Endoribonuclease VII, a novel endoribonuclease from calf thymus, was identified and purified by us. The purified enzyme has $M_r = 74,000$; its homogeneity was checked by analysis in polyacrylamide gels (both in the presence and in the absence of sodium dodecyl sulfate). The nuclease cleaves poly(U) and poly(C) while other single-stranded homopolyribos- as well as polydeoxyribonucleotides are not degraded; poly(A,C) is hydrolyzed to a smaller extent, while poly(U), poly(A) is not degraded at all. Poly(A) modulates the poly(U)-degrading activity; at a molar ratio of approximately 1 [poly(A)]:10 [poly(U)], a more than 100% stimulation of the enzyme activity was achieved, while at lower ratios an almost complete inhibition of the enzyme activity resulted. Binding studies revealed that endoribonuclease VII has a marked affinity for poly(A) and poly(U). During hydrolysis, oligo(U)$_{12}$ fragments with $3'$-OH and $5'$-P termini are formed. The basic enzyme (pI = 8.5) has its activity optimum at pH 7.2, requiring neither monovalent nor divalent cations; the enzyme is not inhibited by thiol group reagents. Several lines of evidence suggest a role of endoribonuclease VII in mRNA processing are presented.

Most mRNA molecules carry on their $5'$ termini a cap structure (reviewed in Ref. 1) and on their $3'$ termini a poly(A) sequence (reviewed in Ref. 2). These two segments of mRNA are synthesized by template-independent enzymes. Poly(A) synthesis is mediated by a poly(A) polymerase (3) which is assumed to exist in several forms (4). The size of poly(A), which varies between 50 and 225 AMP units (reviewed in Ref. 5), is determined by the activities of poly(A)-polymerizing enzymes (poly(A) polymerase(s), Ref. 6) and poly(A)-hydrolyzing enzymes (endoribonuclease IV, Ref. 7; poly(A)-specific exoribonuclease, Ref. 8; and perhaps endoribonuclease V, Ref. 9). However, little is known about the regulatory mechanisms which must exist to control the activities of the poly(A) anabolic and poly(A) catabolic enzymes during the polyadenylation of hnRNA. Evidence is presented that this control may be performed first by poly(A)-binding proteins (10, 11), second by tubulin and G-actin (12), third by poly(A)-hydrolyzing enzymes (endoribonuclease IV, Ref. 6) and poly(A) polymerase(s), Ref. 7; fourth by modified nucleotides in the poly(A) tract (13), and fifth by thiol group reagents; the enzyme is not inhibited by thiol group reagents. Several lines of evidence suggest a role of endoribonuclease VII in mRNA processing are presented.

**EXPERIMENTAL PROCEDURES**

Materials—Materials were obtained as follows. [H]Poly(U) with an average $M_r = 45,000$ (= 135 UMP units) was determined according to Studier (22) (specific activity, 15 mCi/mmol of phosphate); [H] poly(C) (15 mCi/mmol), [H]poly(G) (15 mCi/mmol), [H]poly(A) (37.4 mCi/mmol), [H]poly(U) (22 mCi/mmol), poly(U)$_{25}$ (19.3 mCi/mmol), poly(A)$_{25}$ (21 mCi/mmol), oligo(A)$_{12}$ [poly[(methyl-$^3$H)dT]] (7 mCi/mmol), poly(G)$_{12}$ and poly(A)$_{12}$ were from Miles Laboratories, Inc., Elkhart, IN; bovine serum albumin, phosphodiesterase I (from Crotalus durissus terrificus, 1.5 units/mg; EC 3.1.4.1), phosphodiesterase II (from spleen, 2 units/mg; EC 3.1.16.1), polyribo- as well as polydeoxyribonucleotides, ribonucleic acid (bovine pancreas; specific activity, 50 Kunitz units/mg), 5 S, 16 S, and 23 S, RNA (from Escherichia coli), ferritin, and marker proteins were from Boehringer Mannheim, Mannheim, Germany; GF/C filters were from Hormuth and Vetter, Heidelberg, Germany; Aquasol DNA (thymidine-methyl-$^3$H-labeled from E. coli; 0.1 mCi/µg), and tRNA ($^14$C-labeled from E. coli; 25 mCi/mg) were from New England Nuclear, Dreieichenhain, Germany; Sephadex G-15, Sephadex G-50, Sephadex G-100, and dextran blue were from Deutsche Pharmacia, Freiburg, Germany; Amphotile (pH 3.5–10) was from LKB Bromma, Sweden; DEAE-cellulose (type 23 SS), phosphocellulose (type P 30), and Stains All were from Serva, Heidelberg, Germany; (dA)$_{12}$, (dA)$_{12}$, and poly[(methyl-$^3$H)dc] (2.8 mCi/mmol) were prepared in P-L Biochemicals, Milwaukee, WI; nitrocellulose filters (HAWP 0.45 µm) from Millipore, Bedford, MA. Oligo(A)$_{12}$ was prepared from poly(A) by a controlled hydrolysis under alkaline conditions (23). Poly(dA)$_{12}$ and poly[(H)poly(dA)] (specific activity, 42 mCi/mmol) were prepared enzymatically (24).

The abbreviations used are: GF/C, glass fiber discs; C, RNP, ribonucleoprotein; snRNP, small nuclear ribonucleoprotein.

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1. Polynucleotides and nucleotides are abbreviated according to the recommendations of the Commission on Biochemical Nomenclature (57).

2. The abbreviations used are: GF/C, glass fiber discs; C, RNP, ribonucleoprotein; snRNP, small nuclear ribonucleoprotein.
**Endoribonuclease VII Assay**—Unless stated otherwise, all reactions were carried out in a final volume of 100 μl at 37 °C for 10 min. Each reaction mixture contained 100 mM Tris-HCl (pH 7.2), 2.5 mM MgCl₂, 20 mM EDTA, 400 mM urea, 50 μg of bovine serum albumin, and 8 nmol of [³H]poly(U) (specific radioactivity, 40,000 dpm/nmol)/90 μl, and 10 μl of the enzyme preparation (containing 3 enzyme units in maximum). For determination of enzymatic activity, 40-μl aliquots were routinely taken and acid-insoluble material was collected, applying the GF/C filter technique (25). The details of these procedures were given earlier (26). 40% of [³H]poly(U) was found to be bound at time 0 of incubation to the filters. Determinations revealed that oligo(U)₂₀₆ fragments were not bound to GF/C filters. Under these assay conditions, the reaction velocity increased linearly during the first 15 min. One unit of enzyme is defined as the amount which converts 1 nmol of [³H]poly(U) into an acid-soluble form during a 10-min period under the standard conditions.

**Phosphodiesterase Assays**—Phosphodiesterase assays were composed basically as described (27). 0.4 nmol of [³H]oligo(U) (1.4 × 10⁶ dpm) were incubated (37 °C, 10 min) in a reaction volume of 200 μl with 5 μg of phosphodiesterase I in a 200 mM Tris-HCl buffer (pH 8.8, 10 mM MgCl₂). 40-μl aliquots were taken at 0 and 10 min, and analyzed by gel chromatography on Sephadex G-50 (9). The monoribonucleotides appeared at a Vₜ/Vₘ of 4.0–4.4, while the oligoribonucleotides eluted at a Vₜ/Vₘ of lower than 3.5. The phosphodiesterase II assay contained 0.4 nmol of [³H]oligo(U), 5 μg of phosphodiesterase II in a 2.2 mM sodium succinate HCl buffer (pH 6.5). Incubation was performed at 37 °C and the product was analyzed with respect to the amount of oligoribonucleotides and monoribonucleotides (9).

**Binding Assay of Nucleic Acids to Endoribonuclease VII**—Complex formation between nucleic acids and endoribonuclease VII was determined applying the nitrocellulose filter binding technique of Jones and Berg (28). The binding mixture (total volume, 100 μl) contained 20 mM Tris-HCl (pH 7.5), 300 mM KCl, 2 nmol of labeled RNA or DNA, and 1.2 μg of enzyme protein. After incubation (5 min, 37 °C), the samples were placed onto the filters and washed with 30 ml of the binding buffer supplemented with 1% dimethyl sulfoxide. The radioactivity retained on the filters was determined. In the control assays, the enzyme was replaced by 5 μg of bovine serum albumin.

**Analytical Methods**—Protein concentration was determined spectrophotometrically at 230 and 260 nm (29).

The sedimentation coefficient of endoribonuclease VII complex was determined by velocity gradient centrifugation as described (30). The enzyme samples were applied to 10–30% sucrose gradients (in a 10 mM Tris-HCl buffer, pH 8.0, supplemented with 10 mM NaCl and 400 mM urea) and centrifuged for 4 h at 4 °C and 40,000 rpm in an SW 41 rotor.

Analytical isoelectrofocusing was carried out on a 15-mL column. After dialysis against 0.9% NaCl, 4 mM urea, 2 mM dithiothreitol, and 5% (w/v) sucrose, samples of 300–400 μg of protein were added in the middle of a linear 5–50% sucrose gradient (9 ml) containing 5% carrier ampholytes (pH 3.5–10), 1 mM dithiothreitol, 4 mM urea, and 100 μg of ferritin as marker. Focusing was performed at 4 °C for 19 h with 300-V constant voltage and for 1 additional h with 400 V. As buffer systems, 10 mM phosphoric acid (anode) and 20 mM NaOH (cathode) were used. Fractions of 0.4 ml were collected and the pH of each fraction was measured at 4 °C. Endoribonuclease VII activity of each fraction was determined in the standard assay.

Preparative isoelectrofocusing was carried out on a 60-ml column and a 50-ml linear sucrose gradient under otherwise identical conditions as described for the analytical procedure. After focusing, fractions of 2 ml were collected.

The chain length of the reaction product was determined by gel chromatography using Sephadex G-50 (9). A column (1.1 × 34.5 cm) was equilibrated with 100 mM Tris-HCl (pH 7.5) and 2 mM urea. After application of 1 ml samples, fractions of 0.45 ml were collected and counted in 10 ml of Aquasol each.

The nature of the monoribonucleotides- and nucleotides was determined by ion exchange chromatography using DEAE-Sephadex A-25 (9, 31). Applying a bicarbonate buffer, the monomers appeared at the following mobility of the marker: UT 0.06 M; P-2'-5'-UT, 0.18 M; 5'-UMP, 0.26 M; and 3'-UMP, 0.32 M. Electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the method of Weber and Osborn (32). Protein samples were heated for 5 min at 100 °C in the presence of sodium dodecyl sulfate and mercaptoethanol. A 30-μg sample of protein was analyzed on 10% gels. The gels were stained with Coomassie brilliant blue.

Polyacrylamide gel electrophoresis (10% separation gel, 6% spacer gel) was performed in tubes in the presence of 6 mM urea (33), using a 120 mM acetic acid, 300 mM β-alanine (pH 4.3) buffer system. Prior to electrophoresis, the enzyme samples were dialyzed (12 h, 2 °C) against the electrophoresis buffer (pH 4.3, supplemented with 6 mM urea). The gels were stained with Coomassie brilliant blue. In order to detect endoribonuclease VII activity in situ, the 7.4-cm gels were cut into 2-mm slices. Each slice was incubated in 200 μl of endoribonuclease VII incubation mixture for 30 min at 37 °C under shaking; after incubation, acid-insoluble material was collected using the GF/C filter technique (7).

**RESULTS**

**Purification of the Enzyme**

All procedures were carried out at 0–4 °C unless stated otherwise; a summary of the steps is given in Table I.

**Step 1: Crude Extract**—200 g of frozen calf thymus from 2-month-old animals were cut into pieces, thawed, and suspended in 1 liter of 0.2 M NaCl, 5 mM MgCl₂, 10 mM Tris-HCl, pH 8.0. The suspension was mixed in a Waring Blender for 10 min and subsequently homogenized in an Ultra Turrax (Janke-Kunkel, Staufen) for 15 min. After standing for 30 min, the extract was centrifuged for 15 min at 12,000 × g. About 950 ml of supernatant fraction (fraction I) with 13.3 mg/ml of protein were obtained. The ratio A₅₀₋₂₈₀ was 0.77.

**Step 2: Ammonium Sulfate Precipitation**—Fraction I (950 ml) was brought to 0.45% saturation with solid ammonium sulfate under stirring and allowed to stand for 60 min. The supernatant was collected by centrifugation (15 min; 12,000 × g) and ammonium sulfate was added again to bring the solution to 0.75% saturation. The precipitate formed was obtained by centrifugation (15 min; 12,000 × g), then dissolved in 50 ml of 100 mM Tris-HCl (pH 8.0; 5 mM MgCl₂, 10 mM D-galactose), and dialyzed for 24 h against 100 mM Tris-HCl.

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**Table I**

| Purification step | Total protein | Total enzyme activity | Specific activity | Purification |
|------------------|---------------|-----------------------|------------------|-------------|
|                  | mg            | units × 10⁻⁴          | units × 10⁻⁴/mg  | %  fold     |
| Fraction I. Crude extract | 12,613        | 391.0                 | 3.1              | 0.19        | 100       | 1           | 1            |
| Fraction II. Ammonium sulfate | 2,183        | 255.0                 | 11.7             | 65          | 3.8        |
| Fraction III. Sephadex G-150 | 764           | 190.2                 | 24.9             | 49          | 8.0        |
| Fraction IV. DEAE-cellulose | 205          | 142.1                 | 69.3             | 36          | 22.4       |
| Fraction V. Phosphocellulose | 37            | 42.5                  | 114.8            | 11          | 37.0       |
| Fraction VI. Preparative isoelectrofocusing | 0.38          | 4.58                  | 1,205.4          | 1.2         | 389        | 6,344      |
Poly(A)-modulated, Pyd-specific Endoribonuclease VII

Polyribonuclease VII (Endoribonuclease VII) is a protein isolated from rat liver that degrades polyadenylate (Poly(A)) chains specifically by action on the non-Pyrimidine (Py) residues within the Poly(A) chain. The enzyme catalyzes the cleavage of Poly(A) at Py residues to produce oligonucleotides.

**Purification Procedures**

1. Extraction: The enzyme was extracted from rat liver by homogenization in a buffer containing 10 mM Tris-HCl, 10 mM MgCl₂, 10 mM d-galactose, and 1 M urea.

2. Gel Filtration: The extract was passed through a G-150 gel filtration column to remove large protein aggregates.

3. Isoelectric Focusing: The enzyme was further purified by isoelectric focusing in a sucrose density gradient in the presence of carrier ampholytes in the range of 3.5-10 as described under "Experimental Procedures." The enzyme activity was determined by the standard assay containing 1 X 10⁵ to 1 X 10⁶ units of Poly(A).

4. DEAE-Cellulose Fractionation: The enzyme was further purified by DEAE-cellulose chromatography.

5. Phosphocellulose Adsorption: The enzyme was further purified by phosphocellulose chromatography.

6. Poly(U)-degrading Enzyme Activity: The enzyme activity was determined by the standard assay containing 1 X 10⁵ to 1 X 10⁶ units of Poly(A).

**Characterization of the Enzyme**

- The enzyme has a specific activity of 2000 units/mg.
- The enzyme is active at pH 8.5 and has a PI of 4.1.
- The enzyme is resistant to EDTA and MgCl₂.
- The enzyme is sensitive to sodium dodecyl sulfate (SDS).

**References**

1. Ref. 1
2. Ref. 2
3. Ref. 3
4. Ref. 4
5. Ref. 5
6. Ref. 6
7. Ref. 7
8. Ref. 8
9. Ref. 9

**Figures**

- Fig. 1: Characterization of the products released from [³H]poly(U) by endoribonuclease VII. According to a described method (9), the products were separated on a Sephacryl S-50 column (1 X 30 cm) in the presence of 2 M urea. Fractions of 1.5 ml were collected and the radioactivity was determined. The arrows mark the positions of the authentic compounds. [³H]Poly(U) was incubated in the standard assay containing 1 X 10⁵ to 1 X 10⁶ units of endoribonuclease VII for 10 min at 37°C. The reaction products were separated by preparative isoelectric focusing with 310 ml of fraction IV. Fractions of 1 ml were collected. After washing with 3 column volumes of the equilibration buffer, the poly(U)-degrading enzyme was eluted from the column with the equilibration buffer, supplemented with 100 mM NaCl. Fractions of 2 ml were collected during elution. Each fraction was assayed for protein (A₂₈₀·A₂₆₀ ratio changed to greater than 1.5, indicating that most nucleic acids have been removed (35)). The specific poly(U)-degrading enzyme activity was determined to be 6930 units/mg.

- Fig. 2: Purification of endoribonuclease VII. A, purification on phosphocellulose. A column (1.5 X 16 cm) equilibrated with 50 mM Tris-acetate (pH 4.2; 5 mM MgCl₂, 15% (v/v) glycerol) was loaded with 310 ml of fraction IV. Fractions of 6 ml were collected. After washing with the equilibration buffer, the poly(U)-degrading enzyme activity was eluted with 300 mM Tris-HCl (pH 8.0; 5 mM MgCl₂) (arrow). 10-pl aliquots were assayed for enzyme activity (O-O) and protein content (O-O). B, final purification by preparative isoelectric focusing. 37 mg of fraction VI were subjected to preparative isoelectric focusing in a sucrose density gradient in the presence of carrier ampholytes in the range of 3.5-10 as described under "Experimental Procedures." The enzyme activity was determined in the standard assay at pH 8.5 (O-O; pH gradient, O-O). The enzyme with a basic pl (fractions 28-30) is termed endoribonuclease VII (pl 8.5 enzyme) and that with an acidic pl (fractions 16-21) is the pl 4.1 enzyme.
The purification of endoribonuclease VII (pI 8.5 enzyme) was about 390-fold and that of the pI 4.1 enzyme about 270-fold after six purification steps, if the calculation was based on the overall poly(U)-degrading activities which are present in the crude extract. Because the crude extract contained besides endoribonuclease VII additional poly(U)-degrading enzymes, e.g. phosphodiesterase, ribonuclease A (EC 3.1.27.5), and endoribonuclease V, we calculated the degree of purification in a second way as follows. The specific activity of the purified enzyme was related to that portion of enzyme activity in the crude extract, which was measured after electric focusing around the characteristic pI range (pH 8.2–9.2) of the endoribonuclease VII (in Table I termed pI 8.5 activity). In the crude extract, 6.1% of the total poly(U)-degrading enzyme activity was localized within a pI range of 8.2–8.8. A calculation on this basis revealed a 6340-fold purification of endoribonuclease VII (Table I). The calculation of the yield of the purification of the enzyme, which amounts to 1.2%, is based on the overall poly(U)-degrading activity and the total protein content, present in the crude extract.

Stored in 50% (v/v) glycerol, Fraction VI was stable for more than 5 months without measurable change of its enzyme activity. Freezing of the enzyme in the absence of glycerol at -20 °C resulted in a 85% loss of activity.

**Enzymic Contamination**

The purified endoribonuclease VII was free of nucleotidase and exoribonuclease activity. This conclusion must be drawn from experiments in which the nature of the oligo(U) products formed by this enzyme was determined. As summarized later, only oligo(U)_{n<14} fragments with 3'-OH and 5'-phosphate termini were detected as end products during the reaction with step VI enzyme in the absence of EDTA. The possible existence of a phosphodiesterase in the preparation can also be excluded, because no DNA-degrading activity was detectable (Table II).

**Properties**

If not stated otherwise, the experiments were performed with Fraction VI.

**Sedimentation Property and Molecular Weight**—Fraction VI of endoribonuclease VII was analyzed by velocity sucrose gradient centrifugation (Fig. 3). The enzyme was recovered in the top fractions (58%) and in the bottom fractions (42%); the latter had a sedimentation coefficient of approximately 45 S.

The two activity fractions (bottom fraction and top fraction) were analyzed by gel electrophoresis both under denatured and native conditions. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed (Fig. 4A) the bottom fraction (lane b) to be composed of six major proteins (one with an M_r = 74,000, which comprised about 5% of the total protein and five species with M_r < 30,000) while the top fraction (lane a) contained only one protein species with M_r = 74,000. That protein component in the preparations that was associated with the endoribonuclease VII activity was successfully identified after separation in urea-polyacrylamide gels (Fig. 4B). After separation of the bottom fraction (lane b), two main bands were detected in the separation gel (R_S 0.05 and 0.85); approximately 40% of the protein migrated into the separation gel. In contrast, in the top fraction, only one band had been visualized after electrophoresis (lane a), which had the same relative electrophoretic mobility as the fast migrating band from the bottom fraction. The in situ determination of nuclease activity in urea gels revealed (Fig. 4C) that the two protein bands in the separating gel from the bottom fraction and the single band from the top fraction coincide with the nuclease activity.

From these findings, the hint is taken that the enzyme which was present in the top fractions after sucrose gradient centrifugation was purified to homogeneity. Its apparent M_r = 74,000. It is presently assumed that the second nuclease-associated band identified in urea gels (R_S 0.05) represents a relatively stable protein complex.

**Isoelectric Point**—The pI of fraction VI of endoribonuclease VII was determined in a 15-ml sucrose density gradient, containing 4 M urea. The enzyme activity was recovered in a single sharp peak at a pH of 8.5. The enzyme activity coincides with the protein band (not shown).

**Substrate Specificity**—The specificity of endoribonuclease VII was tested in assays using different natural and synthetic polyribonucleotides as substrates (Table II). The enzyme was found to hydrolyze preferentially poly(U) and poly(C) and, with a slightly lower affinity, poly(A,C) as well. No other
Poly(A)-modulated, Pyd-specific Endoribonuclease VII

Single-stranded deoxyribo- and ribopolymers were degraded. Poly(U) hybridized to poly(A) was not a substrate for the enzyme. In a 1:1 molar mixture between poly[^H]U and poly(A), the poly(U) substrate was hydrolyzed only to 13% (Table II). This result already indicates that poly(A) inhibits the endoribonuclease VII activity. A more detailed description of this outcome is given later. DNA was not hydrolyzed, while tRNA was degraded with an efficiency of 44%, if compared with poly(U) (100%).

Kinetics of Poly(U) Degradation—Under the experimental conditions used (addition of 1.3 enzyme units), the kinetics of poly(U) degradation during the first 15 min was linear and without a lag phase (Fig. 5A); then the curve leveled off when high molecular weight substrate was exhausted.

The Michaelis constant with poly(U) as substrate (2 to 10 mM phosphate in the reaction mixture) was found to be 11.3 mM with respect to phosphate. The maximal velocity was 13.2 nmol of acid-soluble material/µg protein × min.

The molecular activity of the enzyme estimated from the results described in Fig. 1 shows that 1.4 enzyme units (= 12 ng of enzyme complex, corresponding to 1 ng of the 74,000-Da enzyme protein) degrades 1 nmol of poly(U)_{380} (with respect to phosphate) or 7.5 pmol of poly(U)_{380} (with respect to entire polymer) during an incubation period of 10 min to oligo(U)_{12} fragments. This means that 8.4 × 10^9 enzyme molecules have cleaved approximately 4.9 × 10^{13} phosphodiester bonds within 10 min, or that 1 molecule of endoribonuclease VII caused 180 cleavages/min under the experimental conditions.

Product Analysis—Endoribonuclease VII degraded [^H]poly(U) to oligo(U) fragments. These are characterized by a V_c/V_o ratio (34) of 2.26 ± 0.25 after Sephadex G-50 separation (Fig. 1). The average chain length of the fragments was estimated graphically, using the semilogarithmic plot procedure, in which the chain lengths of the oligoribonucleotides are correlated with their V_c/V_o values. Calibration was performed with marker oligoribonucleotides (9). By this approach, the oligo(U) fragments were determined to consist of 11.5 ± 2.3 UMP residues.

The chemical nature of the oligo(U) fragments at their 3' and 5' termini was determined enzymatically using phosphodiesterases I and II. Oligo(U) fragments were formed during incubation of [^H]poly(U) with 12 units of endoribonuclease VII for 60 min. Subsequently the samples were heated (2 min, 95 °C) to destroy the nucleic activity and the reaction products were separated on Sephadex G-50 (Fig. 1). Fractions 14–19 (V_c/V_o, 1.84–2.5) were taken, pooled, and concentrated in a rotary evaporator (at 40 °C) to 100 µl. The residue was desalted by a Sephadex G-15 gel filtration column (0.8 × 12 cm) and the oligoribonucleotides were collected. 0.4 nmol of oligo(U) was analyzed in both the phosphodiesterase I and II assays (see “Experimental Procedures”). After incubation, 94% of the oligomers were converted to monoribonucleotides after incubation with phosphodiesterase I, while only 2% were degraded in the presence of phosphodiesterase II (Table III).

The nature of the monoribonucleotides, released after incubation with phosphodiesterase I, was analyzed by ion exchange chromatography (see “Experimental Procedures”) and determined to be to 96% 5'-UMP. These data prove that the oligo(U)_{11} products, formed by endoribonuclease VII are 3'-hydroxy-terminated and 5'-phosphate-terminated oligoribonucleotides (27), and indicate that this enzyme is a 3'-endoribonuclease.

Effect of Enzyme Concentration—Poly(U) degradation was proportional to the enzyme concentration within a 0.005–0.02-µg range in the standard assay containing 8 nmol of poly(U). Again, at higher concentrations, the rate decreased due to the limitation of the substrate (Fig. 5B).

Effect of pH and Selected Compounds—The pH optimum was determined in a 50 mM Tris-acetate buffer system between pH 4.0 and 8.0. The maximal activity was measured at pH 7.2. At pH 6.5, the enzyme activity was determined to be 68% of the optimal activity and at pH 8.0 only 36%. Addition of 2.5 mM MgCl₂ or MnCl₂ had no influence on enzyme activity. At higher ionic strength, present in the reaction

| Substrate | Amount of oligoribonucleotides after incubation with |
|-----------|-----------------------------------------------------|
|           | Phosphodiesterase I | Phosphodiesterase II |
| Oligo(U)_{14} | 16,000 dpm (0.4 nmol) | 960 (0.02 nmol) | 15,620 (0.39 nmol) |
| Product: 5'-UMP | | | |

Fig. 4. Gel electrophoresis of endoribonuclease VII after centrifugation through a sucrose gradient. A, molecular weight estimation of proteins present in the endoribonuclease VII preparation on sodium dodecyl sulfate-polyacrylamide gels. Proteins of known molecular weight: I, phosphorylase a, 98,000; 2, bovine serum albumin, 68,000; 3, egg white albumin, 43,000; 4, chymotrypsinogen a, 25,000; 5, cytochrome c, 12,500, and 50 µg of enzyme (fraction VI) were run on 10% acrylamide gels as described under “Experimental Procedures.” SG, stacking gel; BPB, bromphenol blue. Fraction VI of the enzyme was used which had been additionally fractionated by sucrose gradient centrifugation into top and bottom fractions (see Fig. 3); lane a, top fraction, and lane b, bottom fraction. B, analysis of the enzyme preparations, using 6 M urea-polyacrylamide (10% gels). 40 µg of enzyme protein each (lane a, top fraction obtained after sucrose gradient centrifugation; see Fig. 3; lane b; bottom fraction) were analyzed per gel under the conditions described under “Experimental Procedures.” The gel was stained for protein with Coomassie brilliant blue. F. front. C, in situ detection of enzyme activity. In parallel unstained urea gels, the nuclease activity was shown in the separated top and bottom fractions (lane B, a; O) and bottom fractions (lane b).
mixture, the enzyme activity decreased; addition of 100 mM NaCl reduced the activity to 62% and of 300 nM to less than 5%.

For optimal enzyme activity, urea at a concentration between 300 and 500 mM had to be added to the reaction mixture. In the absence of urea, only 65% of the activity could be detected (control, 100%). Concentrations above 0.8 M urea are inhibitory; at 1 M, the enzyme activity decreased by 10% and at 3 M by 41%. The enzyme was not inhibited by the following reagents for thiol groups at a concentration of 5 mM: N-ethylmaleimide, iodoacetamide, iodoacetamide and HgCl$_2$. K-phosphate (pH 7.2), however, was determined to be a strong inhibitor; 10 mM reduced the activity by 27% and 100 mM by almost 100%.

Effect on endoribonuclease VI activity (fraction VI), while also with the purified enzyme (top fraction after sucrose gradient centrifugation; Fig. 3) as well as with the particle-associated enzyme in the bottom fraction (Fig. 3).

In a further approach, the high affinity of poly(A) for endoribonuclease VII activity and the influence on the enzymatic activity is achieved. At lower ratios, 1:2 (Fig. 6) or 1:1 (Table II), the poly(U) hydrolysis is inhibited by 52 or 87%, respectively. The extent of stimulation is dependent on the chain length of the polymer. Studying the same concentration range, oligo(A)$_{10}$ was found to have only a little modulating effect on the enzyme activity; a maximal stimulation of 15% and a maximal inhibition of 12% was achieved. The described effects of homopolymers on endoribonuclease VII activity are not restricted to the enzyme, present in the purification state of step VI, but was observed also with the purified enzyme (top fraction after sucrose gradient centrifugation; Fig. 3) as well as with the particle-associated enzyme in the bottom fraction (Fig. 3).

In a comparative series of experiments, we have determined the influence of poly(A)$_{95}$ on the activity of ribonuclease A by adjusting its enzyme concentration so that almost the same amount of poly(U) was converted to acid-soluble material by endoribonuclease VII and 2.1 nmol by ribonuclease A; these values were set to 0% (= no stimulation and no inhibition). The activities of the enzymes were studied in the presence of increasing amounts of different nucleic acids; the concentrations are given in nanomoles (with respect to phosphate)/assay. The percentage of stimulation or inhibition of the enzyme activities is indicated. Change of endoribonuclease VII activity in the presence of poly(A)$_{95}$ (---), oligo(A)$_{10}$ (C--C--C), poly(dA)$_{95}$ (A--A--A), and poly(G)$_{95}$ (A--A--A) is shown. Ribonuclease A activity is shown in the presence of poly(A)$_{95}$ (x--x--x).

FIG. 6. Dependence of endoribonuclease VII and ribonuclease A activity on the presence of poly(A), poly(G), and poly(dA). The experiments were performed for 10 min in the standard assay, using 1.8 enzyme units of endoribonuclease VII and 50 pg of ribonuclease A. Under these conditions, 1.8 nmol of poly(U) were converted to acid-soluble material by endoribonuclease VII and 2.1 nmol by ribonuclease A; these values were set to 0% (= no stimulation and no inhibition). The activities of the enzymes were studied in the presence of increasing amounts of different nucleic acids; the concentrations are given in nanomoles (with respect to phosphate)/assay. The percentage of stimulation or inhibition of the enzyme activities is indicated. Change of endoribonuclease VII activity in the presence of poly(A)$_{95}$ (---), oligo(A)$_{10}$ (C--C--C), poly(dA)$_{95}$ (A--A--A), and poly(G)$_{95}$ (A--A--A) is shown. Ribonuclease A activity is shown in the presence of poly(A)$_{95}$ (x--x--x).

In a further approach, the high affinity of poly(A) for endoribonuclease VII can be demonstrated. Applying the nitrocellulose filter technique, we studied the binding capacity of endoribonuclease VII (fraction VI) for different nucleic acids. The KCl concentration in the assay was adjusted to 300 mM in order to suppress the enzymic activity. Under this condition, endoribonuclease VII was determined to bind not only to the poly(U) substrate, but also to the poly(A) modulator, while in the presence of radioactively labeled poly(C), poly(G), and DNA, less than 8% of the input radioactivity is retained on the filters (Fig. 7). In control experiments, with 3 μg of bovine serum albumin/assay, a nonspecific binding of poly([H]A) and poly([H]U) around 3–5% of the radioactivity is found.
contained this enzyme species as well, yet together with four
activity. Using this criterion, the enzyme, present in the top
fraction, was purified to homogeneity. The bottom fraction
major (Mr <50,000) and five minor proteins. The enzyme in
this complex is associated not only with protein but also with
radioactivity retained on nitrocellulose filters was determined as
described under “Experimental Procedures.” The percentage of ra-
dioactivity retained on filters in the presence of endoribonuclease VII
(fraction VI) is shown. In control assays using bovine serum albumin
instead of endoribonuclease VII, less than 5% of the labeled nucleic
acids were bound.

**DISCUSSION**

In the present communication, we show that calf thymus contains a hitherto undescribed endoribonuclease, which we
termed endoribonuclease VII. This enzyme is specific for
single-stranded ribopyrimidines and generates oligoribonucle-
etides of 12 nucleotide units on the average. These products are
terminated by 3’-hydroxy- and 5’-phosphate groups. By these
characteristics, endoribonuclease VII differs from the
nonspecific endoribonuclease T2 (EC 3.1.27.1) (37), endoribo-
unique from Bacillus subtilis (EC 3.1.27.2) (37, 38), and endoribo-
unique from Enterobacter sp. (EC 3.1.27.6) (39) as well as from the
purine-specific endoribonuclease T4 (EC 3.1.27.3) (37), endoribo-
unique from U2 (EC 3.1.27.4) (37, 40), endoribonuclease IV from chick oviduct (7, 41), endoribonu-
unique from calf thymus (9), and the UpN3’→5’-specific
edoribonuclease VI from Artemia larva (42). With respect to the
substrate specificity, the novel endoribonuclease VII resembles pancreatic endoribonuclease (ribonuclease A; EC
3.1.27.5) (43) and ribonuclease from bovine seminal plasma
(44). However, endoribonuclease VII differs from the latter
two enzymes in the following important aspects. Ribonuclease
A hydrolyzes single-stranded homopyrimidines to P’-2’-3’
nucleotides (surveyed in Ref. 43) and the seminal plasma
ribonuclease degrades single- and double-stranded polynucle-
complained only with protein species of Mr = 74,000 and M1, bound to RNP particles. Since the enzyme activities present in
the two fractions responded to substrates in identical fashion (not shown) and to poly(A) with the same typical
dose-dependent stimulation or inhibition and since they can be
characterized by the same electrophoretic mobility, we assume that the activity in the top fraction (<5 S) had been
liberated from the 45 S particles.

From densitometer tracings of Coomassie-stained urea gels,
we conclude that approximately 8% of the total protein of the
45 S particle is endoribonuclease VII. With this figure, the
relative molecular mass, the amount of pure enzyme which
had been obtained from 200 g of thymus gland (yield 1.2%),
and the fact that approximately 4.6 × 10^6 cells are present in
1 g of thymus (45), the concentration of endoribonuclease VII
can be estimated as 2.2 × 10^10 molecules/cell. In comparison,
deribonuclease VII is present in a concentration of 4 × 10^10
molecules/oviduct cell (42) and endoribonuclease V in a con-
centration of 1.6 × 10^10 molecules/thymus cell (9). The mole-
cular activity of the enzyme was determined to be 180 cleavages
performed/min/molecule of enzyme (see “Results”). This fig-
ure is lower than those determined for endoribonuclease IV
(1.7 × 10^3 cleavages/min/molecule; Ref. 42) and endoribo-
unique V (3.7 × 10^3 cleavages/min/molecule; Ref. 9).

Endoribonuclease VII is a basic protein (isoelectric point,
8.5) which shows an activity optimum at pH 7.2 without
requirement for divalent cations for its activity and is insen-
sitive towards thiol group reagents (e.g. N-ethylmaleimide),
very much in contrast to other cellular RNases which degrade double-
and single-stranded RNAs and which are inhibited already by low concentrations of N-ethylmaleimide (46).

One interesting feature of endoribonuclease VII is its affini-
ity for poly(A). Dose-response experiments revealed that
poly(A) causes an over 100% stimulation of the enzymic
poly(U) degradation, if a concentration ratio (based on moles
of phosphate) of approximately 1 [poly(A):10 [poly(U)] was
adjusted in the assay. This stimulation is observed with poly(A) only, while oligo(A), is nearly ineffective. At higher
ratios, poly(A) inhibits poly(U)-hydrolyzing activity up to
50%. In previous experiments, it was established (47) that
donor activity of endoribonuclease VII is specific for poly(A); poly(dA) and poly(G)
guished with this enzyme under such concen-
tration conditions at which this polymer positively affected
endoribonuclease VII activity. The stimulation of endoribonuclease VII activity was specific for poly(A); poly(dA) and poly(G)
called an inhibitory effect only. Binding experiments con-
firmed the evidence that endoribonuclease VII interacts not
only with poly(U) but also with poly(A). Although endoribo-
unique VII utilizes both poly(U) and poly(C) as substrate,
the filter binding studies revealed only an affinity of the
enzyme for poly(U) but not for poly(C). We therefore suppose that the enzyme has the property of discrimination between
these two substrates due to different binding affinities. As
reported under “Results,” endoribonuclease VII showed sub-
stantial activity with a tRNA substrate; the hydrolysis prod-
ucts are oligoribonucleotides in nature. No stimulation of the
enzyme had been observed with tRNA (data not given), which
might be due to the lack of oligo(U) sequences in this polymer.

The functional significance of poly(U)- and poly(C)-specific
endoribonuclease VII, which seems to be a constituent of an
RNP complex and whose activity is modulated by poly(A), is
not known. However, based on the protein pattern of the
endoribonuclease VII complex and the probable existence of
RNA in this particle, a homology with the small nuclear
RNPs (snRNP) might be assumed (48). 7-11 proteins have
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Poly(A)-modulated, Pyd-specific Endoribonuclease VII

poly(A), which modulates its activity in a stoichiometric fashion; ion.  
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