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Purification and Properties of a Novel Nucleolar Exoribonuclease Capable of Degrading both Single-Stranded and Double-Stranded RNA†

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Received July 25, 1984

ABSTRACT: A ribonuclease that hydrolyzes either linear duplex or single-stranded RNA in an exonucleolytic manner has been partially purified from Ehrlich ascites tumor cell nucleoli and is free from other ribonucleases. The enzyme will also degrade the RNA complement of an RNA·DNA duplex; however, no nuclease activity is observed on linear duplex or single-stranded DNA. The exonuclease acts on RNA nonprocessively from the 3′ end releasing 5′-mononucleotides. The enzyme has a broad pH optimum around pH 8.0, requires Mg^{2+} or Mn^{2+} (0.06 mM) for optimum activity, and is sensitive to ethylenediaminetetraacetic acid and *N*-ethylmaleimide inhibition. Monovalent cations including K^+, Na^+, and NH_4^+ are inhibitory. Gel filtration studies of this enzyme gave a Stokes radius of 40 Å. Sedimentation velocity measurements in glycerol gradients yield a $s_{20,w}$ of 6.0 S. From these values a native molecular weight of 100 000 was calculated. Copurification of the single- and double-stranded activities, identical reaction requirements, and identical heat-inactivation curves strongly suggest that both activities reside with the same enzyme.

The mouse ribosomal RNA system has proven extremely useful for studies of ribosomal RNA transcription and processing both in vivo and in vitro. These studies have included specific transcription of cloned mouse rRNA genes (Miller & Sollner-Webb, 1981; Grummt, 1981; Bach et al., 1981; Grummt et al., 1982; Mishima et al., 1982; Cizewski & Sollner-Webb, 1983), identification of control regions important for rRNA synthesis, (Miller & Sollner-Webb, 1981; Grummt, 1981; Bach et al., 1981; Grummt et al., 1982; Mishima et al., 1982; Cizewski & Sollner-Webb, 1983), definition of the type and order of processing events occurring during maturation of rRNA precursors (Hamada et al., 1980; Michot et al., 1982, 1983; Walker et al., 1982; Bachellerie et al., 1983; Bowman et al., 1983; Goldman et al., 1983), and elucidation of the various steps on the route to synthesis of a functional rRNA (Bowman et al., 1983; Goldman et al., 1983; Eichler & Eales, 1983; Lasater & Eichler, 1984). However, despite the extensive effort that has gone into the study of rRNA metabolism, relatively little is yet known about the enzymes actually involved in the various processing steps or in the turnover of discarded RNA sequences.

In our initial studies of ribonucleases associated with mouse nucleoli, we have used a variety of substrates in an attempt to define the multiplicity of nucleases present. Thus far we have identified four distinct nucleolar ribonucleases. Two of these enzymes were single-strand specific endonucleases which we have designated nucleolar RNase I and II, respectively (Eichler & Tatar, 1980; Eichler & Eales, 1982; Eichler & Eales, 1983). A third single-strand specific activity, nucleolar RNase III, was recently shown to be a 5′ → 3′ exoribonuclease (Lasater & Eichler, 1984). In this paper, we report on a nucleolar exoribonuclease that acts on both single-stranded and double-stranded RNA and describe its purification as well as some of its properties which differentiate and distinguish this novel ribonuclease.

† These studies were supported by National Institutes of Health Grant R01 GM 29162-03.

Materials and Methods

Chemicals. Labeled [^{3}H]CDP (16 Ci/mmol) and [^{3}H]ADP (16 Ci/mmol) were obtained from Amersham. Hydroxylapatite was purchased from Bio-Rad Laboratories, DEAE-cellulose 52 was from Whatman, and Sephacryl S-200 and DEAE Sephadex A-25 were from Pharmacia.

Enzymes. Polynucleotide phosphorylase was purchased from P-L Biochemicals. T_4 RNA ligase, T_4 terminal transferase, and bacterial alkaline phosphatase were purchased from Bethesda Research Laboratories.

RNAs. ^{32}P-Labeled f_2RNA was prepared essentially according to the procedure of Glitz (1971) for MS2 RNA. The specific radioactivity was 3×10^4 cpm/nmol. [^{3}H]Poly(C) (specific activity 2×10^3 cpm/nmol) and [^{3}H]poly(A) (specific activity 9×10^3 cpm/nmol) were synthesized with polynucleotide phosphorylase according to the procedure of Moses & Singer (1970). [^{3}H]Poly(U) (specific activity 2×10^3 cpm/nmol) was purchased from Miles Chemical Corp. [^{3}H]Poly(C)·poly(I) and [^{3}H]poly(A)·poly(U) were prepared as described by Eichler & Tater (1980). Double-labeled poly(C) was prepared as follows: [^{3}H]poly(C) (14×10^3 cpm/nmol) was treated with bacterial alkaline phosphatase to remove terminal phosphate residues and then purified by phenol extraction and concentrated by ethanol precipitation. The 3′-^{32}P labeling was accomplished following the procedure of Peattie (1979) using T_4RNA ligase and [5′-^{32}P]pCp (2.5×10^8 cpm/nmol). The double-labeled [3′-^{32}P,^{3}H]poly(C) was purified by phenol extraction, ethanol precipitation, and spun-column chromatography (Maniatis et al., 1982). [3′-

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^{32}P,^{3}H]Poly(C)·poly(I) was prepared as described above.

DNA Substrates. [^{3}H]Poly(dT) (6×10^3 cpm/nmol) was synthesized by primer extension using T_4 terminal transferase. T_7 [^{3}H]DNA was prepared according to the procedure of Richardson (1966). The specific radioactivity was 4×10^4 cpm/nmol. Heat-denatured T_7 DNA was prepared by heating T_7 [^{3}H]DNA in 0.01 M NaCl and 0.015 M sodium citrate for 5 min in a boiling water bath and quick chilling to 0 °C.

RNase Assay. The assay measures conversion of labeled RNA to acid-soluble nucleotides. The reaction mixture (0.05 mL) contained 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, 0.06 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, and 1.5 nmol of labeled RNA. After 30 min at 37 °C the mixture was chilled at 0 °C in an ice bath, and 0.125 mL of 2.5 mg/mL transfer RNA and 0.075 mL of 25% perchloric acid and 0.75% uranyl acetate were added. Acid-insoluble material was removed by centrifugation in a Beckman B microfuge for 2.5 min. The radioactivity of 0.2 mL of supernatant fluid was determined by mixing with 4 mL of Triton X-100 scintillation fluid [1 L of Triton X-100 and 2 L of 3a20 toluene scintillation fluid (Research Products International Corp.)]. One unit of enzyme is that amount which produces 1 nmol of acid-soluble nucleotide in 30 min.

Kinetic Determination for the Nucleolar Ribonuclease. The reaction mixtures (0.05 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, and [^{3}H]poly(C) with concentrations ranging from 5 to 30 μM and either [^{3}H]poly(C) only, [^{3}H]poly(C) plus 5 μM poly(C)·poly(I), or [^{3}H]poly(C) plus 10 μM poly(C)·poly(I). Reactions were started by the addition of 0.12 unit of enzyme, and samples were processed as described for a standard RNase assay.

Hydrolysis of Synthetic Homoribopolynucleotides and f_2 RNA by the Nucleolar RNase. The reaction mixture (0.2 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 0.9 unit of enzyme, and 6 nmol of either f_2[^{32}P]RNA, [^{3}H]poly(U), [^{3}H]poly(A), or [^{3}H]poly(C). Aliquots (0.02 mL) were removed at 0-, 5-, 10-, 20-, 30-, and 60-min intervals, processed, and counted as described for a standard RNase assay.

Determination of the Nature of Termini. The reaction mixture (0.05 mL) contained 50 mM Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 46 nmol [^{3}H]poly(C) (14×10^3 cpm/nmol), and 2.5 units of ribonuclease. At 0, 30, and 60 min, aliquots (0.005 mL) of the reaction mixture were spotted on a poly(ethylenimine) impregnated cellulose thin-layer plate (Merck) along with 3′- and 5′-cytidine mononucleotide UV markers. The thin-layer plate was developed with 1 M acetic acid to 4 cm and then with 0.3 M LiCl to 16 cm. Samples (1 cm^2) were cut from each lane and counted in 8 mL of toluene scintillation fluid.

Measurement of Protein. Protein was determined by the method of Schaffner & Weissman (1973).

Purification of Nucleolar Ribonuclease. All steps for this purification of the RNase were carried out at 0–4 °C unless otherwise indicated. The preparation of nucleoli from Ehrlich ascites cells was as described earlier by Lasater & Eichler (1984).

Nucleolar Extraction. Purified Ehrlich cell nucleoli (from approximately 4×10^{10} nuclei) were suspended in 100 mL of buffer A [0.2 M potassium phosphate, pH 8.2, 1 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM dithiothreitol]. The suspension was mixed gently for 1 h, and the chromatin was collected by centrifugation at 20000g_{av} for 30 min. The supernatant was saved, and the chromatin was suspended in half the previous volume (50 mL) of buffer A. After 30 min of gentle mixing, the chromatin was collected by centrifugation, and the supernatant was pooled with that obtained from the first extraction. The pooled extract was dialyzed against two changes of 1 L of buffer B (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 5 mM dithiothreitol) over a 20-h period. This material was clarified by centrifugation at 105000g for 60 min. The supernatant represents fraction I (195 mL).

Hydroxylapatite Chromatography. A hydroxylapatite column (2.5 × 30 cm) was equilibrated against 1 mM potassium phosphate, pH 6.8. Fraction I was loaded at approximately 50 mL/h. The column was washed with 300 mL of buffer B and then eluted with 0.3 M potassium phosphate, pH 6.8, 5 mM dithiothreitol, and 20% glycerol. The peak activity fractions which eluted with the high-salt step were pooled and brought to 70% saturation with a saturated ammonium sulfate solution. After 1 h, the precipitate was collected by centrifugation at 20000g for 20 min. The pellet was suspended in a minimal volume (20 mL) of 0.05 M Tris-HCl, pH 8.0, 0.1 M KCl, 5 mM dithiothreitol, 1 mM EDTA, and 20% glycerol and dialyzed overnight against 1 L of this same buffer. The dialyzed material was clarified by centrifugation for 15 min at 20000g and represents fraction II (33 mL).

DEAE-cellulose Chromatography. A DEAE-cellulose column (2.5 × 40 cm) was equilibrated against buffer C (0.05 M KCl, 0.05 M Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol, and 20% glycerol). Fraction II was diluted approximately 2-fold such that its conductivity equaled that of buffer C and was loaded. The column was washed with 400 mL of buffer C, and the activity that passed through the column was pooled and represents fraction III (100 mL).

Ammonium Sulfate Fractionation. A neutralized saturated solution of ammonium sulfate was added to fraction III with stirring, bringing the mixture to 35% saturation. After 30 min of gentle mixing the precipitate was collected by centrifugation, and the supernatant was brought to 55% saturation with a solution of saturated ammonium sulfate. Again after 30 min, the precipitate was collected by centrifugation and the supernatant discarded. The 35–55% pellet was resuspended in a minimal volume of buffer D (50 mM Tris-HCl, pH 8.0, 0.3 M KCl, 1 mM EDTA, 5 mM dithiothreitol, and 20% glycerol) and represents fraction IV (9 mL).

Sephacryl S-200 Chromatography. A Sephacryl S-200 column (2.5 × 90 cm) was equilibrated against buffer D. Fraction IV was loaded onto the sizing column, and fractions (3 mL) were collected. Active peak fractions were pooled and represent fraction V (27 mL).

Hydroxylapatite Chromatography. A hydroxylapatite column (1 × 6.4 cm) was equilibrated against buffer D. Fraction V was loaded, and 1-mL fractions were collected. The column was washed with 10 mL of buffer D and eluted with 0.3 M potassium phosphate, pH 6.8, 5 mM dithiothreitol, and 20% glycerol. The peak activity fractions which eluted with the potassium phosphate step were pooled and represent fraction VI (3.6 mL).

A summary of the purification and yield of the nucleolar RNase is presented in Table I.

RESULTS

Physical Properties. To determine the apparent native molecular weight for the partially purified nucleolar ribonuclease, the enzyme fraction was chromatographed on a Sephacryl S-200 molecular sieving column that was standardized with proteins of known molecular weight. As shown in Figure 1, both the single-stranded and double-stranded

Table I: Purification of Ehrlich Nucleolar Ribonuclease[a]

	purification step	volume (mL)	units/mL	mg/mL	units/mg	yield
I	0.2 M KPO_4, pH 8.0, extract	198		2.4		
II	hydroxylapatite chromatography	33	1430	5.8	246	100
III	DEAE-cellulose chromatography	100	487	0.7	482	79
IV	ammonium sulfate fractionation	9	2143	2.7	794	46
V	Sephacryl S-200 chromatography	27	243	0.09	2641	16
VI	hydroxylapatite chromatography	3.6	1438	0.34	4241	12

[a] Purification was carried out as described in the text, starting with nucleoli derived from 4×10^{10} nuclei. One unit of enzyme is equivalent to 1 nmol of nucleotide solubilized in acid in 30 min at 37 °C in standard assay conditions with [^{3}H]poly(C)·poly(I) as the substrate.

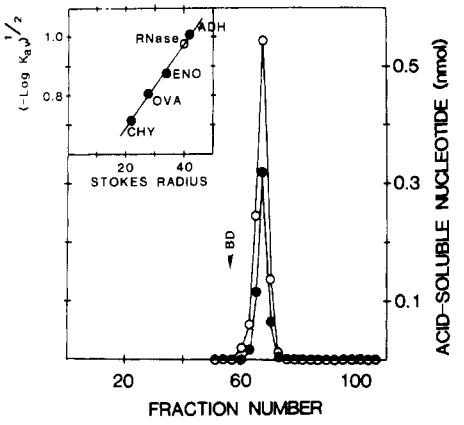

FIGURE 1: Gel filtration of the nucleolar ribonuclease. A Sephacryl S-200 column (1.5 × 95 cm) was equilibrated against 0.05 M Tris-HCl, pH 8.0, 0.3 M KCl, 10% glycerol, 1 mM EDTA, and 5 mM dithiothreitol. Fractions (1.2 mL) were collected and 5-μL aliquots were assayed with [^{3}H]poly(C)·poly(I) (●), and 2-μL aliquots were assayed with [^{3}H]poly(C) (○). BD, blue dextran. Inset: The fractional elution volumes (K_{av}) of various standard proteins used to calibrate the column were determined by the Bio-Rad dye-binding protein assay of each fraction. ADH, yeast alcohol dehydrogenase (41.7 Å); OVA, ovalbumin (27.6 Å); CHY, chymotrypsinogen (22 Å) (○) The fractional elution position of the nucleolar ribonuclease.

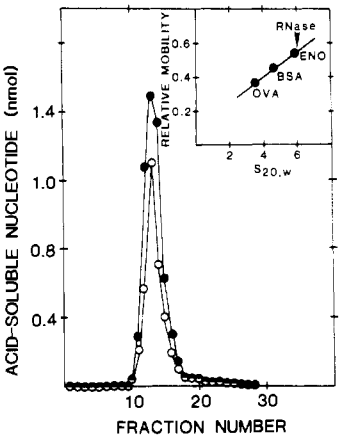

FIGURE 2: Glycerol gradient centrifugation of nucleolar ribonuclease. An aliquot (0.07 mL) of fraction VI was brought to 0.12 mL with 0.05 M Tris-HCl, pH 8.0, 0.05 M KCl, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, and 0.1 mM EDTA. This sample was layered on a 3.8-mL 10–30% (v/v) glycerol gradient containing 0.05 M Tris-HCl, pH 8.0, 0.05 M KCl, 1 mM dithiothreitol, and 0.1 mM EDTA. The gradients were centrifuged 18 h at 50 000 rpm at 4 °C in a SW60 rotor. After centrifugation, fractions (0.15 mL) were collected from the bottom of the tube. An aliquot (4 μL) of each fraction was assayed for double-stranded RNase activity (●) with [^{3}H]poly(C)·poly(I) as the substrate, and 2-μl aliquots of each fraction were assayed for single-stranded RNase activity (○) with [^{3}H]poly(C) as the substrate. Inset: Protein standard peaks were determined by the Bio-Rad dye-binding protein assay of each fraction with yeast enolase, 5.9S (ENO), bovine serum albumin, 4.3S (BSA), and ovalbumin, 3.6S (OVA), as markers in parallel gradients.

ribonuclease activities eluted as a single peak, and from the elution position a Stokes radius of 40 Å (see Figure 1, insert) was determined. A sedimentation coefficient of 6 S for the nucleolar ribonuclease was estimated according to the method of Martin & Ames (1961) using enolase, bovine serum albumin, and ovalbumin ($s_{20,w}$ = 5.9, 4.3, and 3.6, respectively) as markers on a glycerol gradient. Again, both the single-stranded and double-stranded ribonuclease activities of this enzyme fraction cosedimented on the glycerol gradient (Figure 2). By substitution of the value for the Stokes radius (40 Å) and the sedimentation coefficient (6 S) into the Svedberg equation (Siegal & Monty, 1966), an apparent native molecular weight of 100 000 was calculated by assuming a partial specific volume of 0.73.

Temperature Inactivation. In order to support the proposal that both the single-stranded and double-stranded ribonuclease activities of this fraction reside on the same protein, the enzyme was incubated at 48 or 52 °C for 2.5–25 minutes and then assayed for ribonuclease activity remaining. As shown in Figure 3, the rates of heat inactivation were identical with either a single-stranded or double-stranded RNA substrate at the two different temperatures.

Requirements of the Ribonuclease Activities. Both activities of this nucleolar ribonuclease fraction were found to be optimally active in Tris buffer at pH 7.5–8.5 and required $MgCl_2$ or $MnCl_2$ for optimal activity. The maximum stimulation for either the single-stranded or double-stranded RNase activity occurs at concentrations of 50–60 μM for Mg^{2+} (Figure 4). $CaCl_2$ could not serve to fulfill the divalent cation requirement. Both activities were completely inhibited by 5 mM EDTA or

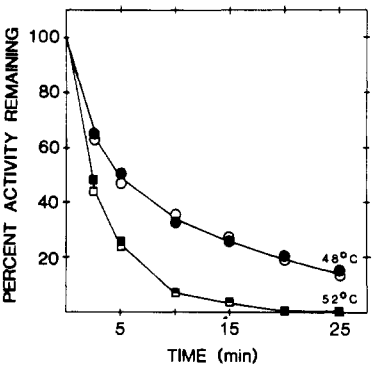

FIGURE 3: Heat inactivation of the nucleolar ribonuclease. An aliquot (0.01 mL) of fraction VI was brought to 0.2 mL with 0.05 M Tris-HCl, pH 8.0, 0.05 M KCl, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, and 10% glycerol. This mixture was incubated at 48 °C (circles) or at 52 °C (squares). At the times indicated, aliquots (0.01 mL) were withdrawn and immediately added to a reaction mixture (0.05 mL) containing 0.05 M Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, and 1.5 nmol of either [^{3}H]poly(A) (open symbols) or [^{3}H]poly(A)·poly(U) (closed symbols). The reactions were processed and counted as described for a standard RNase assay under Materials and Methods.

1 mM *N*-ethylmaleimide. Although a sulfhydryl reagent such as dithiothreitol had little effect on enzyme activity in assay, the mercaptan was routinely maintained during purification

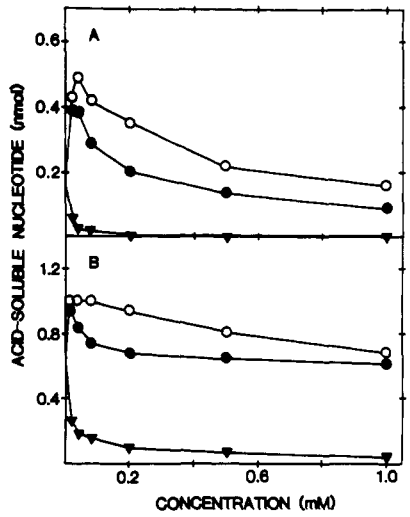

FIGURE 4: Effect of divalent cation on the hydrolysis of [^{3}H]poly(C)·poly(I) and [^{3}H]poly(C) by the nucleolar ribonuclease. The reaction mixtures (0.05 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, divalent cation as indicated, and 1.5 nmol of either (A) [^{3}H]poly(C)·poly(I) or (B) [^{3}H]poly(C). The reactions were started by the addition of 1 unit of enzyme, and the samples were processed and counted as described for a standard RNase assay under Materials and Methods. (○) $MgCl_2$; (●) $MnCl_2$; (▼) $CaCl_2$.

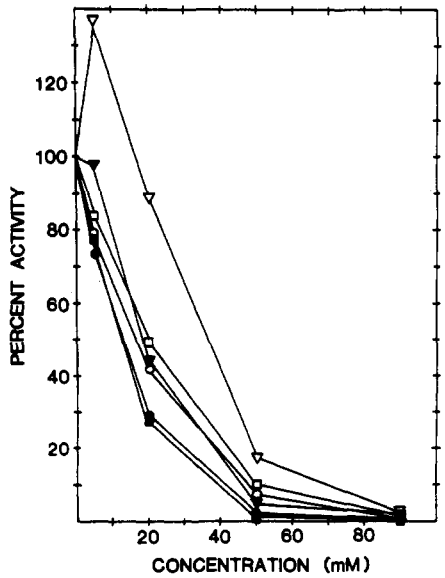

FIGURE 5: Effect of monovalent cations on the hydrolysis of single- and double-stranded RNA by the nucleolar RNase. The reaction mixtures (0.05 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 1.5 nmol of either [^{3}H]poly(C) (open figures) or [^{3}H]poly(C)·poly(I) (closed figures), and monovalent cation as indicated. Reactions were started by the addition of 0.4 unit of enzyme, and samples were processed as described for a standard RNase assay. (circles) NaCl; (squares) KCl; (triangles) NH_4Cl.

and storage to improve enzyme stability.

Monovalent cations such as Na^+, K^+, and NH_4^+ were generally inhibitory (Figure 5); although at concentrations of 10 mM or less, NH_4Cl differentially stimulated the single-stranded RNase activity. The rate of hydrolysis declines rapidly for any of the monovalent cations at concentrations greater than 10 mM and falls to less than 5% of the control values at 90 mM.

Kinetics of Ribonuclease Activity. The effect of enzyme and substrate concentrations on the degradation of single- or double-stranded RNA was examined. The reaction was linear

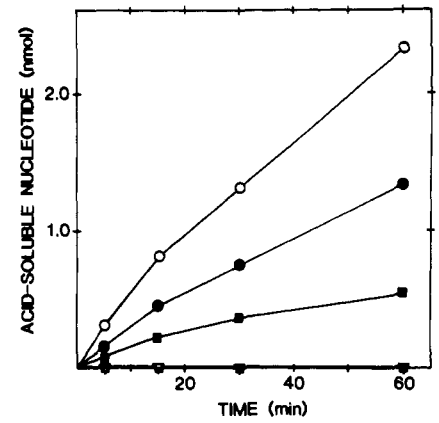

FIGURE 6: Comparative degradation of various substrates by the nucleolar ribonuclease. Reaction mixtures (0.2 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 1.3 units of enzyme, and 6 nmol of either (○) [^{3}H]poly(A), (●) [^{3}H]poly(A)·poly(U), (■) [^{3}H]poly(A)·poly(dT), (□) [^{3}H]poly(dT), and (▽) [^{3}H]poly(dT·poly(A). Reactions were run at 37 °C and started by the addition of enzyme. Aliquots (0.03 mL) were removed at the times indicated, processed, and counted as described for a standard RNase assay.

over an enzyme concentration of 0.1–1.2 units for at least 30 min in a 0.05-mL assay containing 1.5 nmol of substrate. The nucleolar ribonuclease gave an apparent K_m for single-stranded RNA of approximately 3 μM calculated on a nucleotide basis. In addition, double-stranded RNA acted competitively to inhibit the degradation of the single-stranded RNA substrate which further supports the proposal that both single-stranded and double-stranded ribonuclease activities are associated with the same enzyme.

Specificity of Degradation. When the base specificity of the single-stranded ribonuclease activity was examined with ^{3}H-labeled poly(C), poly(U), poly(A), and f_2 [^{32}P]RNA as substrates, the enzyme was found to be capable of degrading any one of these single-stranded substrates. In addition, a comparison between the degradation of single- and double-stranded substrates is shown in Figure 6. Although, the extent of degradation for the single-stranded substrate is greater than twice that for the double-stranded substrate, this difference, in actuality, may not exist. If one considers that only one strand of the RNA duplex is labeled and that it is the release of this label that is monitored as acid-soluble nucleotide released, it can be seen that degradation of the complementary strand does not register in the assay. Since the enzyme can degrade poly(U), as well as poly(A), then the extent of degradation on a total nucleotide basis would generate results nearly equal to that observed for the single-stranded substrate alone.

The nucleolar ribonuclease did not degrade single-stranded DNA or the DNA strand of a RNA·DNA hybrid duplex. However, the enzyme would attack the RNA strand of a RNA·DNA duplex but at a rate substantially lower than that observed for a RNA duplex (Figure 6). Further analysis was carried out by using native or denatured T_7 [^{3}H]DNA as a substrate. Under conditions that would render single-stranded RNA 50% acid soluble, less than 3% of either the native or denatured T_7 DNA was made acid soluble (data not shown).

Mode of Attack. To determine whether the nucleolar ribonuclease acts as an endonuclease or exonuclease, timed aliquots of [^{3}H]poly(C) or [^{3}H]poly(C)·poly(I) digests were analyzed on a DEAE-Sephadex A-25 column. Regardless of the extent of degradation, the only acid-soluble product identified was mononucleotide (Figure 7). These results would be expected if the nuclease degraded RNA exonucleolytically.

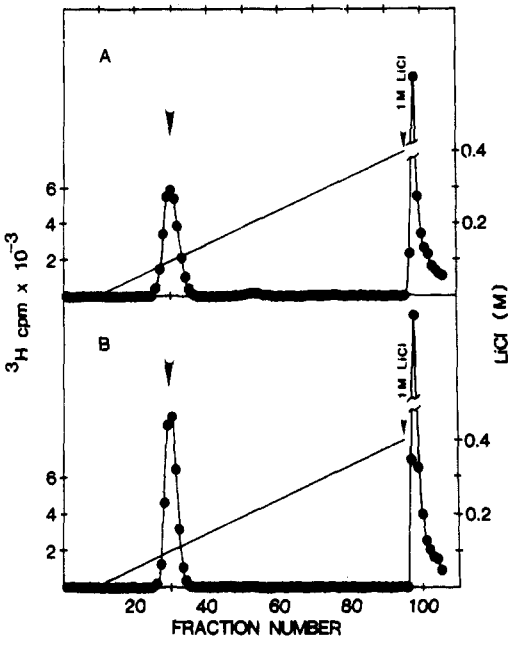

FIGURE 7: Product distribution. A reaction mixture (0.3 mL) containing 0.05 M Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 5.8 units of enzyme, and 9 nmol of either (A) [^{3}H]poly(C) or (B) [^{3}H]poly(C)·poly(I). Aliquots (0.02 mL) were taken at 0, 5, 15, 30, and 60 min and were processed and counted as described for a standard RNase assay as described under Materials and Methods. The remaining reaction material was brought to 10 mM in EDTA and diluted 10-fold with 5 mM Tris-HCl, pH 7.5, and 7 M urea. The sample was loaded onto a 1-mL DEAE-Sephadex A-25 column (0.7 × 2.6 cm) jacketed at 65 °C and equilibrated against the same buffer. The loaded column was washed with 3 mL of buffer, and a 50-mL gradient from 0.0 to 0.4 M LiCl was run followed by a 1 M LiCl step. Fractions (0.5 mL) were collected and counted in 4 mL of Triton X-100 scintillation fluid. The arrow indicates the position of mononucleotide elution.

Mode of Phosphodiester Bond Cleavage. To determine whether the enzyme cleaves on the 3′ or 5′ side of the phosphodiester bond, the mononucleotide product of the degradation of [^{3}H]poly(C) was chromatographed on a poly(ethylenimine)-impregnated cellulose acetate thin-layer plate under conditions which distinguish a 5′- from a 3′-mononucleotide. The radiolabel released as mononucleotide moved exclusively with the 5′-mononucleotide UV marker (data not shown). The exonuclease, therefore, cleaves on the 3′ side of the phosphodiester bond, releasing 5-′mononucleotides

Direction of Attack. In order to define the direction of attack by the nucleolar ribonuclease, [^{3}H]poly(C) containing a 3′-^{32}P end label was used as a substrate. As shown in Figure 8A, the rate and extent of ^{3}H-label release is less than that of the ^{32}P label. Similar results were obtained with [3′-^{32}P,^{3}H]poly(C)·poly(I) (Figure 8B). These results indicate that the ribonuclease degrades an RNA polymer, whether single or double stranded from the 3′-terminal end in a nonprocessive manner.

DISCUSSION

A unique ribonuclease activity capable of degrading either single-stranded or double-stranded RNA has been partially purified from nucleoli of Ehrlich ascites tumor cells. The results presented above indicate that the single- and double-stranded activities were similar in their sensitivity to *N*-ethylmaleimide and heat inactivation. The catalytic requirements were also identical. Both activities were stimulated by the addition of divalent cation (Mg^{2+} or Mn^{2+}) while monovalent cations, for the most part, were inhibitory. EDTA was also inhibitory. In both cases RNA was degraded in an

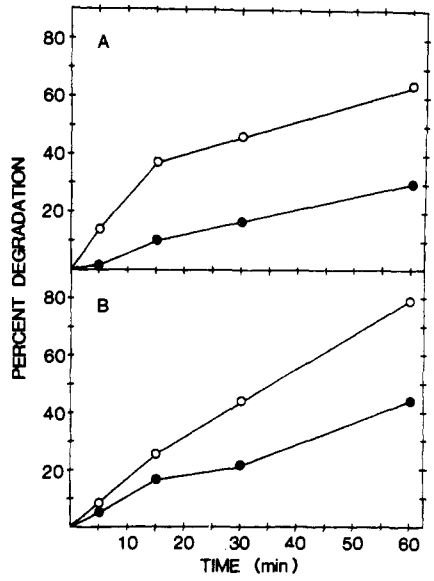

FIGURE 8: Digestion of [3′-^{32}P,^{3}H]poly(C) and [3′-^{32}P,^{3}H]poly(C)·poly(I) by the nucleolar ribonuclease. The reaction mixture (0.3 mL) contained 0.05 M Tris-HCl, pH 8.0, 0.06 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 5.8 units of enzyme, and 9 nmol of either (A) [3′-^{32}P,^{3}H]poly(C) or (B) [3′-^{32}P,^{3}H]poly(C)·poly(I). At the indicated times, aliquots (0.02 mL) were removed and processed for counting as described for a standard RNase assay under Materials and Methods. (●) ^{3}H label; (○) ^{32}P label.

exonucleolytic fashion producing 5′-mononucleotides. Polarity studies suggested that the enzyme initiates the exonucleolytic attack of RNA from the 3′ end of the strand being degraded. The degradation is nonprocessive so that the polymer population is randomly shortened by the action of the nuclease. Although the enzyme will degrade RNA hybridized to DNA, it will not attack single- or double-stranded DNA alone. Molecular weight determinations demonstrated that both activities cosediment in a glycerol gradient and cochromatograph on a molecular sieving column. Taken with the fact that both the single-stranded and double-stranded ribonuclease activities copurify, these results strongly support the conclusion that the two ribonuclease activities are due to a single enzyme molecule.

By several criteria, this enzyme bears little resemblance to any double-stranded or single-stranded RNase previously described in the literature. Saha & Schlessinger (1978) described two activities from Hela cell nuclei that degraded double-stranded RNA. Only one of these activities, designated PCII, degraded RNA in an exonucleolytic fashion. However, that enzyme had an apparent molecular weight of only 20000, was stimulated by 50–80 mM NaCl, and poorly degraded either poly(C)·poly(I) or poly(A)·poly(U). Hall & Crouch (1977) described two ribonuclease activities from chick embryos also capable of degrading double-stranded RNA. However, both enzymes attacked double-stranded RNA endonucleolytically. Similarly, Ohtsuki et al. (1977) described the purification and properties of a double-strand specific RNase from calf thymus nuclei, but the mode of attack was again endonucleolytic. A highly purified single-strand-specific 3′ → 5′ exonuclease was previously described by Lazarus & Sporn (1967). That ribonuclease preferentially attacked poly(A) in a processive manner, releasing 5′-AMP. No activity was observed with double-stranded RNA as substrate.

The processing of ribosomal RNA precursors is a nonconservative process since nonutilizable products are degraded. Under the assumption that the ribonuclease described in this paper is truly nucleolar, it could conceivably function in the

turnover of discarded RNA sequences during ribosomal RNA processing. Moreover, when protein synthesis is inhibited, synthesis and partial processing of precursor rRNA still continues, but little or no mature rRNA emerges from the nucleolus (Mirault & Scherrer, 1972). Similarly, Cooper & Gibson (1971) found that in resting lymphocytes the synthesis of ribosomal RNA appears to exceed the capacity of the cell to utilize the RNA in ribosome assembly. Therefore, processing of nucleolar RNA not only involves the specific cleavage of larger precursor RNA molecules but also includes the degradation of cleavage products discarded during biogenesis and the degradation of nonutilizable transcripts. It is in this latter role that this nucleolar RNase seems particularly suited. The lack of specificity for either sequence or structure makes the RNase ideally suited for the degradation of discarded RNA to replenish ribonucleotide pools.

Registry No. Exoribonuclease, 37288-24-7; Mg, 7439-95-4; Mn, 7439-96-5.

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