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Characterization of an Endoribonuclease from *Xenopus* Oocytes. Possible Role in Ribonucleic Acid Turnover†

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ABSTRACT: On the basis of kinetic and base ratio data, a "4S" cytoplasmic RNA fraction may be an intermediate in the degradation of heterogeneous nuclear RNA (Aronson, A. (1972), *Nature (London), New Biol. 235*, 40). An endoribonuclease has therefore been purified and characterized from *Xenopus* oocytes. This enzyme preferentially hydrolyzes at pyrimidine residues splitting poly(U) 30 times faster than poly(C). There is no detectable hydrolysis of double-stranded poly(A)·poly(U) nor of DNA. The 3′ end of oligonucleotides produced by enzyme digestion are primarily uridine. The 3′ ends of "4S" RNA isolated from sea urchin embryo cytoplasm are also primarily uridine suggesting an *in vivo* function for this enzyme. The enzyme does not appear to be localized in the nucleus.

A very substantial fraction (85–94%) of the RNA synthesized by early sea urchin embryo turns over rapidly (Aronson and Wilt, 1969; Brandhorst and Humphreys, 1971). In fact, the time of synthesis of these molecules is very close to their half-life (A. I. Aronson, unpublished results). Most of the RNA synthesized by these embryos is rather large (about 3×10^6–10^7 daltons) with a base ratio close to that of the sea urchin DNA (Wilt *et al.*, 1969) and is classified as heterogeneous nuclear RNA (HnRNA). There is little if any detectable rRNA synthesis in these embryos prior to gastrulation (Emerson and Humphreys, 1970). Similar turnover of HnRNA in mammalian cells has also been described (Darnell *et al.*, 1973).

We had previously found a population of RNA molecules of approximately 4 S in the cytoplasm of hatched blastula embryos which on the basis of base ratio analysis and kinetic properties could have been an intermediate in the degradation of HnRNA (Aronson, 1972). The existence of this "4S" RNA suggested endonucleolytic cleavage of HnRNA so we began a search for an endonuclease which could be involved in turnover (Aronson, 1972). At the same time, we found an endonuclease in *Xenopus laevis* oocytes which appeared to

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have very similar properties to the urchin activity. Because of the greater ease of obtaining *Xenopus* oocytes, we have purified the endonuclease from stage 6 oocytes and have evidence that the enzyme is unique in several properties, especially specificity. We also have some preliminary data which is consistent with a role for such an enzyme in HnRNA turnover.

Materials and Methods

Preparation of RNA. High molecular weight RNA was prepared from sea urchin (*Strongylocentrotus purpuratus*) hatched blastula embryos that had been labeled for 30 min at 14° with [8-^{3}H]guanosine (5–25 Ci/mol, 2–2.5 μCi/ml). The embryos were washed three times with 1.5 M dextrose at 4° and the resulting pellet was suspended in 0.1 M Tris-HCl (pH 9). The suspension was brought to 0.6% (w/w) sodium deoxycholate–1% (w/w) sodium dodecyl sulfate, and an equal volume of a 1:1 mixture of EDTA-treated phenol containing 0.1% 8-hydroxyquinoline and 50% chloroform was added. After shaking for 15–30 min at 22°, the extract was centrifuged at 12,000*g* for 20 min in a Sorvall RCIIB centrifuge, and the aqueous phase was removed, reextracted with phenol–chloroform, and precipitated with two volumes of ethanol at −17°. The pellet was dissolved in buffer containing 10 mM Tris-HCl and 10 mM 2-(*N*-morphilino)ethanesulfonic acid adjusted to pH 7 (Tris-MES Buffer), made 1 mM in $MgCl_2$ and treated with 10 μg/ml of DNase (EC 3.1.4.5, Worthington Biochemical Corp.) at 37° for 15 min. After a second treatment with DNase, the pH was adjusted to 7.5, nuclease-free Pronase (Calbiochem) was added to 100 μg/ml, and the mixture was incubated at 37° for 30 min. The RNA was reextracted with phenol–chloroform and further purified by precipitation with cetyltrimethylammonium bromide (CTAB, Ralph and Bellamy, 1964) plus 2 M NaCl. Most of the radioactive RNA (60–90%) had a sedimentation coefficient greater than 30 S as determined by acrylamide–sodium dodecyl sulfate gel electrophoresis (Berridge and Aronson, 1973). Less than 2% of the radioactivity remained undegraded after treatment with alkali indicating little contamination with DNA.

Poly(A)·Poly(U) Formation. Equimolar amounts of poly(A) and poly(U) (Miles Laboratories) were incubated at 22° for 30 min in Tris-MES buffer (pH 7), containing 1 mM $MgCl_2$. Under these conditions, annealing is almost instantaneous as determined by the decrease in hyperchromicity at 260 nm, and the major reaction product is double-stranded RNA (Steiner and Beers, 1961; Michelson *et al.*, 1967).

Isolation and Purification of Endonuclease: Preparation of Crude Cytosol and S100. All procedures involved with enzyme extraction and purification were carried out at 4°. Ovary (150 ml) was excised from mature *Xenopus laevis* females, cut into small pieces, and gently homogenized in a loosely fitting Dounce homogenizer with an equal volume of Tris-Mes buffer (pH 7.0), containing 2 mM $MgCl_2$ and 1% (v/v) Triton X-100. The homogenate was centrifuged at 12,000*g* for 30 min and the light yellow extract between the yolk pellet and yellow lipid phase was recovered and is referred to as crude cytosol.

Alternatively, oocytes were prepared from fresh ovary by the collagenase procedure of Dumont (1972). This treatment was followed by incubation with Pronase (100 μg/ml, 30°/5 min) to remove the vitelline membrane and adhering follicle cells but since the Pronase step did not appear to significantly alter initial enzyme yields and could explain poor enzyme preservation in early preparations, it was not used routinely. Oocytes were washed two to three times with Steinberg's medium (Ecker and Smith, 1971) and homogenized, and cytosol was prepared as described above.

Crude cytosol was centrifuged at 105,000*g* for 2 hr and the clear supernatant was carefully removed with a syringe. This fraction is referred to as S100 enzyme. The material remaining after removal of the S100 was divided into three fractions: (i) liquid material which could be removed by gentle swirling with Tris-Mes buffer, (ii) the upper pellet of ribosomes that could be removed with more vigorous agitation, and (iii) the residual light amber pellet of glycogen (personal communication, L. D. Smith).

Ammonium Sulfate Fractionation. The S100 preparation was brought to 35% $(NH_4)_2SO_4$ by the slow addition of either solid or 100% $(NH_4)_2SO_4$ solution at 4°. The solution was allowed to stand at 4° for 1 hr with gentle stirring and the precipitate was removed by centrifugation. The supernatant was brought to 65% saturation with solid $(NH_4)_2SO_4$ and stirred for 1 hr at 4°. The resulting precipitate was collected by centrifugation, dissolved in a small volume of AGSMN buffer (pH 6) (20 mM NaAc, 5% v/v glycerol, 10 mM mercaptoethanol, 2 mM $MgCl_2$, and 50 mM NaCl), and dialyzed overnight *vs.* 20 volumes of this buffer.

Column Chromatography. The dialyzed preparation was diluted to about 60 ml with the AGSMN and applied to a 45 × 1.4 cm column of carboxymethyl-Sephadex A-50 (Pharmacia) that had been equilibrated with AGSMN buffer (pH 5.5). The enzyme was washed onto the column with 300 ml of buffer and eluted with 300 ml of 0.05–0.6 M linear NaCl gradient made up in AGSM buffer with a flow rate of about 15 ml/hr. Fractions (7 ml) were collected and those containing enzyme activity (see bar on Figure 1A) were pooled and dialyzed against a saturated solution of Poly(ethylene glycol) 6000 made up in PGSM buffer (pH 6) (as for AGSM but containing 0.1 M sodium phosphate instead of sodium acetate).

The concentrated enzyme solution was dialyzed overnight against 1 l. of PGSM buffer (pH 6) and applied to a 40 × 1.4 cm column of hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) that had been equilibrated with PGSM buffer (pH 6). The enzyme was washed onto the column with 100 ml of PGSM buffer and eluted with a 400-ml, 0.1–0.8 M sodium phosphate gradient in GSM. Active fractions (Figure 1B) were pooled, concentrated by dialysis against poly(ethylene glycol) in AGSMN, and dialyzed overnight against 1 l. of AGSMN (pH 5.5).

The concentrated enzyme (3.8 ml) was made up to 15% (v/v) with glycerol and applied to an 80 × 2.5 cm column of Sephadex G-100 (Pharmacia) that had been equilibrated with AGSMN buffer. The column was eluted with 350 ml of AGSMN (pH 5.5) (3.4-ml fractions, 15 ml/hr) and the active fractions (Figure 1C) were pooled, dialyzed against poly(ethylene glycol) in AGSMN (pH 5.5) as above, and then for 16 hr *vs.* AGSMN.

For some enzyme preparations, the sequence of chromatographic columns was altered and a phosphocellulose column included in the purification, but the procedure as described gave the highest specific activity enzyme.

Purified enzyme was stored in small aliquots at −70° in AGSMN buffer (pH 5.5) containing 15% v/v glycerol. Under these conditions, the enzyme retained full activity for many months even after freeze–thawing several times.

Assays for Endonuclease Activity, Poly(U) Assay. Enzyme activity was usually determined by a modification of the method described by Zimmerman and Sandeen (1965). Each assay mixture contained 2.0 A_{260} units of poly(U) (Miles

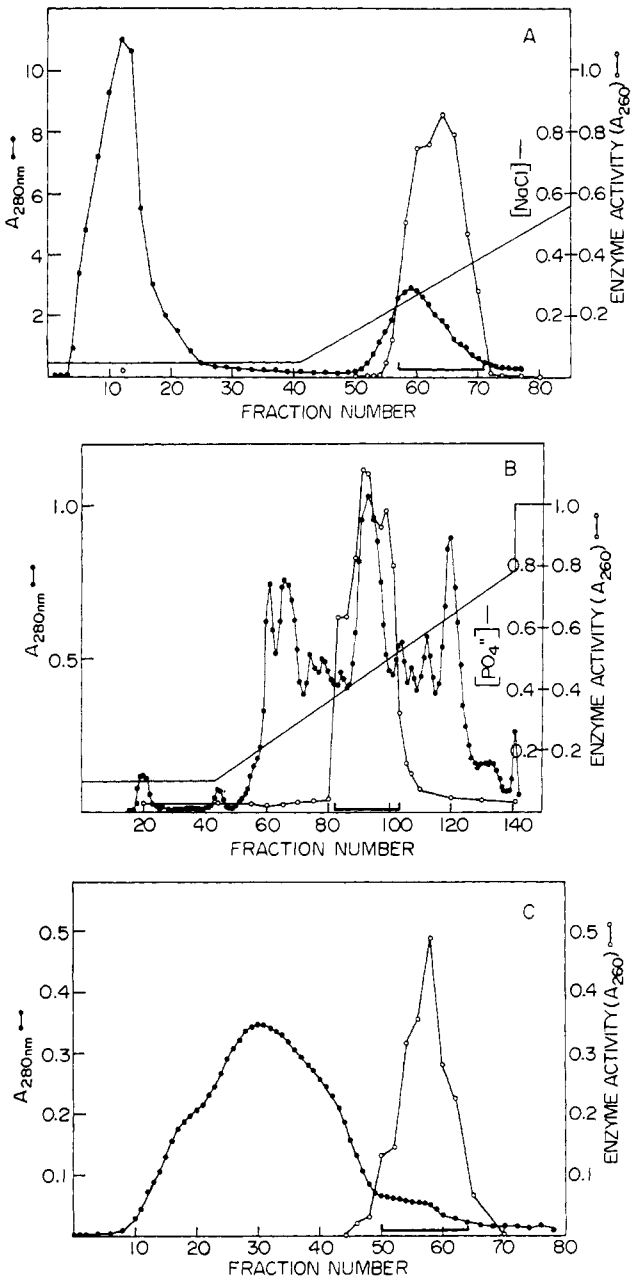

FIGURE 1: Column chromatography of *Xenopus* endonuclease enzyme activity was determined by the poly(U) assay described in Materials and Methods, except that each assay mixture contained 1 A_{260} unit of poly(U) and 5-μl samples from each fraction were incubated for 5 min at 37°. The bar under the fractions containing enzyme activity represents those fractions pooled for further purification: (A) Carboxymethyl-Sephadex A-50; (B) hydroxylapatite (Bio-Gel HTP); (C) Sephadex G-100.

Laboratory), 2 μmol of Tris-Mes buffer (pH 7.2), 5 μmol of mercaptoethanol, 12% (v/v) glycerol, 0.4 μmol of $MgCl_2$, and enzyme in a total volume of 0.25 ml. The tubes were incubated for 20 min at 37° and chilled on ice, and 1 mg of recrystallized bovine serum albumin was added. Residual nucleic acid was precipitated with 0.6 ml of 4% (v/v) $HClO_4$. Following centrifugation at 12,000*g* for 10 min, the A_{260} of the supernatant was measured. One unit of enzyme activity is defined as the release of 1.0 A_{260} unit of acid-soluble oligonucleotide in the above assay.

Electrophoretic Assay. Although the poly(U) assay measures general ribonuclease activity and is not specific for endonuclease, enzyme preparations could be tested specifically for endonucleolytic activity by an electrophoretic assay previously described (Berridge and Aronson, 1973). This assay measures endonuclease activity as a percentage loss of radioactivity in RNA from the top 6 mm (*i.e.*, $\geq$30S region) of an 8-cm, 2.6% acrylamide gel under conditions where all of the RNA remains acid insoluble (see Figure 5).

Both assays have similar sensitivity although the poly(U) assay is effective over a much greater range of enzyme concentrations, is much more convenient for assaying fractions from chromatography columns and more accurate for quantitating enzyme activity.

Isolation of 3'-Nucleoside Ends from RNA. RNA which had been treated with 1 unit of RNase-free (Wimmer, 1972) alkaline phosphatase (3.1.3.1, Worthington) was dissolved in 1 M KOH and incubated at 22° for 24 hr. The resulting hydrolysate was applied to Dowex AG50W resin (NH_4^+ form) and ammonia was removed from the eluate with a stream of air. Nucleosides were separated from nucleotides as described by Winslow and Lazzarini (1969) and chromatographed in two dimensions on thin-layer cellulose with fluorescent indicator (Eastman) in the solvent system of Randerath (1965). The appropriate regions were eluted with 10 mM HCl and the A_{260} was determined in a Zeiss spectrophotometer.

Labeling of Nucleoside Ends. Either bulk RNA degraded to about 4 S by purified endonuclease or "4S" RNA isolated from sea urchin hatched blastula embryos was used. To purify the latter fraction, crude sea urchin cytoplasm (Aronson and Wilt, 1969) was centrifuged first at 12,000*g* for 20 min in a Sorvall RCIIB centrifuge and the supernatant was centrifuged at 45,000 rpm for 90 min in an SW-50 rotor in a Spinco L2-65 ultracentrifuge. The supernatant was carefully removed and recentrifuged as above. The RNA was extracted from the supernatant and contained >98% "4S" material on the basis of gel electrophoresis profiles. The "4S" degradation RNA (Aronson, 1972) was separated from tRNA on hydroxylapatite columns (0.9 $\times$ 10 cm) employing an exponential gradient of 0.005–0.5 M potassium phosphate (pH 6.8) (see Figure 8).

RNA preparations were treated with nuclease-free alkaline phosphatase, oxidized with periodate, and reduced with [^{3}H]$NaBH_4$ as described (De Wachter and Fiers, 1967). The labeled RNA was purified on a P2 column (Bio-Rad, 0.9 $\times$ 20 cm) eluting with the void volume in 0.01 M sodium acetate (pH 7.0). Following alkaline hydrolysis (0.3 N KOH, 37°, 16 hr) and neutralization with Dowex AG-50 (NH_4^+ form), the hydrolysate was concentrated *in vacuo* and absorbed to acid-washed charcoal. Nucleotides and nucleosides were eluted with 50% ethyl alcohol–1% NH_3, dried *in vacuo*, and fractionated as described above. Nucleosides which had been oxidized with periodate and reduced with unlabeled $NaBH_4$ were included as markers. The appropriate ultraviolet absorbing spots were cut out and placed into scintillation vials containing 5 ml of Omnifluor (New England Nuclear Corp.) for counting in a Nuclear-Chicago Isocap scintillation counter.

Protein Determination. All protein determinations were performed on material that had been precipitated with Cl_3CCOOH. Protein was determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Results

Enzyme Purification. The results of a typical enzyme purification scheme are shown in Table I and Figure 1A–C. Although initial centrifugation resulted in a considerable frac-

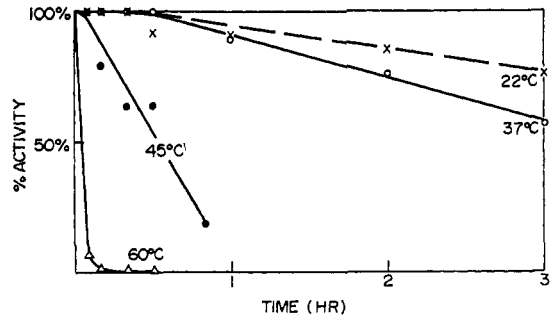

FIGURE 2: Heat stability of the endonuclease. Enzyme activity was determined by the poly(U) assay (Materials and Methods) except that dithioerythritol (2 mM) was used instead of mercaptoethanol. Each assay tube contained 0.25 μg of purified enzyme protein. The tubes were incubated at the temperatures indicated for periods up to 3 hr. Enzyme activity is expressed as a percentage of zero time controls.

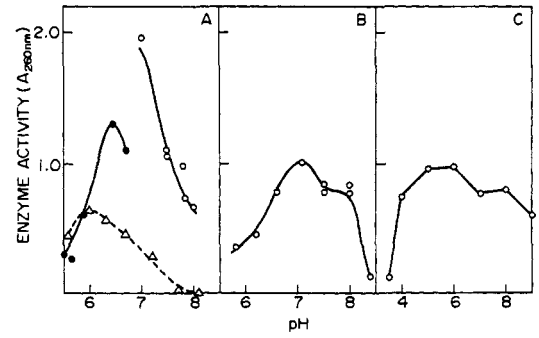

FIGURE 3: Optimal pH for enzyme activity and stability. Each assay mixture contained 2.0 A_{260} units of poly(U), 4 μmol of mercaptoethanol, 0.4 μmol of $MgCl_2$, 10% v/v glycerol, and 51 μg of an enzyme preparation that had been partially purified by high-speed centrifugation and ammonium sulfate precipitation (see Table I). Buffers were adjusted to respective pH's at 37°. Assays were performed as described in Materials and Methods and enzyme activities are plotted on the ordinate as A_{260} values: (A) (○) 20 mM Tris-HCl; (●) 20 mM MES; (△) 20 mM sodium phosphate; (B) variation of enzyme activity with pH in 10 mM Tris-MES buffer; (C) enzyme stability at various pH's. Enzyme was incubated in Tris-HCl or 20 mM sodium acetate buffers at 4° for 16 hr prior to assaying in 20 mM Tris-HCl buffer (pH 7.65) in the standard poly(U) assay.

tion of enzyme activity being associated with the pellet, even in the presence of Triton X-100 or deoxycholate, this step was necessary to provide a soluble enzyme that could be fractionated. In general, 40–60% of the total enzyme activity in the cytosol was in the pellet after centrifugation at 105,000*g* (Table I). Almost all of this activity could be removed from the ribosomal fraction of the pellet by washing with 0.5 M NH_4Cl. Although we have not examined the ribosomal activity in detail, it appears to have many of the properties of the purified soluble endonuclease, *i.e.*, ability to hydrolyze primarily at UMP residues, inhibition by EDTA, and ability to hydrolyze HnRNA to "4S" RNA without the loss of Cl_3-CCOOH-precipitable material. Attempts to purify this activity have been frustrated by the requirement of high salt concentrations for enzyme solubility.

Purification varies from 150- to 500-fold with 5–10% recovery of activity. Loss of activity is partly due to instability of the enzyme, especially at low protein concentrations. The purified enzyme is heat labile with a half-life of 30 min at 45° and about 1 min at 60° (Figure 2).

Properties of the Purified Enzyme. Molecular weight standards, bovine serum albumin (monomer and dimer), and cytochrome *c* (monomer and dimer) were run on the Sephadex G-100 column as in Figure 1C. The molecular weight of the *Xenopus* endonuclease was estimated to be about 43,000 daltons on the basis of a plot of the logarithm of the molecular weights of these standards *vs.* elution volume.

Figure 3A shows the effects of pH on enzyme activity in several different buffer systems. The pH optimum appears to depend to some extent on the buffer used. The optimum in phosphate buffer was 6 but in both MES and Tris buffers a pH optimum of about 7 was found. Because monovalent metal ions strongly inhibit enzyme activity in the poly(U) assay, and may thus alter the optimal pH for enzyme activity, we have used 10 mM Tris-MES buffered at pH 7 in most subsequent enzyme assays. The pH optimum in this buffer is 7 with a slight shoulder at 8 (Figure 3B).

The effect of maintaining enzyme solutions at various pH's for 16 hr at 4° prior to assaying in Tris-HCl buffer (pH 7.65) is summarized in Figure 3C. Since the activity was most stable between pH 5 and 6, the enzyme was maintained in this range during fractionation.

Monovalent cations such as NH_4^+, Na^+, and K^+ inhibit enzyme activity 60–90% at 20–100 mM in the poly(U) assay, but not in the gel electrophoresis assay employing [^{3}H]HnRNA. For the latter assay, 50 mM KCl was optimal suggesting that these monovalent cations inhibit by altering poly(U) structure rather than the enzyme.

A divalent cation (only Mg^{2+} was tested) appears to be an absolute requirement for enzyme activity, both in the poly(U) assay (Figure 4A) and in the electrophoretic assay (Figure 4B), since 10 mM EDTA completely inhibited enzyme activity

TABLE I: Purification of *Xenopus* Endonuclease.[a]

Vol (ml)	Fraction	Total Units of Enzyme	Protein (mg)	Units[b]/mg of Protein	Yield (%)
300	Crude homogenate	58,266	20,640	2.8	
184	Cytosol	38,860	3,422	11.3	66.7
124	105,000*g* supernatant	19,322	1,767	10.9	33.2
	105,000*g* pellet	14,877			
60	35–65% $(NH_4)_2SO_4$ precipitate	13,400	765	17.5	23.0
13.5	CM-Sephadex[c]	9,700	176	55	16.6
3.8	Hydroxylapatite[c]	6,419	31	207	11.0
6.6	Sephadex G-100[c]	3,268	2	1634	5.6

[a] See Materials and Methods for details of procedures. [b] Assayed with poly(U) as described in Materials and Methods. [c] See Figure 1 for elution profiles.

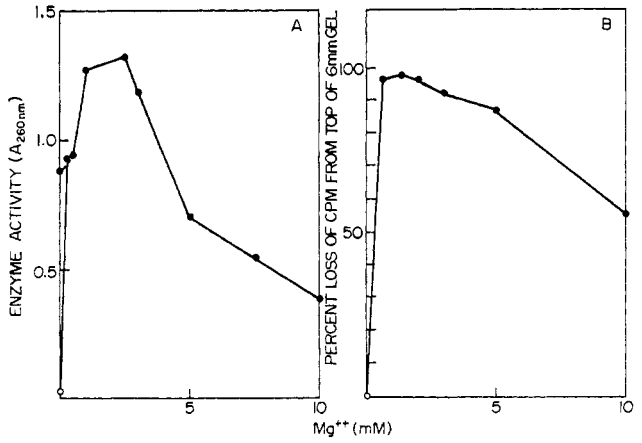

FIGURE 4: Effects of Mg^{2+} on enzyme activity in the poly(U) and electrophoretic assays. (A) Poly(U) assays were performed as described in Materials and Methods. Each mixture contained 1.14 μg of purified enzyme protein. (B) Electrophoretic assay: each tube contained 0.2 μmol of Tris-MES buffer (pH 7.0), 10% v/v glycerol, 0.1 μmol of diethioerythritol, 1 μmol of NaCl, 0.87 A_{260} of [^{3}H]-HnRNA (150,000 cmp), $MgCl_2$ as required, and 0.029 μg of purified enzyme protein in a total volume of 30 μl. Assays were incubated for 20 min at 37°. One tube in each experiment also contained 10 mM EDTA (○).

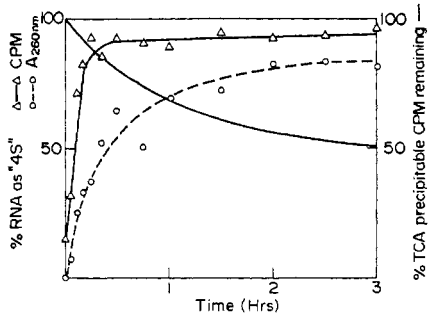

FIGURE 6: Kinetics of appearance of "4S" RNA. [^{3}H]HnRNA (0.2 ml, 17.4 A_{260} units) was incubated at 37° with purified *Xenopus* endonuclease (3 μg) in 0.8 ml of Tris-MES buffer (pH 7), containing 15% v/v glycerol, 10 mM mercaptoethanol, 2 mM $MgCl_2$, and 50 mM NaCl (T-MGSMN buffer). At various times, 50-μl samples were removed into chilled tubes containing 25 μl of 60% sucrose plus 5 mM $CuSO_4$ and frozen at −70°. From each sample, 25 μl was electrophoresed on 8 cm, 2.6% sodium dodecyl sulfate acrylamide gels (see legend to Figure 5), while 10 μl was precipitated with 7% Cl_3COOH in the presence of 100 μg of bovine serum albumin, filtered, and counted. (○) A_{260} units in 4–6S RNA as a percentage of total absorbance units recovered from each gel; (△) radioactivity in 4–7.5S RNA as a percentage of total radioactivity recovered from each gel;—percentage of radioactivity remaining Cl_3COOH precipitable.

Magnesium concentrations greater than 2 mM inhibited enzyme activity in both assays.

The enzyme has a temperature optimum of about 37° being four times more active than at 30° and three times more active than at 42°.

Enzyme Specificity: Kinetics of RNA Hydrolysis. The principle nuclease activity in crude cytosol preparations from *Xenopus* ooctyes appears to be an endoribonuclease which first cleaves [^{3}H]HnRNA to a species sedimenting at 4–7 S without the loss of Cl_3CCOOH-precipitable material (Figure 5). More extensive hydrolysis results in the slow loss of radioactivity from the "4S" peak which appears to be a relatively stable intermediate hydrolysis product.

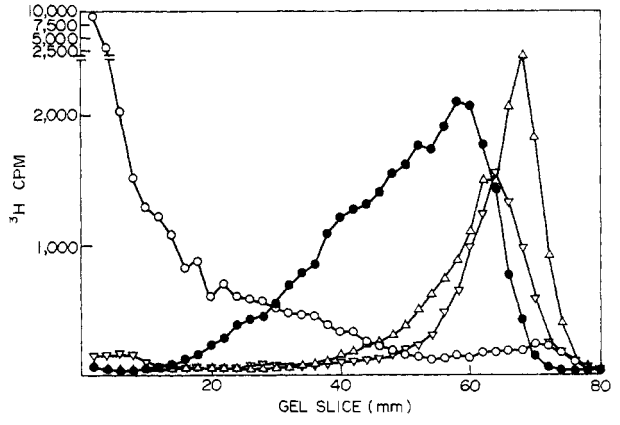

FIGURE 5: Endonuclease activity in *Xenopus* cytosol. [^{3}H]HnRNA (10 μl, 0.52 A_{260} unit; 40,000 cpm) was incubated at 22° with 25 μl of a cytosol preparation from *Xenopus* oocytes which had been diluted in 10 mM Tris-HCl (pH 7.8)–2 mM $MgCl_2$. Following incubation, the tubes were chilled on ice and a 10-μl sample was removed for Cl_3CCOOH precipitation. The remaining solution was mixed with 10 μl of 60% sucrose that had been saturated with sodium dodecyl sulfate and 25 μl was applied to 8 cm, 2.6% acrylamide–sodium dodecyl sulfate gels (Berridge and Aronson, 1973). The gels were electrophoresed at 5 mA/gel for 2 hr: (○) control RNA without enzyme; (●) incubated 90 min with 4 μg of protein; (▽) incubated 120 min with 40 μg of protein; (△) incubated 24 hr with 40 μg of protein. Cl_3CCOOH-precipitable cpm in each 10-μl sample removed prior to electrophoresis were control. 7529; 90 min, 7699; 2 hr, 5206; 24 hr, 2733. Total counts recovered from each gel closely parallel these values.

Specificity of Enzyme for Nucleotide Polymers. The effect of crude *Xenopus* endonuclease on each of four RNA homopolymers in the presence or absence of 2 mM $MgCl_2$ is summarized in Table II. For these assay conditions, the enzyme selectively hydrolyzes poly(U) in the presence of $MgCl_2$.

Experiments with purified enzyme confirmed that poly(A) and poly(G) are completely resistant to hydrolysis (Table IIB), *i.e.*, less than 0.3% of the activity of poly(U). Under optimal assay conditions, however, there is hydrolysis of poly(C) at about 3% the rate with poly(U). Neither poly(A)·poly(U) annealed under conditions which produce primarily double-stranded RNA nor heat-denatured DNA was hydrolyzed (Table II). The enzyme was also inactive on native salmon sperm DNA.

To further investigate the base specificity of the enzyme, the oligonucleotide tRNA fragment rTψCAAUUCCCCG$_p$ from yeast aspartyl-tRNA (obtained from Dr. G. Keith) was partially hydrolyzed with purified *Xenopus* endonuclease and the resulting oligonucleotides resolved by chromatography on DEAE-cellulose columns, equilibrated with 7 M urea (Tomlinson and Tener, 1963). The primary products of digestion are fragments 5–10 nucleotides long (Figure 7A). The penta-, hexa-, and heptanucleotide fragments must have resulted from scission near the middle of the molecule, at either AMP or UMP residues. More extensive digestion (Figure 7B) yielded fragments 1–5 nucleotides long. The 3′ ends and nucleotide compositions of each of these fractions were determined (Table III). Only pyrimidines and the original 3′-guanosine are found as nucleosides which is consistent with cleavage only at pyrimidine residues.

Extensive incubation of poly(U) or of the dinucleotides UpU or UpA with crude *Xenopus* endonuclease resulted in

TABLE II: Effects of Crude and Purified Endonuclease on Homopolymers.[a]

Homopolymer	nmol of Acid-Soluble Oligonucleotide Released/20 min		
	A. Crude Enzyme		B. Purified Enzyme
	10 mM EDTA	2 mM $MgCl_2$	2 mM $MgCl_2$
Poly(C)	8	2	10
Poly(A)	4	1	0
Poly(U)	6	170	312
Poly(G)		0	0
Poly(A)·poly(U)[b]			3
Denatured DNA[b,c]			0

[a] Each assay mixture in A contained 0.2 μmol of polynucleotide, 2 μmol of Tris-HCl (pH 7.8), and 0.12 mg of crude cytosol protein (Table I) in a total volume of 0.25 ml. Each assay mixture in B contained 0.2 μmol of polynucleotide, 2 μmol of Tris-MES buffer (pH 7), 2 μmol of mercaptoethanol, 12% v/v glycerol, and 1.14 μg of purified enzyme protein in a total volume of 0.25 ml. Tubes were incubated 20 min at 37° and nuclease activity was determined (see Materials and Methods). Acid-soluble material released from poly(A) and poly(U) was measured at 260 nm, poly(C) at 280 nm, and poly(G) at 250 nm. Extinction coefficients of nucleotides at pH 2 were used for conversion of acid-soluble material to mμmoles nucleotide. [b] Incubated for 2 hr at 37°. [c] Salmon sperm DNA heated to 100° for 15 min.

TABLE III: Nucleotide Composition of Aspartyl-tRNA Oligonucleotide Hydrolysis Products.[a]

Peak	Nucleoside End	Nucleotides	Major Components
I		Up, ψp or rTp	Up, ψp or rTp
IIA	C (U or ψ)	Tp, ψp	ψ Cp, rT ψp
IIB	G, C	Cp	C Cp, C Gp
III	G (U,C)	Ap, Cp, Up	C C Gp....
IV	G (U,C)	Ap, Cp, Up	C C C Gp...
V	G (U)	Ap, Cp, Up	C C C C Gp...

[a] Peaks I and II from Figure 7B were electrophoresed at pH 3.5 in 0.05 M ammonium formate buffer. Peak I gave one component running a little ahead of Up while peak II separated into two main bands, IIA and IIB. These two bands and peaks III–V were treated with alkaline phosphatase, hydrolyzed with 15% piperidine at 100° for 2 hr in a sealed tube, and the nucleoside and nucleotide components were determined by chromatography in two dimensions on thin-layer cellulose with a fluorescent indicator in solvent I: NH_4OH (23% NH_3)–1-propanol–water (3:6:1, v/v) and solvent II: concentrated HCl–isopropyl alcohol–water (17.6:68:14.4, v/v) of Randerath (1965). The nucleosides in parentheses are minor components; only the major nucleotides of peaks III–V are indicated. The major components in these peaks were deduced from the facts that the principle nucleoside was guanosine and these peaks contained tri-, tetra-, and pentanucleotides, respectively.

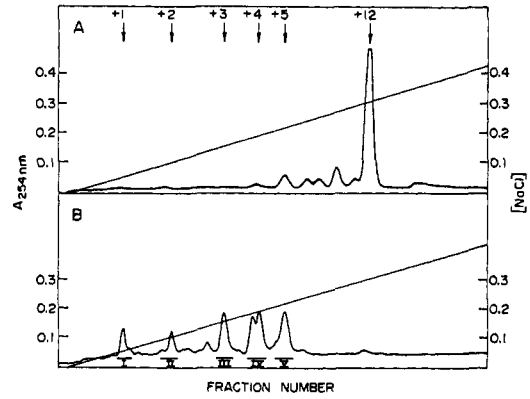

FIGURE 7: Hydrolysis of an oligonucleotide from yeast aspartyl-tRNA. (A) 4.4 A_{260} units of the oligonucleotide rTψCAAUUCCCCGp were incubated at 37° for 2 hr with 36 μg of purified enzyme diluted in TMGSM buffer (pH 7) in a total volume of 1.25 ml. The resulting hydrolysate was fractionated on a 7 M urea–DEAE-cellulose column (Tomlinson and Tener, 1963; 60 × 0.6 cm). The column was eluted with a linear 0–0.5 M NaCl gradient containing 0.02 M Tris-HCl (pH 7.4) (300-ml total volume) and the absorbance was monitored at 254 nm. The numbers at the top of the figure refer to the number of phosphate groups in each peak. (B) As for A except that 8.4 A_{260} units of the oligonucleotide was incubated in buffer containing 5 mM dithioerythritol (instead of mercaptoethanol), and 50 mM NaCl at 22° for 18 hr.

hydrolysis to Up and not pU as determined by chromatography of the products on fluorescent thin-layer cellulose in a borate buffer system (Plesner, 1955). Under similar conditions, there was no hydrolysis of U>p suggesting that the endonuclease does not hydrolyze by way of cyclic intermediate. High concentrations of either the crude or purified enzyme hydrolyzed poly(U) to Up implying that the same activity hydrolyzes RNA both to "4S" fragments and to pyrimidine mononucleotides. The inability to hydrolyze U>p and the Mg^{2+} requirement indicate that the enzyme is a phosphodiesterase.

Subcellular Localization of Endonuclease Activity. Preliminary investigation of the subcellular localization of endonuclease activity using the electrophoretic assay showed that both isolated germinal vesicles and cytosol from stage 6 oocytes contained endonuclease activity. To quantitate this localization and to minimize the effects of cytoplasmic "tags" on the germinal vesicles, the contents of 20 germinal vesicles isolated from defolliculated stage 6 oocytes were withdrawn with a microinjection apparatus and the total nuclease activity was determined by the poly(U) assay (Table IV). As a control, 20 stage 6 oocytes were disrupted in a Vortex mixer in TMGSM buffer (pH 7) and an aliquot was diluted and assayed for activity (Table IV). The contents of the germinal

TABLE IV: Subcellular Localization of Endonuclease.[a]

Fraction	Units/ Volume	Total Units/ Oocyte or per GV
1. Whole oocyte	1120	1.02
2. Germinal vesicle (GV) contents	2380	0.094
3. Whole oocytes + 10 mM EDTA	212	
4. Germinal vesicle + 10 mM EDTA	100	

[a] Whole oocyte extracts were diluted 5900 times and germinal vesicle (GV) contents 2860 times. The poly(U) assay was used (see Materials and Methods).

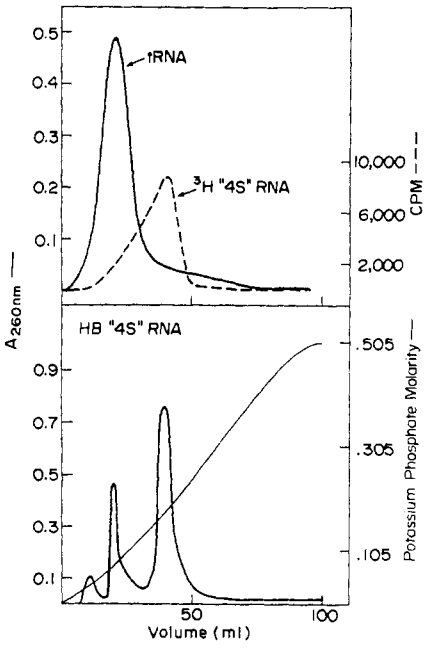

FIGURE 8: Fractionation of "4S" RNA on hydroxylapatite. Fifty micrograms of total *Bacillus subtilis* tRNA (General Biochemical Corp.) plus ^{3}H-labeled "4S" RNA (25,000 cpm: <0.1 A_{260} unit) prepared as in Figure 5 in 0.005 M potassium phosphate (pH 6.8) were absorbed into a 0.9 × 10 cm hydroxylapatite column. After 30 min, the column was eluted with an exponential gradient of 0.005–0.5 M potassium phosphate (pH 6.8) (100-ml total volume). The A_{260nm} and Cl_3COOOH precipitable cpm of each 3-ml fraction were determined and are plotted in the top half. Recoveries for both tRNA and ^{3}H-labeled "4S" RNA were greater than 98%. In the lower half, "4S" RNA prepared from hatched blastula sea urchin embryos (see Materials and Methods) was fractionated as described above.

vesicles contained about twice the endonuclease activity per unit volume as did whole oocytes. Since germinal vesicles comprise only 4–5% of the total volume of the oocyte, however, they contain only 8–10% of the total activity. Since both germinal vesicle and whole oocyte activities were inhibited by EDTA (Table IV) and cleave endonucleolytically, it is likely that they are similar if not identical enzymes. In addition, we have no evidence for more than one endonuclease in crude extracts or purified fractions.

Enzyme activity is present at about the same level per unit volume in both stage 4 and stage 6 oocytes.

End-Group Analysis of "4S" RNA Produced in Vitro and in Vivo. Unlabeled sea urchin high molecular weight RNA was treated with partially purified *Xenopus* endonuclease until all RNA was degraded to about 4 S (see Figure 5). The RNA was then reisolated by phenol extraction and ethanol precipitation. Following treatment with RNase-free alkaline phosphatase, the nucleoside moiety at the 3′ end of the RNA was determined either by periodate oxidation and labeling with [^{3}H]-$NaBH_4$ (see Materials and Methods) or by determining the ultraviolet absorbance of the isolated nucleosides (Table V). Following [^{3}H]$NaBH_4$ labeling, all of the radioactivity was present in uridine residues, and as determined by absorbance at 260 nm, greater than 80% of the end groups were uridine (Table VA). Because the [^{3}H]$NaBH_4$ procedure defines the end group prior to hydrolysis and purification of the nucleoside and is not dependent on possible errors in measuring small amounts of ultraviolet-absorbing material, it is probably a more accurate measure of the end-group composition of "4S" RNA.

Since there is very little information on HnRNA turnover

TABLE V: Analysis of 3′ End Groups of RNA Fractions.[a]

	% of Total Recovered as mmol or cpm			
	A	G	U	C
A. *In vitro* "4S" product				
(a) [^{3}H]$NaBH_4$ labeling			100	<1
(b) A_{260} absorbance	2.6	16.1	81.3	<1
B. *In vivo* product [^{3}H]$NaBH_4$ labeling				
(c) "4 S" (phosphatase)	5–8	3–6	74–84	8–12
(d) Deacylated tRNA	29	3	19	49
(e) Crude nuclear RNA	23	13–16	49–54	10–18

[a] *In vitro* "4S" RNA produced by treatment of 96 A_{260} units of unlabeled sea urchin hatched blastula high molecular weight RNA with 3.6 μg of purified *Xenopus* endonuclease for 2 hr at 37° in TMGSMN buffer (pH 7) (1-ml total volume). The "4S" was reisolated by phenol extraction, treated with alkaline phosphatase and the 3′-nucleosides isolated and characterized as described in Materials and Methods. For the *in vivo* fractions, embryos were fractionated as described in Materials and Methods. The RNA was extracted from the initial low-speed pellet to provide (e). tRNA (d) and "4S" RNA (c) were separated as in Figure 8. The tRNA was deacylated by incubation at pH 9.0 for 60 min at 37°. The labeling of "4S" RNA without prior phosphatase treatment was less than 10% of the treated preparations. Values for (c) and (e) are ranges for two experiments. Only a single set of values is given for (a), (b), and (d).

in *Xenopus* oocytes, RNA was prepared from hatched blastula sea urchin embryos. The low molecular weight RNA was fractionated on a hydroxylapatite column (Figure 8). About 70% eluted as did *in vitro* degraded ^{3}H "4S" RNA and 25–30% as did *Bacillus subtilis* tRNA. The initial peak has not yet been characterized. Pooled fractions were precipitated with two volumes of ethanol and dissolved in either 0.03 M Tris (pH 9.0) to deacylate tRNA or 0.03 M Tris (pH 8.0) for alkaline phosphatase treatment (see Materials and Methods). The RNAs were then oxidized, labeled, and hydrolyzed to provide nucleosides (Table VB). There was very little labeling of the "4S" RNA without alkaline phosphatase treatment but 70–80% of the total recovered ^{3}H-labeled nucleosides was uridine as opposed to 74–84% with prior phosphatase treatment. The tRNA labeling was primarily in cytidine and adenosine whereas all four nucleosides were labeled in the crude nuclear RNA with a predominance of uridine. The latter values do depart from a random labeling on the basis of base ratios (30% UMP) and indicate possible endonucleolytic cleavage of the RNA during preparation or contamination of the nuclear RNA with other populations (unlysed cells, etc.).

Discussion

The primary ribonuclease activity in *Xenopus* oocytes appears to be a phosphodiesterase which cleaves RNA endonucleolytically at UMP residues and to a much lesser extent at CMP residues to produce 2′- or 3′-phosphate oligonucleotides. The borate buffer system used cannot distinguish between 2′- and 3′-nucleotides so both possibilities remain. Although it is difficult to rule out a transient 2′,3′-cyclic intermediate, the fact that U>p is totally resistant to hy-

drolysis to 2′- or 3′-UMP suggests that the enzyme is not of the cycling variety but is rather a phosphodiesterase as suggested by its absolute requirement for a divalent cation (Barnard, 1969).

There have been few reports in the literature of phosphodiesterases that produce 3′-terminal oligonucleotides and are specific for RNA molecules, and to the knowledge of the authors, no class of phosphodiesterase with the high degree of base specificity shown by the *Xenopus* endonuclease although a bacterial ribonuclease with some specificity for UMP residues has been described recently (Levy *et al.*, 1973). It should be remembered, however, that the specificity for homopolymers and specific bases in RNA may not be absolute, but rather a function of the secondary structure of the RNA as determined by the ionic conditions of the assays. In this context, it is of interest to note the changes in salt requirement for optimal enzyme activity between the poly(U) assay and the gel electrophoretic assay.

Xenopus endonuclease that has been partially purified by ammonium sulfate precipitation and column chromatography contains no detectable DNase activity nor alkaline phosphatase activity as determined by the method of Garen and Levinthal (1960). In addition, we were not able to detect exoribonuclease activity following extensive degradation of the oligonucleotide rTψCAAUUCCCCGp (see Table III). Small amounts of nuclease activity with a pH optimim of about 5 have been detected in acid homogenates of *Xenopus* oocytes and sea urchin embryos prepared by the method described by Fernlund and Josefsson (1968) and are probably exonucleases.

Although enzyme activity appears to be linear over a wide range of enzyme concentrations using the poly(U) assay, we have been unable to obtain reliable kinetic parameters with the purified enzyme. Part of the problem is that poly(U) (even EDTA treated and dialyzed preparations) above 20 A_{260} units/ml appears to inhibit enzyme activity.

Although hydrolysis of sea urchin RNA occurred primarily at UMP residues (Table V), prolonged treatment of the oligonucleotide rTψCAAUUCCCCCp shows considerable hydrolysis at CMP residues. This result is supported by the fact that under ideal conditions a small amount of hydrolysis of poly(C) does occur (Table II).

It is of interest that the primary ribonuclease activity in both *Xenopus* oocytes and in sea urchin embryos (Aronson, 1972) is an endoribonuclease that first cleaves RNA to fragments 150–300 nucleotides long with little loss of Cl_3CCOOH-precipitable material. The fact that these fragments appear to be relatively stable to further degradation probably reflects the highly ordered secondary structure of both the labeled RNA, most of which is HnRNA and of the rRNA. There are probably ribonuclease sensitive regions in RNA at somewhat regular intervals. The fact that *Xenopus* endonuclease is specific for pyrimidine residues of single-stranded RNA (Tables II) suggests that these RNase-sensitive regions are probably single-stranded RNA loops which are rich in pyrimidines, especially UMP residues.

That "4S" RNA is an intermediate product in the turnover of HnRNA *in vivo* in developing sea urchin embryos (Aronson, 1972) suggests that the *in vitro* studies described above may be relevant to RNA turnover within the oocyte. In addition, most of the "4S" RNA molecules prepared from sea urchin embryos contain uridine at the 3′ end (Table V) which is also consistent with a function of this endonuclease in HnRNA turnover.

The lack of localization this enzyme in *Xenopus* oocyte nuclei (Table IV) as well as sea urchin nuclei (Aronson, 1972) appears to conflict with this role. There is very little known about HnRNA turnover in *Xenopus* oocytes, however, so the lack of high activity in the germinal vesicle may reflect a low rate of RNA metabolism in these eggs (La Marca *et al.*, 1973). It is also possible that much of the HnRNA degradation occurs immediately upon passage of the RNA to the cytoplasm with mainly synthesis occurring in the nucleus. Further characterization of this enzyme and the process of HnRNA turnover should help to resolve its *in vivo* function.

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