides were eluted with water, and the nucleotides were eluted with 0.05 N HCl. The 1-ml fractions were dissolved in 10 ml of scintillation fluid and counted in a liquid scintillation counter. The ratio of the total nucleotide plus nucleoside counts to the nucleoside counts was used to calculate the average chain length of the product. No radioactivity was found in the nucleoside fraction in samples incubated without endonuclease for 0, 20, or 120 min.

Table V: Differentiation of the Two Major Rat Liver Nuclear Nucleases.[a]

Inhibitor	Adenylate Residues Released (nmol/15 min)		Rel Hydrolysis	
	Endo-nuclease	Exo-nuclease	Endo-nuclease	Exo-nuclease
	45.9	33.4	1.00	1.00
3 mM NaF	36.4	8.2	0.79	0.25
8 mM $Na_4P_2O_7$[b]	17.5	28.2	0.38	0.84

[a] Purified endonuclease (0.19 unit) or exonuclease (0.13 unit) purified through DEAE-cellulose was assayed under standard conditions except for the addition of inhibitor as indicated. [b] An equal concentration of magnesium chloride was added to eliminate inhibition due to pyrophosphate binding Mg^{2+}.

in the presence of sodium pyrophosphate was for endonuclease 2.08 and for exonuclease 0.30. The two nucleases could also be distinguished by thin-layer chromatography of the products as described above.

Nuclear Localization of the Two Major Rat Liver Nucleases. Since the two major nucleases of nuclei could be partially differentiated, it was possible to obtain some information concerning the subnuclear localization of these two enzymes. By using 3 mM sodium fluoride or 8 mM sodium pyrophosphate and a calculation factor (ratio described above) determined from the relative activities of the two enzymes with either inhibitor (see Table V), the approximate amounts of the two nucleases in nuclei, nucleoplasm, and nucleoli were determined (Table VI). There was no loss of the nuclease activities after the sonication of nuclei, which was used in the isolation of nucleoli. Endonuclease and exonuclease activities were present in nuclei in approximately equal quantities. The bulk of both these enzymes was found in the nucleoplasm, with only about 5 and 20%, respectively, present in the nucleoli. The small percentage of endonuclease activity found in nucleoli may be related to the activ-

Table VI: Nuclear Localization of the Two Major Nucleases.[a]

	Rel Nuclease Act.	Rel Hydrolysis with NaF/Rel Hydrolysis with $Na_4P_2O_7$	% Nuclease Act. Due to Endonuclease	Rel Act.	
				Endonuclease	Exonuclease
Nuclei	1.00	1.14	48	1.00	1.00
Nucleoplasm	0.89	1.32	57	0.96	0.81
Nucleoli	0.11	0.66	20	0.04	0.19

[a] The three sources of enzyme were assayed in the absence of inhibitor, with 3 m*M* sodium fluoride or 8 m*M* sodium pyrophosphate as described in Table V, and the percent of nuclease activity due to the endonuclease was determined from the ratio of the relative hydrolysis in the presence of NaF to the relative hydrolysis in the presence of sodium pyrophosphate. Nuclei were isolated as described for the purification of the endonuclease. Nucleoli and nucleoplasm were isolated by the aqueous sonication method of Busch and Smetana (1970).

ity seen by Boctor et al. (1974). At the present time we have not eliminated the possibility that the activities seen in the nucleoplasm leaked out of the nucleoli during the aqueous extraction procedure.

Discussion

The results presented here describe the properties of a major endonuclease activity of rat liver nuclei. Although most liver nucleases have not been well characterized, we have attempted to relate the enzyme reported here to other known activities. Thus, the nuclear endonuclease is similar to the mitochondrial endonuclease in that both release 5′-oligonucleotides, require a divalent cation for activity, show no absolute base or sugar specificity, and show preference for single-stranded polynucleotides (Curtis et al., 1966). Further comparisons of these two activities appear warranted.

Due to the increased interest in RNA processing a variety of nuclease activities from mammalian nuclei have been described recently. Two reports dealing with endonucleases isolated from either nucleolar ribonucleoprotein particles of Novikoff hepatoma ascites cells (Prestayko et al., 1973a,b), or from L cells (Winicov and Perry, 1974) indicate that these activities differ from the enzyme described here. These nucleolar activities release 3′-phosphoryl-terminated oligonucleotides, were inactive with poly(A), and do not require a divalent cation for activity. Other nucleolar endonucleases from HeLa cells (Kwan et al., 1972; Mirault and Scherrer, 1972) and rat liver (Boctor et al., 1974) have not been sufficiently examined for proper comparison to the enzyme described here, although their subnuclear localization suggests that they are different proteins. Likewise, the endonuclease present in 30S ribonucleoprotein particles of rat liver nuclei (Niessing and Sekeris, 1970) needs further examination.

In contrast to the activities described above, it seems likely that the endonuclease isolated from pig liver nuclei by Heppel (1966) and the endonuclease detected by Sporn (Lazarus and Sporn, 1967; Sporn et al., 1969) are related to the enzyme described here. All three endonucleases require a divalent cation for activity and release 5′-oligonucleotides using poly(A) as the substrate. Further studies are required, however, to prove that these activities are identical.

The nucleoplasmic exonuclease (Lazarus and Sporn, 1967) and the endonuclease described here appear to be the most abundant nuclease activities in nuclei and are the most likely candidates for the major salvage nucleases of heterogeneous nuclear RNA (HnRNA). The endonuclease releases products with 3′-hydroxyl end groups which the exonuclease can use as substrate (Sporn et al., 1969). HnRNA appears to be processed in the nucleoplasm with 90% of the polyribonucleotide material never reaching the cytoplasm (Weinberg, 1973). The major breakdown products of HnRNA are 5′-nucleotides (Hurlbert and Hardy, 1972; Harris et al., 1962). One possible piece of evidence supporting the involvement of these two enzymes in HnRNA breakdown is the finding that ammonium sulfate (0.4 *M*) completely inhibits the degradation of HnRNA (Hurlbert and Hardy, 1972; Steiner 1966) and also completely inhibits the breakdown of poly(A) by these two nucleases in vitro. The first specific cleavages of HnRNA processing may be by an endonuclease such as one specific for double-stranded regions, but the discarded segments are probably degraded by these two enzymes which prefer single-stranded polynucleotides (Lazarus and Sporn, 1967). Even the reasonably large intranuclear turnover of the poly(A) regions of HnRNA (LaTorre and Perry, 1973; Perry et al., 1974) may be attributable to these two nucleases. The exonuclease has already been shown to degrade the isolated poly(A) regions of HnRNA (Sheldon et al., 1972).

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Calorimetric and Potentiometric Characterization of the Ionization Behavior of Ribonuclease A and Its Complex with 3′-Cytosine Monophosphate†

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ABSTRACT: The proton association behavior of ribonuclease A and its complex with 3′-cytosine monophosphate has been thermodynamically characterized in the pH range 4–8 at 25°, μ = 0.05. Calorimetric and potentiometric titration data have been used to estimate the apparent pK values and enthalpy values for protonation of the four histidine residues of the protein, ΔH_p. In the free enzyme the pK values were deduced to be 5.0, 5.8, 6.6, and 6.7 and ΔH_p deduced to be −6.5, −6.5, −6.5, and −24 kcal/mol for residues 119, 12, 105, and 48, respectively. For the nucleotide–enzyme complex it was concluded that the apparent pK values of residues 119, 12, and 48 increased to an average value of about 7.2, the ΔH_p values remaining constant for all histidine groups except 48. It was also concluded that only the dianionic phosphate form of the nucleotide inhibitor is bound to the enzyme in this pH range. These results are consistent with a thermodynamic model for the binding reaction in which inhibitor–enzyme association is coupled to the ionization of three imidazole residues (12, 119, and 48) and the interaction between the negative phosphate moiety of the inhibitor and the positively charged residues 12 and 119 is purely electrostatic. However, the "interaction" with residue 48 probably involves a conformational rearrangement of the macromolecule.

Many investigations have been directed toward the elucidation of the mechanism of the catalytic action of ribonuclease A (for a general review see Richards and Wyckoff, 1971; Usher and Richardson, 1970; Usher et al., 1972). These several studies have established that two histidine residues, 12 and 119, located at the active site are directly involved in the catalytic reaction, one acting as a proton acceptor and the other as a proton donor. In addition, it has been postulated that the reaction proceeds via a dianionic pentacoordinated phosphate intermediate (Witzel, 1963) which, in principle, can be thermodynamically stabilized by electrostatic interaction with the positively charged surface of the enzyme. It is thus apparent that a thermodynamic description of the ionization states of the enzyme and of specific substrate–protein interactions is vital to the development of a quantitative understanding of the enzymatic reaction.

Potentiometric titration data (Tanford and Hauenstein, 1956; Bull and Breeze, 1965) have provided a phenomenological description of the ionization behavior of the free enzyme. More recently, nuclear magnetic resonance (NMR) studies (Meadows et al., 1968; Ruterjans and Witzel, 1969;

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