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Endoribonuclease from Bovine Adrenal Cortex Cytosol†

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ABSTRACT: An endoribonuclease which digests a variety of synthetic homoribopolymers and poly(A)-rich mRNA has been identified and purified >500-fold with respect to specific activity from bovine adrenal cortex cytosol. Enzymatic digestion of synthetic poly(riboadenylic acid) was stimulated by Mn^{2+} and Mg^{2+} and the enzyme exhibited broad pH and salt optima. Poly(cytidylic acid) and poly(uridylic acid), but not poly(guanylic acid), served as substrates for the enzyme preparation; double-stranded RNA, DNA, and DNA-RNA hybrids were not digested by the enzyme. Digestion generated oligonucleotides with 3′-hydroxyl and 5′-monophosphoester termini. On isoelectric focusing, the enzymatic activity banded at pH 8.3 ± 0.2. An initial preferential cleavage of the poly(A) tract of poly(A)-rich RNA is suggested by the rapid appearance of a 4-6S digestion product highly enriched for adenylic acid; however, progressive digestion of the RNA occurs with additional incubation.

A region rich in poly(riboadenylic acid) has been identified in many eukaryotic and prokaryotic mRNAs (Lee et al., 1971; Darnell et al., 1971a,b; Edmonds et al., 1971; Lim and Canellakis, 1971; Aviv and Leder, 1972; Comstock et al., 1972; Lai and Duesberg, 1972; Prescott et al., 1971; Armstrong et al., 1972), and it has been proposed that most mRNAs in the eukaryotic cell contain a poly(A)[1] tract (Adesnik et al., 1972) at the 3′ terminus (Yogo and Wimmer, 1972; Molloy et al., 1972). The poly(A) tract may serve a regulatory role (Darnell et al., 1973), and regulation of its synthesis and degradation have been widely studied. Several eukaryotic ribonucleases which can digest poly(A) have been identified including a processive nuclear exoribonu-

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[1] Abbreviations used are: MEM, minimal essential medium; SDS, sodium dodecyl sulfate; poly(A), poly(riboadenylic acid); poly(C), poly(ribocytidylic acid); poly(G), poly(riboguanylic acid); poly(U), poly(ribouridylic acid).

clease purified from several tissues (Lazarus and Sporn, 1967; Sporn et al., 1969; Lazarus et al., 1968) and an endoribonuclease first purified from rat liver nuclei (Heppel, 1966).

The observation that a ribonuclease which cleaved the poly(A) tract appeared to copurify with lymphocyte mRNA (Rosenfeld et al., 1972b) prompted a search for an enzyme with similar or identical properties in quantities large enough to be amenable to study. We report here the identification and partial purification of an endoribonuclease from bovine adrenal cortex cytosol which digests synthetic poly(A) and other homoribopolymers and studies of digestion of synthetic poly(A) and poly(A)-rich RNA by this enzyme.

Experimental Procedures

Materials

[^{3}H]Adenosine (15.5 Ci/mmol), [^{3}H]uridine (15 Ci/mmol), and *Escherichia coli* [^{3}H]tRNA used as a marker for gradient analysis were purchased from Schwarz/Mann; [^{32}P]orthophosphate in H_2O was from New England Nuclear. Protease-free pancreatic RNase was obtained from Sigma Chemical Co. and was treated by heating at 60° for 30 min at pH 5 prior to additional use. Insoluble pancreatic RNase attached to Sepharose beads (enzyme concentration 6.7 units/ml) was purchased from Miles Laboratories, Inc. Takadiastase RNase T_1 (Sankyo preparation), RNase T_2 essentially free of RNase T_1 activity, and actinomycin D were purchased from Sigma Chemical Co. Poly(ribouridylic acid) and poly(riboadenylic acid) were purchased from Schwarz/Mann. [^{3}H]Poly(riboadenylic acid) (3–10 Ci/mmol) and trypsin immobilized on Sepharose beads (enzyme concentration 5.9 units/ml) were obtained from Miles Laboratories, Inc. Electrophoretically purified DNase 1 was obtained from Worthington Biochemical Corp.

Methods

Preparation of Poly(A)-Rich RNA Free of Associated Ribonuclease Activity. [^{3}H]Adenylate or [^{3}H]uridylate rapidly labeled polyribosome associated poly(A)-rich RNA was isolated from cultures of highly purified human peripheral lymphocytes by the nitrocellulose binding method previously described (Rosenfeld et al., 1972a). Specific activity was calculated on the assumption of 40 μg of RNA/1 A_{260} unit. [^{32}P]RNA was prepared in a similar manner except that the medium was changed to phosphate-free MEM with 10% dialyzed autologous serum 36 hr prior to addition of carrier-free [^{32}P]orthophosphate (20 mCi) after which incubation was continued for an additional 90 min. The poly(A)-rich RNA was freed of any copurifying ribonuclease activity by heating and sedimentation through linear SDS–sucrose density gradients as previously described (Rosenfeld et al., 1973) and isolated poly(A) tracts were separated following pancreatic and RNase T_1 digestion of the RNA as has been described (Rosenfeld et al., 1973).

Ribonuclease Purification. Fresh bovine adrenal cortex was homogenized in 4 v/w of 10 m*M* Tris-HCl (pH 7.4), 10 m*M* KCl and 1 m*M* $MgCl_2$, using 10 strokes with a motor-driven Teflon pestle, filtered through cheesecloth, and spun at 10,000*g* for 15 min. All procedures were carried out at 4°. The supernatant was adjusted to 50% saturation with ammonium sulfate, stirred for 2 hr, and centrifuged for 90 min at 15,000*g*. The precipitate was resuspended and dialyzed in an excess of 10 m*M* Tris-HCl (pH 7.4), 10% glycerol, and 6 m*M* 2-mercaptoethanol (buffer A). Calcium phosphate was eluted twice with 0.25 *M* potassium phosphate (pH 8.0), 10% glycerol, and 6 m*M* 2-mercaptoethanol; the eluates were dialyzed 12 hr against buffer A and then adjusted to 50% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 20,000 rpm for 15 min, resuspended in, and dialyzed against an excess of buffer A. The sample was applied to a 3 × 37 cm DEAE column and eluted using a 0.05–0.5 *M* linear sodium chloride gradient. The fractions with maximal specific activity (0.06–0.09 *M* NaCl) were pooled and concentrated, and dialyzed against 20 m*M* Tris-HCl (pH 7.4 at 25°), 100 m*M* KCl, 5 m*M* $MgCl_2$, and 6 m*M* 2-mercaptoethanol; 4–6 mg of the sample was applied to a 5–20% linear sucrose gradient in the same buffer and centrifuged for 40 hr at 39,500 rpm in an SW40 rotor. The peak activity migrated at 7 S and the fractions with peak activity were stored at −80° until used. Protein concentrations were quantitated by the method of Lowry et al. (1951).

Assay of RNA Cleavage. Radiolabeled poly(A)-rich RNA or synthetic poly(A) was digested by incubation at 37° in 30 m*M* Tris-HCl (pH 8.3), 20 m*M* ammonium sulfate, and 0.8 m*M* $MnCl_2$ (buffer B) in the presence of purified endoribonuclease or appropriate balanced buffer. Following incubation, a 40-fold excess of 10 m*M* Tris-HCl (pH 7.6), 500 m*M* KCl, and 1 m*M* $MgCl_2$ was added and the solution was slowly filtered through a Millipore filter. The filter was then washed with an additional 20 ml of the same buffer, dissolved in 10 ml of Bray's solution (Bray, 1960) and counted in a Beckman liquid scintillation counter. This assay provided a sensitive evaluation of digestion of synthetic poly(A) or the poly(A) tract of poly(A)-rich RNA; poly(A)-rich RNA from which the poly(A) had been cleaved was no longer retained on the Millipore filter. Alternatively, the samples were applied to Whatman DE81 paper, then washed and counted as previously described (Blatti et al., 1970). In the experiments described in Table II, conversion of polynucleotide from Cl_3CCOOH precipitable to Cl_3CCOOH soluble was employed as the criterion for digestion since the Millipore binding assay could not be employed for assay of digestion of all of the substrates.

Isoelectric Focusing of the Ribonuclease. A 110-ml (KKB 810) isoelectric focusing column was used in all experiments. Carrier ampholites to produce a pH 3–10 gradient and the sample to be focused were added to the ice-cold sucrose solutions before generating the sucrose density gradients. Isoelectric focusing was accomplished by application of a 500-V current for 48 hr with appropriate increases in voltage during the run to compensate for increased resistance. Temperature of the jacketed column was maintained at 4°. The column was collected and the pH and enzymatic activity of the fractions were measured.

Chromatography of Enzymatic Digestion Products. Following digestion of [^{3}H]poly(A) by the purified endoribonuclease (0.8 μg/ml) at 37° for a time sufficient to produce 50–90% digestion, as determined by the Millipore binding assay, 75 μg of snake venom phosphodiesterase in 0.1 *M* glycine (pH 9.0) was added and incubations were continued for an additional 10 min. This time was minimal compared to the time of the first incubation which was designed to be 120–200 min. The reaction mixtures were subjected to descending paper chromatography in *n*-propyl alcohol–ammonia–water (55:10:35) to separate the oligomers of AMP (Lapidot and Khorana, 1963). Oligomers containing eight or more nucleotides remain at the origin. One-

Table I: Purification of Endoribonuclease.[a]

	Total Protein (g)	% of Total Activity Recovered	Increase in Specific Activity (*X*-fold)
Homogenate	100	100	1
Ammonium sulfate precipitate	46	96	2.1
Calcium phosphate elution	30	84	2.8
DEAE chromatography (pooled peak fractions)	2.7	68	25.2
Rate-zonal sedimentation (peak fractions)	0.041	32.2	782

[a] Poly(A) ribonuclease was purified as described under Methods and activity was determined by digestion of synthetic [^{3}H]poly-(riboadenylic acid)(62.5 mCi/mol). Specific activity was computed from picomoles of [^{3}H]poly(A) digested/microgram of protein during a 20-min incubation at 37° in buffer B by the Millipore binding assay described under Methods. The recovery of protein following rate-zonal sedimentation was extrapolated since only a percentage of the protein from the previous step was applied to the gradient step. This was one of five separate preparations with calculated increases in specific activity of the enzyme preparation varying from 460- to 820-fold above that of the 100,000g supernatant material.

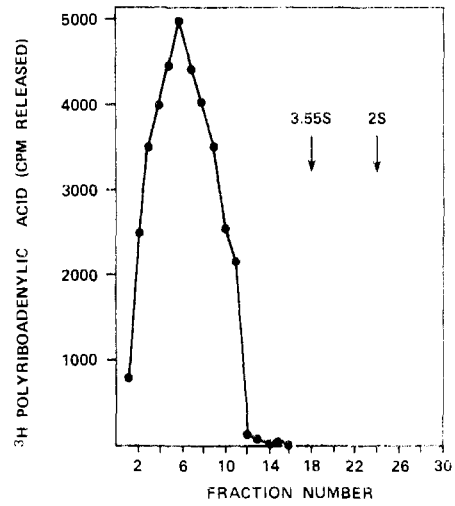

FIGURE 1: Sucrose density gradient purification of purified endoribonuclease. Four milligrams of dialyzed protein from the DE52 column chromatography fractions of highest specific activity was applied to a linear 5–20% sucrose gradient in 30 m*M* Tris-HCl (pH 7.4), 100 m*M* KCl, 5 m*M* $MgCl_2$, and 6 m*M* 2-mercaptoethanol, and centrifuged for 40 hr at 39,500 rpm in an SW56 rotor. An aliquot of each fraction was assayed for enzymatic activity as described under Methods, and activity is expressed as cpm [^{3}H]poly(riboadenylic acid) digested. Ovalbumin and myoglobin were used are marker proteins. In addition, the migration of the cAMP receptor protein (4.9 S) at fraction 14 was observed on parallel gradients.

centimeter strips were cut from the chromatography and assayed for radioactivity. 5′-AMP and adenosine markers were run with each sample.

Base Analysis. [^{32}P]Poly(A)-rich RNA was incubated for various times in buffer B with or without ribonuclease and adjusted to 0.5% SDS. Samples were then subjected to rate-zonal centrifugation through linear 5–20% sucrose density gradients containing 10 m*M* Tris-HCl (pH 7.6), 10 m*M* KCl, 0.5% SDS, in an SW56 rotor at 56,000 rpm for 360 min. The 4–6S regions of the gradients were collected, 20 μg of carrier tRNA was added, and RNA was precipitated by addition of 4 vol of cold ethanol. Aliquots were subjected directly to base analysis or were used to prepare poly(A) tracts for analysis. [^{32}P]RNA to be analyzed was dissolved in 0.3 *N* KOH and incubated at 37° for 28 hr. The samples were applied to Whatman No. 1 paper and subjected to high voltage electrophoresis in pyridine acetate buffer, pH 3.5 (Smith, 1967). High specific activity marker [^{32}P]tRNA was digested and subjected to electrophoresis in parallel and the position of Cp, Ap, Gp, and Up was determined by autoradiography. Appropriate regions of the papers were removed and counted in Liquifluor–toluene, and base composition was expressed as percentage of the total counts. Protein content was quantitated by the method of Lowry et al. (1951).

Results

Purification of the Endoribonuclease. Purification of an enzyme which digested synthetic poly(riboadenylic acid) (poly(A)), poly(cytidylic acid) (poly(C)) and poly(uridylic acid) (poly (U)) was accomplished as described under Methods. Using digestion of synthetic poly(A) as an assay, activity and recovery of enzymatic activity were determined. The ribonuclease was purified more than 500-fold with respect to specific activity (Table I). Following DEAE-cellulose chromatography, sedimentation of the peak fractions through linear sucrose density gradients separated the ribonuclease activity from most other proteins (Table I, Figure 1, and unpublished data). The ribonuclease was not purified to homogeneity; analysis of the most highly purified preparations on polyacrylamide gels (buffer system B of Rodbard and Chrambach (1971)) showed three or four bands of protein staining (Coomassie Brilliant Blue); activity was not recoverable from slices of these gels (data not shown). The enzyme was stable for at least 6 months when stored at −70° in buffer B containing 10 m*M* dithiothreitol and 20% glycerol.

Digestion of Synthetic Poly(A) by Purified Ribonuclease. The enzyme digested synthetic poly(A) in a time- and concentration-dependent manner. Digestion of poly(A) is observed whether decreased retention on Millipore filters or conversion to Cl_3CCOOH solubility is used as the criterion of digestion; the former method provided the more sensitive assay of digestion. Confirmation of degradation was obtained by sedimentation analysis of synthetic poly(A) and its digestion products (Figure 2).

Enzyme Properties. Addition of 20 m*M* EDTA to a reaction mixture containing no divalent cation diminished activity of the enzyme threefold but did not entirely abolish enzyme activity; however, the synthetic polynucleotide used in this experiment was not dialyzed against EDTA and traces of divalent cation may have been present in the reaction mixture. The response of enzymatic activity to variation of divalent cation concentration with natural or synthetic substrate was compared; this is illustrated in Figure 3. The enzyme exhibited a broad salt optimum (Figure 3) and its activity was essentially unchanged by alteration in pH between 6.5 and 9.5 (data not shown). Heating for 10 min at 60° was associated with a 60% decrease of activity whereas heating for 10 min at 90° produced an 85% loss. Preincubation with Pronase or trypsin (250 μg/ml) for 30 min at 37° produced >95% loss of enzymatic activity. Isoelectric focusing of the partially purified enzyme revealed p*I* = 8.3 ± 0.2 (Figure 4).

The ribonuclease was tested for activity on a number of potential substrates. All synthetic single-stranded ribonu-

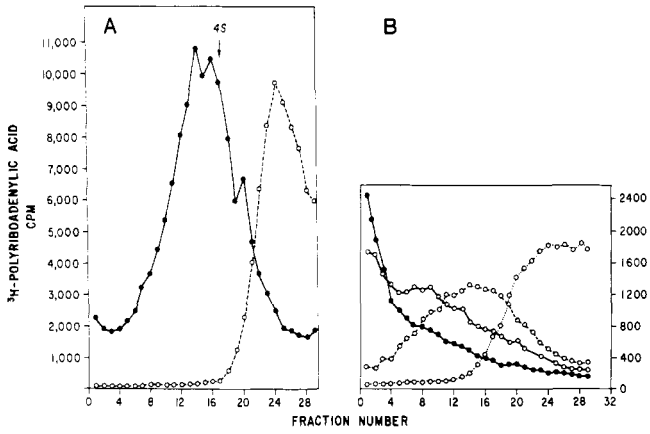

FIGURE 2: Sedimentation analysis of purified ribonuclease digestion of products of synthetic poly(A). Synthetic [^{3}H]poly(A) (62.5 Ci/mol) was incubated with enzyme (0.3 μg/ml) in buffer B at 37° for the times indicated below; the reaction mixtures were adjusted to 0.5% SDS, applied to a linear 5–20% sucrose density gradient containing 10 m*M* Tris-HCl (pH 7.6), 20 m*M* KCl, and 0.5% SDS, and centrifuged at 56,000 rpm in an SW56 rotor at 20°. Centrifugation was for 8 hr in (A) and 16 hr in (B). Fractions were collected from the bottom and counted in 10 ml of Bray's solution with >95% recovery of applied counts. Yeast tRNA was used as a marker. Sedimentation was from right to left. (A) (●—●) Untreated [^{3}H]poly(A) (100 μg); (O - - - O) [^{3}H]poly(A) (1 μg) + ribonuclease incubated for 5 min; (B) (●—●) untreated [^{3}H]poly(A) (0.5 μg); [^{3}H]poly(A) (10 μg) + ribonuclease, incubated: (O—O) 1 min; (O - - - O) 2 min; (O · · · O) 10 min.

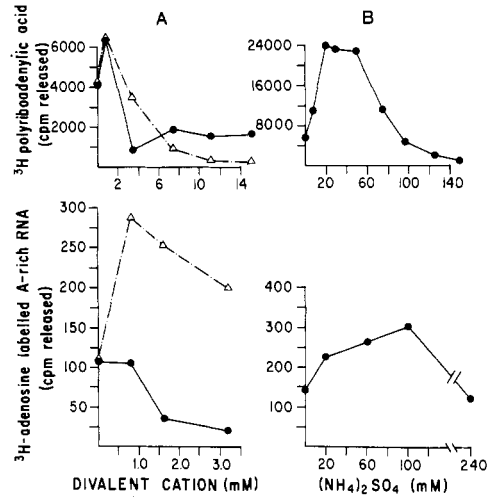

FIGURE 3: Divalent cation and salt requirements of purified ribonuclease digestion of poly(A) and poly(A)-rich RNA. In the assays in the upper panel, reaction mixtures containing enzyme (0.8 μg/ml) and 1.0 nmol of synthetic [^{3}H]poly(A) (62.5 Ci/mol) were incubated for 30 min at 37°. In the assays in the lower panel, reaction mixtures containing ribonuclease (0.08 μg/ml) and 100 ng of [^{3}H]uridylate poly(A)-rich RNA (10,000 cpm/μg) were incubated for 20 min at 37°. Incubations were terminated and processed via the Millipore binding assay described under Methods. All assays were performed in duplicate. (A) Divalent cation concentration was varied as indicated in a reaction mixture containing 30 m*M* Tris-HCl (pH 8.3), 20 m*M* $(NH_4)_2SO_4$. (Δ - - - Δ) Mn^{2+}; (●—●) Mg^{2+}. (B) Ammonium sulfate concentration was varied as indicated in a reaction mixture containing 30 m*M* Tris-HCl (pH 8.3) and 1.6 m*M* $MnCl_2$.

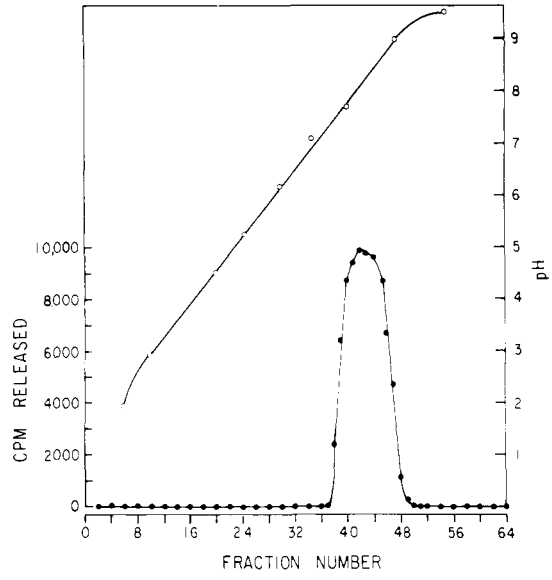

FIGURE 4: Isoelectric focusing of purified endoribonuclease. Two milligrams of enzyme purified through the DEAE-cellulose chromatography step was subjected to isoelectric focusing as described under Methods. An aliquot of each sample was assayed as described under Methods using 1 μg of synthetic [^{3}H]poly(A) (9 Ci/mmol) per assay; incubations were for 120 min at 37° and digestion was quantitated by the Millipore binding assay as described under Methods. (O) pH; (●) [^{3}H]poly(A) digested (cpm released).

cleotide homopolymers tested except poly(G) were digested by the enzyme preparation (Table II). Substrates insensitive to the enzyme included double-stranded RNA, single- or double-stranded DNA, and the RNA portion of DNA–RNA hybrids.

Mode of Cleavage. Several lines of evidence suggest that the enzyme acts as an endoribonuclease. Sedimentation of digestion products of synthetic poly(A) through sucrose density gradients reveals rapid accumulation of large digestion products with concomitant complete disappearance of the substrate (Figure 2A); with continued incubation, these digestion products are progressively cleaved into small oligonucleotides (Figure 2B). Analysis of digestion of synthetic poly(A) by retention on DE81 filters also confirms that <3% of the digested material represents oligonucleotides of less than six to eight bases (data not shown). Additional proof of an endonucleolytic mode of cleavage is provided by chromatographic analysis of digestion products under conditions where oligomers of more than eight nucleotides remain at the origin. Following even extensive enzymatic digestion of poly(A) as determined by the Millipore assay, only a small percentage of the radiolabeled material migrated as small oligonucleotides (Figure 5).

The site of cleavage was studied by chromatographic analysis of the products of *Crotalus adamanteus* venom phosphodiesterase digestion of the oligonucleotides generated by RNase digestion of synthetic poly(A). Oligonucleotides terminating in 3′-OH are sensitive to snake venom phosphodiesterase digestion, while those which terminate in 3′-phosphate are relatively resistant to cleavage by this enzyme (Razzell and Khorana, 1961). Snake venom phosphodiesterase converts the products of digestion of synthetic poly(A) by the purified ribonuclease in an essentially quantitative manner to 5′-AMP (Figure 5). No adenosine was found, indicating the absence of contaminating phosphatase activity in the ribonuclease preparation (Figure 5). The products of digestion of synthetic poly(A) were resistant to digestion by bovine spleen phosphodiesterase, as would be expected for oligonucleotides terminating in 3′-OH and 5′-phosphomonoesters (Hilmoe, 1960).

Digestion of Poly(A)-Rich RNA. The ability of the purified enzyme to utilize poly(A)-rich RNA as substrate was

Table II: Substrate Specificity of the Purified Endoribonuclease.[a]

Substrate	nmol Digested/mg of Protein
[^{3}H]Poly(riboadenylic acid)	6.42
[^{3}H]Poly(riboguanylic acid)	<0.01
[^{3}H]Poly(ribouridylic acid)	6.69
[^{3}H]Poly(ribocytidylic acid)	6.97
[^{3}H]Uridylate-labeled double-stranded RNA	<0.05
[^{3}H]Thymidylate-labeled double-stranded DNA	<0.01
[^{3}H]Uridylate-labeled DNA–RNA hybrid	<0.01
[^{3}H]Poly(A)·poly(dT) hybrid	<0.01

[a] Tritium-labeled synthetic polynucleotides (Miles Laboratories, Inc.) were adjusted to specific activity 50–70 Ci/mmol. [^{3}H]-Thymidylate double-stranded DNA (a generous gift from Dr. M. Goulian) had specific activity of 2.5 Ci/mmol. [^{3}H]Uridylate-labeled double-stranded RNA (t2 replicative form, a generous gift from Dr. T. Hunter, The Salk Institute) had specific activity of 0.3 Ci/mmol. [^{3}H]Poly(A)·poly(dT) hybrid was formed by hybridization of the polynucleotides in 0.2 *M* KCl and had specific activity of 2Ci/mmol. [^{3}H]Uridylate-labeled DNA–RNA hybrid was prepared as previously described (Miller et al., 1973) with specific activity of 1 Ci/mmol. Reaction mixtures contained 100 m*M* $(NH_4)_2SO_4$, 0.8 μg/ml of purified ribonuclease, and 8000–20,000 cpm of the indicated nucleic acid substrate. Incubations were for 240 min at 37° and were terminated by addition of 2 ml of 10% Cl_3CCOOH and 20 μg of carrier tRNA. The precipitate was collected by filtration through glass fiber filters and washed with 30 ml of 6% Cl_3CCOOH and 10 ml of ethanol, and the filter was placed in Liquifluor–toluene for counting. Digestion was estimated by decrease of Cl_3CCOOH-precipitable counts. In none of the assays did >60% digestion of substrate occur. Results represent the average of duplicate determinations differing by less than 4%.

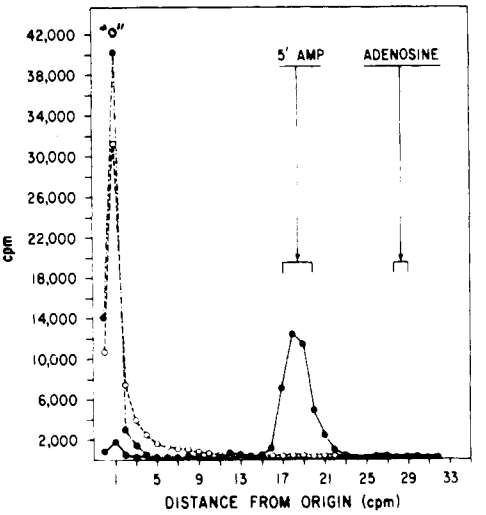

FIGURE 5: Chromatography of the digestion products of the purified ribonuclease. Reactions containing 0.6 μg of [^{3}H]poly(riboadenylic acid) (62.5 Ci/mol) with or without enzyme (0.1 μg/ml) were incubated at 37° for the times indicated below. Under these conditions, ribonuclease produced 80% digestion in 140 min as determined by the standard Millipore binding assay described under Methods. The reaction mixtures were chromatographed as described under Methods. Fraction 1 represents the origin and "5'-AMP" and "ADENOSINE" the position of those markers. (● — · — ●) [^{3}H]Poly(A) without added enzyme; (O - - - O) [^{3}H]poly(A) + ribonuclease for 140 min; (●—●) [^{3}H]poly(A) + ribonuclease, incubation for 140 min, addition of 75 μg of snake venom phosphodiesterase in 0.1 *M* glycine (pH 9.0), then incubation for an additional 5 min.

investigated. As measured by the Millipore assay, equivalent digestion of poly(A)-rich RNA and synthetic poly(A) occurred with 15- to 20-fold higher concentrations of enzyme required for the latter substrate; direct comparison is difficult, however, since in the Millipore assay cleavage of the poly(A) tract from poly(A)-rich RNA is detected with amplified sensitivity compared to digestion of other portions of the RNA. Products of very limited digestion of poly(A)-rich RNA by purified endoribonuclease were analyzed by rate-zonal sedimentation. As shown in Figure 6, digestion of [^{3}H]uridylate-labeled poly(A)-rich RNA by the purified ribonuclease caused a shift to regions of the gradient characteristic of lighter species; however, while digestion of [^{3}H]adenylate-labeled poly(A)-rich RNA was associated with a similar shift, another distinct class of digestion products migrating at approximately 4–6S was observed. These data suggested that, at least initially, preferential digestion of the poly(A) tract of the mRNA was occurring (with higher concentrations of enzyme or longer incubation times, widespread degradation of mRNA was observed); hence, base analysis of the 4–6S digestion product was undertaken. Limited digestion of [^{32}P]poly(A)-rich RNA was achieved using a low concentration of enzyme and short incubation times. The samples were then subjected to rate-zonal sedimentation as described under Methods. One to three percent of the total ^{32}P counts sedimented at 4–6S following digestion under the conditions described under Table III. Very limited digestion was associated with the appearance of material having a marked enrichment of adenylate content but clearly containing a significant percentage of other bases, indicating limited digestion of other portions of mRNA in addition to the poly(A) tract (Table III).

Discussion

Since the description of a poly(A) tract at the 3'-terminus of most eukaryotic mRNAs (Lee et al., 1971; Darnell et al., 1971a,b; Edmonds et al., 1971; Lim and Canellakis, 1971; Aviv and Leder, 1972; Comstock et al., 1972; Lai and Duesberg, 1972; Prescott et al., 1971; Armstrong et al., 1972; Adesnik et al., 1972; Yogo and Wimmer, 1972; Molloy et al., 1972), the regulation of its synthesis and degradation have been widely studied. Although function of the poly(A) tract is not yet completely defined, one proposed role involves transport of mRNA from nucleus to cytoplasm (Darnell et al., 1973). The observation that the poly(A) tract of lymphocyte mRNA was cleaved by a ribonuclease which copurified with the mRNA (Rosenfeld et al., 1972b, 1973) prompted an attempt to isolate a similar enzyme in quantities large enough to permit study.

Identification and partial purification of an endoribonuclease which digests certain homoribopolymers and naturally occurring RNAs are described in this report. The enzyme was purified greater than 500-fold with respect to specific activity. This enzyme generated oligonucleotides with 3'-hydroxyl and 5'-monophosphoester termini. Polymers were hydrolyzed at a relatively rapid rate to oligonucleotides varying in chain length, but digestion products containing less than six to eight nucleotides were observed only following prolonged digestion. With synthetic poly(A) as substrate, the ribonuclease demonstrated a marked stimulation of activity by manganous ion, and an absolute requirement for divalent cation was not rigorously excluded. The ability of the enzyme to digest poly(A)-rich mRNA in addition to all single-stranded homoribopolymers except poly(G) suggests a lack of specificity of nucleotide requirements for cleavage. Digestion of double-stranded RNA, DNA, or DNA–RNA hybrids was not detected.

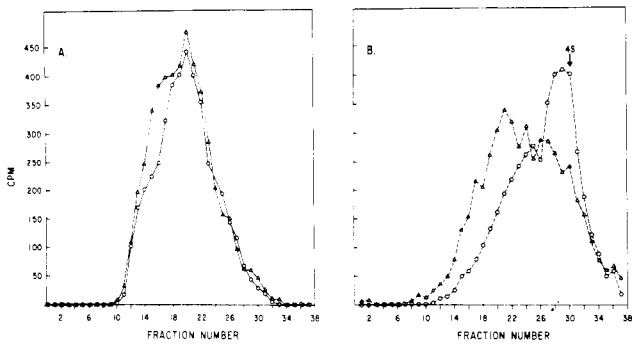

FIGURE 6: Sedimentation of digestion products of [^{3}H]adenylate- or [^{3}H]uridylate-labeled poly(A)-rich RNA. [^{3}H]Adenylate- or [^{3}H]uridylate-labeled poly(A)-rich RNA was prepared as described under Methods. Aliquots containing 5000 cpm of poly(A)-rich RNA (specific activity 120,000 cpm/μg) were incubated for 3 min at 37° in the absence or presence of purified ribonuclease (0.08 μg/ml). Incubations were terminated by adjusting to 0.5% SDS. The samples were then applied to 5–20% linear sucrose gradients containing 10 m*M* Tris-HCl (pH 7.6), 20 m*M* KCl, 0.5% SDS, and centrifuged at 56,000 rpm for 200 min in an SW56 rotor. Yeast tRNA was used as marker. Sedimentation was from right to left. (A) Untreated poly(A)-rich RNA. (Δ—Δ) [^{3}H]uridylate-labeled poly(A)-rich RNA; (O—O) [^{3}H]adenylate-labeled poly(A)-rich RNA. (B) Poly(A)-rich RNA + ribonuclease. (Δ - - - Δ) [^{3}H]uridylate-labeled poly(A)-rich RNA; (O - - - O) [^{3}H]adenylate-labeled poly(A)-rich RNA.

Table III: Nucleotide Composition of the 4–6S Digestion Product.[a]

Nucleotide	Poly(A)-Rich RNA (% of total cpm)	Poly(A) Tract (% of total cpm)	4–6S Digestion Product: Incubation Time 1 min	4–6S Digestion Product: Incubation Time 5 min
Cp	21.7	1.7	6	12
Ap	30.8	97.1	83	63
Gp	22.6	0.6	1	7
Up	25.6	0.6	10	13

[a] [^{32}P]Poly(A)-rich RNA (200,000 cpm) was incubated with purified ribonuclease (0.03 μg/ml) at 37° in buffer B for 1 or 5 min, subjected to rate-zonal sedimentation for 360 min, and the 4–6S regions were pooled (see Methods). Centrifugation followed by base analysis of material in the 4–6S region with no incubation revealed cpm in C, A, G, and U of 388, 1464, 424, and 621, respectively, and represented 30–40% of counts observed after incubation; these background values were subtracted for computation of nucleotide composition shown above. Isolated poly(A) tracts were prepared as described under Methods. Results of base analysis of total poly(A)-rich RNA, isolated poly(A) tracts, and the 4–6S digestion products are averages of duplicates differing by less than 3%.

Many of the characteristics of the enzyme are similar to an endoribonuclease first purified from rat liver by Heppel (1966) and subsequently identified in nuclei of other tissues during purification of a processive exoribonuclease (Lazarus et al., 1967; Sporn et al., 1969). In addition, it has properties similar to a nuclease purified from *Azotobacter agilis* (Stevens and Hilmoe, 1960).

The extensive purification of an endoribonuclease which digested synthetic poly(riboadenylic acid) (among other substrates) permitted study of the digestion of poly(A)-rich RNA by the enzyme. Although extensive degradation of mRNA was consistently observed during prolonged digestion, incubation of mRNA with low concentrations of the enzyme and short incubation times was associated with appearance of a 4–6S digestion product having a high adenylic acid content. The presence of this A-rich segment suggests an initial preferential cleavage of the poly(A) portion of mRNA by the enzyme, although it cannot be determined whether this results from a specific cleavage point at or near the 5′-terminus of the poly(A) tract or from relative resistance of the poly(A) to enzymatic degradation, allowing its recovery intact with very limited digestion. The pH and salt optima and the profiles of activity with varying divalent cation concentration for this enzyme using poly(A)-rich RNA as substrate are similar to those of a ribonuclease copurified with lymphocyte poly(A)-rich RNA which cleaves only the poly(A) tract (Rosenfeld et al., 1972b, 1973). Moreover, a specific protein inhibitor of the digestion of poly(A)-rich RNA by both the endonuclease described herein and the copurified ribonuclease has been identified and purified from guinea pig adrenal cortex ribosomal eluate (Perkins et al., 1974). Further purification to homogeneity of the ribonuclease described here will be required to ascertain whether congruence exists between it and the ribonuclease copurifying with the poly(A)-rich mRNA.

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Photoexcited Bacterial Bioluminescence. Identity and Properties of the Photoexcitable Luciferase†

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ABSTRACT: Properties of photoexcitable luciferase are compared with those of luciferase, both isolated from the bacterium *Beneckea harveyi*. The proteins have the same molecular weight, are similarly charged at pH 8, and can be inactivated, with comparable efficiencies, by antibodies against either pure luciferase (a heterodimeric protein) or individual subunits thereof. Compared with luciferase, photoexcitable luciferase has a broader pH range for optimal activity, is more stable under acidic conditions, is less stable under alkaline conditions, and is more resistant at neutral pH to inactivation by heat, urea, and trypsin. A flavine-like chromophore, designated B, can be isolated from photoexcitable luciferase. The binding of B to luciferase restores all the properties characteristic of photoexcitable luciferase. Moreover, photoexcitable luciferases from mutants selected to have heat labile luciferases are also thermally unstable. It is concluded that photoexcitable luciferase actually consists of a luciferase-B complex which is conformationally distinct from luciferase under certain conditions.

Bacterial luciferase (L)[1] catalyzes the bioluminescent oxidation of $FMNH_2$ and a long chain aldehyde by molecular oxygen (Hastings, 1968; Eberhard and Hastings, 1972). This enzyme (mol wt 79,000) consists of two nonidentical subunits, α and β, with the active center located on the α subunit (Meighen et al., 1971a,b; Cline and Hastings, 1972). Luciferase catalyzes the bioluminescent reaction by reacting sequentially with $FMNH_2$, O_2, and aldehyde to form a series of enzyme intermediates (Hastings and Gibson, 1963; Hastings et al., 1973). The enzyme-bound $FMNH_2$ and aldehyde are believed to be oxidized by the oxygen to generate an excited enzyme intermediate (enzyme-flavine*), and ultimately the products: a long chain carboxylic acid, FMN, H_2O, and light (Eberhard and Hastings, 1972; Shimomura et al., 1972; Dunn et al., 1973).

An interesting feature of bacterial bioluminescence is the existence of two types of in vitro luminescence activity attributable to two distinct protein species. The normal activity is initiated by adding $FMNH_2$ to L in the presence of aldehyde and oxygen. Light emission can also be induced by flash irradiation of photoexcitable luciferase (PL) in the absence of FMN or $FMNH_2$ (Gibson et al., 1965; Mitchell and Hastings, 1970). Both reactions are oxygen- and aldehyde-dependent and utilize these substrates in the same sequence. Both have the same rates for the decay of light when tested with aldehydes of different chain length and emit at the same wavelengths. In contrast to the similarities observed in the two luminescence reactions, the two proteins differ in that PL migrates slightly faster than L on DEAE columns at pH 6.25 and pH 7 and is more resistant to sulfhydryl group modification and heat denaturation than L. The PL contains a flavine-like chromophore designated B while L can be isolated chromophore free.

The flavine-like chromophore B could be dissociated from PL by treating the latter with urea or guanidine. The addition of B to L restored the PL activity, with a concomitant loss of L activity, and changed the chromatographic behavior of L on DEAE-cellulose to a pattern similar to PL. Based on these observations, Mitchell and Hastings (1970) proposed that PL is actually the bacterial luciferase-B complex. Nevertheless, the identity of PL still remained un-

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[1] Abbreviations used are: L, bacterial luciferase; PL, photoexcitable luciferase; LB, reconstituted luciferase-B complex; TSL-mutant, mutant with temperature-sensitive luciferase.