

# The Processive Degradation of Individual Polyribonucleotide Chains

## I. *ESCHERICHIA COLI* RIBONUCLEASE II\*

(Received for publication, September 11, 1967)

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### SUMMARY

The experiments reported here suggest that *Escherichia coli* ribonuclease II, an exonuclease, hydrolyzes a given polyribonucleotide chain to completion before releasing a small, resistant oligonucleotide and initiating hydrolysis of another chain. Investigation of the products of hydrolysis of oligo- and polyribonucleotides indicates that enzymatic degradation starts at that end of the chain bearing a 3'-hydroxyl group. The enzyme shows a strong preference for long polymers as substrates, although oligonucleotides are hydrolyzed if present at sufficiently high concentration.

*Escherichia coli* ribonuclease II catalyzes the degradation of single stranded polyribonucleotides to nucleoside 5'-monophosphates (1-3). The enzyme requires magnesium and in addition is activated by potassium or ammonium ions. Helical forms of RNA are not hydrolyzed. The enzyme has been characterized as an exonuclease, since mononucleotides are the principal products during all stages of digestion, and large oligonucleotides do not accumulate (3). The evidence presented in this paper indicates that the enzyme catalyzes the hydrolysis of a polyribonucleotide chain beginning at the 3'-hydroxyl end, and that it sequentially liberates mononucleotides from the chain until a small oligonucleotide ( $n = 2$  to 4), which is itself comparatively resistant to hydrolysis, is formed. Furthermore, the results suggest that a given enzyme molecule tends to hydrolyze a single RNA chain repeatedly until its 5'-hydroxyl chain end is released as a small oligonucleotide, and only then begins the hydrolysis of a new RNA chain. This mode of attack will be termed processive degradation.<sup>1</sup>

\* This paper is dedicated to Professor Heinrich Brinkmann of Swarthmore College on the occasion of his 70th birthday.

<sup>1</sup> A preliminary report of these findings has been made (NOSSAL, N. G., TOLBERT, G. P. C., AND SINGER, M. F., *Fed. Proc.*, **26**, 612 (1966)).

### EXPERIMENTAL PROCEDURE

<sup>14</sup>C-UDP, <sup>14</sup>C-ADP, <sup>14</sup>C-GDP, <sup>3</sup>H-CDP, and unlabeled mononucleotides were purchased from Schwarz BioResearch. pAp was the gift of Dr. H. G. Khorana. Crystalline BSA,<sup>2</sup> obtained from Pentex, was dialyzed for several days against 1 mM EDTA and then against H<sub>2</sub>O. Radioactive phenylalanine and serine were purchased from New England Nuclear, and <sup>14</sup>C-valine was obtained from Calbiochem. Sephadex G-100 was obtained from Pharmacia. Polyacrylamide gels (Bio-Gel) were products of Bio-Rad Laboratories.

*Paper Chromatography and Paper Electrophoresis*—Paper chromatography on Whatman No. 1 or 3MM paper was carried out with 1-propanol-concentrated NH<sub>4</sub>OH-H<sub>2</sub>O (65:10:35), Solvent 1 (4); 1-propanol-concentrated NH<sub>4</sub>OH:H<sub>2</sub>O (55:10:35), Solvent 2 (5); 95% ethanol-1 M ammonium acetate (40:60), Solvent 3 (6); saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-2-propanol-1 M sodium acetate (80:2:18), Solvent 4 (7); and 2-propanol-H<sub>2</sub>O (70:30) with NH<sub>3</sub> in the vapor phase, Solvent 5 (8). All proportions are by volume. DEAE-cellulose paper chromatography (Whatman DE-81) was carried out for 6 to 9 hours with 1 M or 0.5 M NH<sub>4</sub>HCO<sub>3</sub> (freshly prepared). The latter molarity is preferred for the separation of small oligonucleotides ( $n = 2$  to 5). Paper electrophoresis was carried out in 0.5 M potassium phosphate buffer, pH 7.0, at 13.4 volts per cm, or in 0.05 M ammonium formate buffer, pH 3.5, at 17.8 volts per cm. All the above procedures were carried out at room temperature.

*Analytical Procedures*—For total alkaline hydrolysis, polynucleotides were incubated for 18 hours in 0.3 or 0.4 M KOH at 37°. Solutions then either were neutralized with 0.3 M HCl and subjected to paper electrophoresis or were neutralized with 1 M HClO<sub>4</sub> and chromatographed with Solvent 5.

Aqueous samples were counted in a liquid scintillation counter

<sup>2</sup> The abbreviations used are: BSA, bovine serum albumin; poly A, polyadenylic acid; poly U, polyuridylic acid; poly AU, copolymer of adenylate and uridylylate; poly AG, copolymer of adenylate and guanylate; 1 A<sub>260</sub> unit is that amount of material giving an absorbance of 1 at 260 mμ in 1.0 ml of solution in a 1-cm light path. Abbreviations for specifically labeled polyribonucleotides are described in Table I.

in 10 ml of Bray's solution (9) containing 0.1 ml of 1 N  $\text{NH}_4\text{OH}$ . Squares cut from Whatman No. 1 or DE-81 paper chromatograms were counted in Bray's solution or in a solution containing 2 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per 473 ml of toluene.

Protein was determined by the method of Lowry *et al.* (10).

**Enzymes**—RNase II was a hydroxylapatite fraction (Fraction VII) (3) purified from *E. coli* MRE 600 (11), a mutant lacking RNase I (12). The molecular weight of RNase II was estimated to be approximately 65,000 by using the sucrose density gradient method of Martin and Ames (13) with bovine serum albumin as the reference marker (14). A unit of enzyme produces 1  $\mu\text{mole}$  of AMP per hour at 37° in a reaction mixture containing 2 mM poly A,<sup>3</sup> 0.1 M Tris-HCl (pH 7.5), 0.1 M KCl and 1.5 mM  $\text{MgCl}_2$ . The assay was carried out as previously described (3). Occasionally, 0.1 M ammonium bicarbonate buffer, pH 7.5, was used to replace both the Tris-HCl and the 0.1 M KCl to facilitate the removal of salt before chromatography.  $\text{NH}_4^+$  ions can substitute for  $\text{K}^+$  ions with this enzyme (1).

Polynucleotide phosphorylase was prepared from *Micrococcus lysodeikticus* (15) or from *E. coli* MRE 600 through the DEAE-Sephadex step described by Williams and Grunberg-Manago (16). Two different preparations of enzyme from *M. lysodeikticus* were used: one required a primer in order to polymerize nucleoside diphosphates (15); the other, less purified fraction required no primer (17). The *E. coli* fractions did not require primer. A unit of enzyme is equivalent to the incorporation of 1  $\mu\text{mole}$  of <sup>32</sup>P<sub>i</sub> into ADP in 15 min at 37°, with poly A as a substrate (17).

The *Azotobacter agilis* endonuclease, which degrades poly A to oligonucleotides containing phosphate monoesterified to the terminal 5'-hydroxyl group, was the gift of Dr. Audrey Stevens (18). Pancreatic RNase A was purchased from Sigma. *E. coli* alkaline phosphatase was donated by Dr. Wallace Brockman (ammonium sulfate fraction (see Reference 19)); this enzyme (electrophoretically purified) was also purchased from Worthington. A unit of enzyme produces 1  $\mu\text{mole}$  of P<sub>i</sub> from AMP per hour at 37° when assayed in 0.1 M Tris-HCl, pH 9.0, at a concentration of 0.012 M 5'-AMP.

T1 RNase was prepared by the procedure of Takahashi (20) or was purchased from Calbiochem. A unit of enzyme produces a change of 1 absorbance unit per ml in the following assay. Yeast RNA (0.15 mg), 0.05 M Tris-HCl (pH 7.5), 0.002 M EDTA, and enzyme in a volume of 0.2 ml were incubated for 15 min at 37°. An equal volume of 0.25% uranium acetate in 4% perchloric acid was added, and 0.1 ml of the clear supernatant fluid was diluted to 1.5 ml prior to determining the absorbance at 260 m $\mu$ .

Venom phosphodiesterase was prepared by a modification of the method of Koerner and Sinsheimer (21) or was purchased from Worthington; in the latter case it was freed of contaminating 5'-nucleotidase by the procedure of Keller (22). A unit produces 1  $\mu\text{mole}$  of AMP per hour at 37° when incubated with 2 mM poly A, 1 mM  $\text{MgCl}_2$ , and 10 mM Tris-HCl, pH 8.7. The *E. coli* cyclic diesterase preparation was a cold water wash fraction from shocked *E. coli* (MRE 600) cells, and was the gift of Dr. Leon Heppel (23). One unit produces 1  $\mu\text{mole}$  of P<sub>i</sub> per hour from 2',3'-cyclic UMP under the standard assay conditions (23).

A fraction containing a mixture of aminoacyl-tRNA synthetases

was prepared from *E. coli* B by following the procedure of Muench and Berg (24) through the DEAE-cellulose column step. This preparation still contained 34 units of RNase II per ml when measured by the standard assay for RNase II. The aminoacylation of tRNA was proportional to the concentration of tRNA under the following conditions. Reaction mixtures (0.1 ml) all contained 0.1 M sodium cacodylate buffer (pH 6.9), 4 mM reduced glutathione, 0.01 M  $\text{MgCl}_2$ , 0.01 M KCl, and 20  $\mu\text{g}$  of the synthetase fraction. In addition they contained 0.5 mM <sup>14</sup>C-valine ( $5.5 \times 10^6$  cpm per  $\mu\text{mole}$ ), 0.87 mM ATP, and up to 3  $A_{260}$  units of tRNA; or 0.25 mM <sup>14</sup>C-serine ( $5.9 \times 10^6$  cpm per  $\mu\text{mole}$ ), 0.44 mM ATP, and up to 6  $A_{260}$  units of tRNA; or 0.5 mM <sup>3</sup>H-phenylalanine ( $9.6 \times 10^6$  cpm per  $\mu\text{mole}$ ), 1.3 mM ATP, and up to 2  $A_{260}$  units of tRNA. The reaction was initiated by the addition of the amino acid, and, after 10 min at 37°, duplicate 40- $\mu\text{l}$  aliquots were absorbed onto Whatman No. 3MM paper discs and subjected to a modification (25) of the procedure of Mans and Novelli (26) in order to determine the incorporation of amino acid into tRNA. The discs were counted in 10 ml of Bray's solution. The results were corrected for any radioactivity detected in control mixtures that lacked enzyme.

**Oligonucleotides**—(Ap)<sub>4</sub>A, (Ap)<sub>6</sub>A, and (Up)<sub>7</sub> were obtained from Miles Chemical Company and were characterized by alkaline hydrolysis. The abbreviations used to describe the specifically labeled oligonucleotides and polyribonucleotides synthesized for this work are given in Table I.

(Ap)<sub>5</sub>U and (Ap)<sub>5</sub>C were prepared by using (Ap)<sub>4</sub>A as the primer for the polymerization of UDP and CDP, respectively, by *M. lysodeikticus* polynucleotide phosphorylase in the presence of pancreatic RNase, which preferentially hydrolyzes the phosphodiester bond following pyrimidine residues. Reaction mixtures (2.0 ml), containing 0.1 M Tris-HCl (pH 8.2), 5 mM  $\text{MgCl}_2$ , 2.45 mM (Ap)<sub>4</sub>A, 0.2 mg of BSA, 1.2 mg of RNase A, 0.018 M UDP or CDP, and 1.8 (UDP) or 3.0 (CDP) units of polynucleotide phosphorylase, were incubated at 37° for 1 (UDP) or 2½ (CDP) hours. The resulting phosphorylated hexanucleotides ((Ap)<sub>5</sub>Up and (Ap)<sub>5</sub>Cp) were isolated by chromatography in Solvent 3 and were eluted with H<sub>2</sub>O; 80% yields, based on the (Ap)<sub>4</sub>A input, were obtained. The hexanucleotides were dephosphorylated with *E. coli* alkaline phosphatase as follows. Reaction mixtures, containing 0.2 M Tris-HCl (pH 8.1), 2.5 mM  $\text{MgCl}_2$ , 52  $A_{260}$  units of (Ap)<sub>5</sub>Up, and 21 units of enzyme in a volume of 6.0 ml or 57  $A_{260}$  units of (Ap)<sub>5</sub>Cp and 32 units of enzyme in 7.8 ml, were incubated for 2 hours at 37°. The solutions were deproteinized with phenol and extracted with ether, and the products were then isolated by chromatography with Solvent 3. The yields of dephosphorylated hexamer and the ratios of AMP to nucleoside after alkaline hydrolysis were: for (Ap)<sub>5</sub>U, 24% and 4.1; for (Ap)<sub>5</sub>C, 22% and 4.4. The poor yield was probably primarily due to the partial solubility of oligonucleotides in phenol.

(Ap)<sub>5</sub>G\*p was similarly prepared (by Dr. D. M. Logan) with polynucleotide phosphorylase but in the presence of T1 RNase which is specific for bonds following guanosine residues. The reaction mixture (3.7 ml) contained 0.1 M Tris-HCl (pH 8.2), 2 mM  $\text{MgCl}_2$ , 14.9 mM <sup>14</sup>C-GDP ( $2.8 \times 10^6$  cpm per  $\mu\text{mole}$ ), 1.1 mM (Ap)<sub>4</sub>A, 0.37 mg of BSA, 11 units of phosphorylase, and 750 units of T1 RNase (Calbiochem) and was incubated for 3 hours at 37°. The product, (Ap)<sub>5</sub>G\*p, was separated by chromatography as described above. The yield was greater than 90% and,

<sup>3</sup> Unless noted otherwise, the concentrations of polynucleotides and oligonucleotides are given as concentration of constituent mononucleotides (or phosphorus).

TABLE I  
Abbreviations for polyribonucleotides

The preparation of these polyribonucleotides is described under "Experimental Procedure." An asterisk indicates a radioactive group.

Abbreviation	Structure	Radioactive label and position	Average chain length
(pA) <sub>5</sub> <sup>a</sup>	pApApApApA	None	
(Ap) <sub>6</sub> A <sup>a</sup>	ApApApApApA	None	
(Ap) <sub>7</sub>	ApApApApApApA	None	
(Ap) <sub>5</sub> U	ApApApApApU	None	
(Ap) <sub>5</sub> C	ApApApApApC	None	
(Ap) <sub>5</sub> G <sup>*</sup> p	ApApApApApG <sup>*</sup> p	<sup>14</sup> C; 3'-terminal Gp	
(pA <sup>*</sup> ) <sub>3</sub> (pA) <sub>n</sub>	pA <sup>*</sup> pA <sup>*</sup> pA <sup>*</sup> pA...pA	<sup>14</sup> C; 5'-terminal pApApA	108
(Ap) <sub>n</sub> U <sup>*</sup> b	ApAp...ApU <sup>*</sup>	<sup>14</sup> C; 3'-terminal U	80 < n < 105
(Ap) <sub>n</sub> C <sup>*</sup>	ApAp...ApC <sup>*</sup>	<sup>3</sup> H; 3'-terminal C	38
(Ap) <sub>n</sub> G <sup>*</sup>	ApAp...ApG <sup>*</sup>	<sup>14</sup> C; 3'-terminal G	56

<sup>a</sup> Homologous compounds are similarly abbreviated.

<sup>b</sup> Thirty-four per cent of the chains in this preparation were not dephosphorylated. Thus they had the structure ApAp...ApU<sup>\*</sup>p.

after KOH hydrolysis, AMP and GMP were recovered in a ratio of 4.2:1. The solution of (Ap)<sub>5</sub>G<sup>\*</sup>p had little or no detectable polynucleotide phosphorylase or T1 RNase activity.

<sup>14</sup>C-Labeled oligonucleotides of the general structure (pA)<sub>n</sub>, where n = 2 to 5, were prepared by the digestion of <sup>14</sup>C-poly A (6.5 × 10<sup>4</sup> cpm per μmole of adenosine) with the *A. agilis* endonuclease. The procedure of Stevens and Hilmoe (18) was followed, except that after chromatography of the oligonucleotides with Solvent 1 they were further purified by chromatography with Solvent 3. (pA)<sub>2</sub> accounted for 16.5% of the product, and gave a ratio, after alkaline hydrolysis, of adenosine to 2',3'-AMP to pAp of 1.00:0.0:0.91; (pA)<sub>3</sub>, 37.0%, 1.00:0.88:0.85; (pA)<sub>4</sub>, 31.4%, 1.00:2.08:0.99; and (pA)<sub>5</sub>, 15.4%, 1.00:3.12:1.01.

**Polymers**—Unless specifically noted, all the polymers used in the studies were synthesized in this laboratory, with the use of polynucleotide phosphorylase from *M. lysodeikticus*, and were dialyzed extensively against 1 mM EDTA and then distilled water before use. Their structures and corresponding abbreviations are outlined in Table I.

(pA<sup>\*</sup>)<sub>3</sub>(pA)<sub>n</sub> was prepared by use of <sup>14</sup>C-pApApA as the primer, with a primer-requiring fraction of polynucleotide phosphorylase from *M. lysodeikticus*. The polymer was purified by repeated extraction with phenol followed by extensive dialysis, first against 0.04 M KCl containing 0.005 M disodium EDTA, and then against H<sub>2</sub>O. The average chain length, determined from the ratio of radioactivity in the terminal <sup>14</sup>C-(pA)<sub>3</sub> to the absorbance at 257 mμ, was 108. The polymer was characterized by chromatography for 117 hours with Solvent 3. Five per cent of the radioactivity migrated with (pA)<sub>3</sub>, less than 1 per cent migrated faster than (pA)<sub>3</sub>, and the remainder did not leave the origin. After alkaline hydrolysis in the presence of carrier pAp, the ratio of radioactivity in 2',3'-AMP to that in pAp was 2.4:1, suggesting that the 5'-terminal phosphate had been removed from about 12% of the chains.

(Ap)<sub>n</sub>U<sup>\*</sup> was prepared by controlled digestion of poly AU (labeled with <sup>14</sup>C in the uridylic acid residues) with pancreatic RNase, followed by dephosphorylation with *E. coli* alkaline phosphatase. Poly AU was synthesized with polynucleotide phosphorylase from *E. coli*. The ratio of ADP to <sup>14</sup>C-UDP in the substrate mixture was 30:1, and the ratio of mononucleotides

in the product was 58:1. The polymer was purified by phenol extraction and dialysis. Poly AU (103.5 μmoles) was incubated for 16 hours at 37° with 150 μg of pancreatic RNase, 0.14 M KCl, and 0.054 M Tris-HCl, pH 7.5, in a volume of 14 ml. These conditions produced one break per 46 nucleotides as measured by phosphate labile to alkaline phosphatase. Thus most of the uridine residues and few of the adenosine residues would be expected to be in a terminal position. The digest was adjusted to 0.1 M HCl and incubated for 4 hours at room temperature to cleave the cyclic phosphate esters. The pH was adjusted to 8 with KOH, and Tris-HCl, pH 8.1, was added to give a final concentration of 0.25 M. The polymer was then incubated for 1 hour at 37° with 325 units of alkaline phosphatase (Brockman), a 7-fold excess over an amount which quantitatively dephosphorylated AMP at the same concentration as the polymer terminal phosphate. The dephosphorylated polymer was extracted with phenol and ether to remove the RNase and alkaline phosphatase. The polymer (23.8 μmoles) was fractionated on a column of Bio-Gel P-150 (1.3 × 100 cm), covered with a 1-cm layer of Bio-Gel P-30, by elution with 1 M ammonium bicarbonate. The average chain lengths of the fractions were determined from the ratio of radioactivity (in the terminal uridine) to the absorbance at 257 mμ. Selected fractions were pooled, and the ammonium bicarbonate was removed by repeated lyophilization. When the pooled fractions, containing material of average chain length between 50 and 105, were hydrolyzed with KOH, 66% of the radioactivity was found in uridine and the remainder in UMP, indicating incomplete dephosphorylation of the polymer. After incubation with a 10-fold greater concentration of alkaline phosphatase under the above conditions, more than 96% of the radioactivity was in uridine after alkaline hydrolysis.

(Ap)<sub>n</sub>C<sup>\*</sup> was similarly prepared with polynucleotide phosphorylase (*M. lysodeikticus*) with 400 μmoles of ADP and 20 μmoles of <sup>3</sup>H-CDP. After incubation for 6 hours at 37° with 4 mg of pancreatic RNase and phenol extraction, polymer was isolated by chromatography on Bio-Gel P-4 in 1 mM Tris-HCl, pH 7.5, and lyophilized. The poly (Ap)<sub>n</sub>C<sup>\*</sup>p (31 μmoles) was incubated for 30 min at 37° with 500 μmoles of Tris-HCl, pH 9.0, and 60 units of alkaline phosphatase (Brockman) in a volume of 5.0 ml. The polymer was chromatographed overnight in Solvent 2, and the material remaining at the origin was eluted,

TABLE II  
Hydrolysis of pApApApApA by RNase II

The incubation mixtures contained (per ml) 0.1 M ammonium bicarbonate buffer (pH 7.5), 1.5 mM MgCl<sub>2</sub>, 0.15 mg of BSA, and the indicated concentrations of <sup>14</sup>C-(pA)<sub>n</sub> and enzyme. The total volumes were: Experiment A, 0.5 ml; B, 0.2 ml; C, 0.1 ml. After 30 min at 37°, the reaction mixtures were heated for 5 min at 100°, lyophilized two times to remove salt, and chromatographed for 6 hours on DEAE-cellulose paper with 0.5 M ammonium bicarbonate. The paper strips were cut into suitable sections, with the use of known oligonucleotide markers to aid in location of products, and the sections were counted in the toluene scintillation solution. The radioactivity was corrected for that obtained in control experiments without enzyme.

Experiment	Conditions		Rate of hydrolysis (pA) <sub>n</sub> μmoles AMP/hr/enzyme unit	Products <sup>a</sup>				
	Concentration of (pA) <sub>n</sub> <sup>a</sup> μM	Concentration of enzyme units/ml		(pA) <sub>5</sub>	(pA) <sub>4</sub>	(pA) <sub>3</sub>	(pA) <sub>2</sub>	AMP
				μM	μM	μM	μM	μM
A	2.1	3	0.002	0.6	0.0	1.6	0.0	3.6
B	22.0	3	0.02	5.3	0.6	16.1	0.5	34.0
	22.0	1	0.03	13.4	1.6	6.6	0.5	15.4
C	220	3	0.08	149	15.4	54.0	0.0	127
	220	1	0.06	205	4.0	10.0	0.0	27

<sup>a</sup> Concentrations are expressed as oligonucleotide.

TABLE III  
Hydrolysis of oligonucleotides by RNase II

Reaction mixtures (0.2 ml) containing radioactive oligonucleotide, enzyme, 10 μg of BSA, 0.1 M NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 7.5), and 1.5 mM MgCl<sub>2</sub> were incubated for 7 hours at 37° and then treated as described in Table I.

Oligonucleotide substrate		Substrate undegraded	
Compound	Concentration <sup>a</sup> mM	0.2 RNase unit %	3 RNase units %
(pA) <sub>2</sub>	1	99	98
(pA) <sub>3</sub>	0.67	98	62
(pA) <sub>4</sub>	0.50	41	4
(pA) <sub>5</sub>	0.40	45	3

<sup>a</sup> Concentrations are expressed as oligonucleotide.

lyophilized, and rechromatographed for 48 hours in the same solvent. The polymer eluted from the origin contained 10.1 μmoles of nucleotide and 1.6 × 10<sup>5</sup> cpm, giving an A:C ratio of 38:1. After alkaline hydrolysis, 97% of the radioactivity was in cytidine and the remainder in CMP.

(Ap)<sub>n</sub>G\* was prepared from poly AG which had been made with 200 μmoles of ADP and 10 μmoles of <sup>14</sup>C-GDP. The polymer was extracted with phenol, chromatographed on Bio-Gel P-4 to remove mononucleotides, concentrated by lyophilization, and dialyzed. T1 RNase (4500 units) prepared by the procedure of Takahashi (20) was incubated with 52.4 μmoles of polymer in 9.9 ml for 21 hours at 37°. Toluene was added after 4 hours. The (Ap)<sub>n</sub>Gp was extracted with phenol and chromatographed on Bio-Gel P-4. The polymer (16.9 μmoles) was

dephosphorylated by incubation with 4.8 units of *E. coli* cyclic diesterase for 45 min at 37° with 0.1 mM CaCl<sub>2</sub> and 0.2 M sodium acetate buffer, pH 6.1, in a volume of 4.0 ml. The solution was heated for 10 min at 70°, chromatographed on Bio-Gel P-4, and concentrated. The average chain length, calculated from the ratio of radioactivity to total phosphate, was 56. More than 99% of the radioactivity was found in guanosine after alkaline hydrolysis, and 13% of the chains were found to terminate with adenosine.

*E. coli* B tRNA was prepared and stripped of amino acid by the method of Zubay (27), or was purchased from Schwarz BioResearch.

## RESULTS

### Experiments with Oligonucleotides

**Degradation of Oligonucleotides**—In a previous publication from this laboratory (3), the results of experiments utilizing a series of oligonucleotides of general structure (pA)<sub>n</sub> as substrates for RNase II were described. Those data were interpreted to indicate that (pA)<sub>n</sub> would be degraded only if *n* were 7 or greater. This interpretation was consistent with the fact (1, 3) that oligonucleotides accumulate as resistant end products of the digestion of long chain polyribonucleotides. Recently, the hydrolysis of oligonucleotides was reinvestigated as a function of oligonucleotide concentration, and it became clear that oligonucleotides of chain length less than 7 are indeed hydrolyzed if their concentration in the reaction mixture is sufficiently high. Table II presents data pertaining to the hydrolysis of (pA)<sub>5</sub>. At a concentration of 0.22 mM (expressed as chains of (pA)<sub>5</sub>), the rate of AMP production is 0.06 that of the rate of poly A hydrolysis when the latter is measured at saturating concentration (1.2 μM, expressed as polymer chains; average *n* = 1000). For poly A, the half-maximal rate of hydrolysis was obtained at a concentration of 0.012 μM (as chains), while the data in Table II suggest that the concentration of (pA)<sub>5</sub> required for half-maximal rate is at least three orders of magnitude greater. Table III shows that (pA)<sub>4</sub> is hydrolyzed at about the same rate as (pA)<sub>5</sub>, but that (pA)<sub>3</sub> and (pA)<sub>2</sub> are more resistant to hydrolysis. In the experiments in Table III, all the oligonucleotides were tested at similar concentrations, and the concentration of (pA)<sub>5</sub> was close to the optimal value. Under the conditions of these experiments (pA)<sub>2</sub> was hydrolyzed to a very small extent.

The relative preference of RNase II for long polymers, suggested by the experiments just described, is shown directly by the data in Table IV. The effect of mixing approximately equal concentrations (as numbers of chains, not nucleotide residues) of poly A and <sup>14</sup>C-(pA)<sub>5</sub> on the independent rate of hydrolysis of each compound was investigated. Poly A was present at saturating concentration; the oligonucleotide was not. The molar concentration of enzyme was much lower than the concentration of either substrate. The pentanucleotide had little or no effect on the hydrolysis of poly A, but the presence of poly A inhibited the digestion of (pA)<sub>5</sub> over 90%.

Tables II and IV also contain data concerning the products of degradation of (pA)<sub>5</sub>. Free (pA)<sub>4</sub> does not accumulate as an intermediate during the hydrolysis of (pA)<sub>5</sub>. In fact, at all levels of digestion studied, the yield of (pA)<sub>3</sub> is higher than that of (pA)<sub>4</sub>. It is of interest that, during the digestion of (Ap)<sub>5</sub>A and (Ap)<sub>7</sub> (Table V), more (Ap)<sub>3</sub>A than (Ap)<sub>4</sub>A is produced. These findings will be considered under "Discussion."

**Direction of Hydrolysis of Oligonucleotides by RNase II**—Investigation of the products of digestion of oligonucleotides can establish the direction of hydrolysis by an exonuclease (28–30). The method is diagrammed in Fig. 1, with  $(Ap)_6A$  as an example. Since the products of RNase II digestion are 5'-mononucleotides, adenosine will appear as a product only if hydrolysis starts at the 5'-hydroxyl end. The products found upon digestion of  $(Ap)_6A$  are shown in Table V. No adenosine was detected; the only products were 5'-AMP and oligonucleotides of chain length less than 6. Thus, the direction of hydrolysis is 3' to 5'. In a similar experiment,  $(Ap)_3A$  was degraded to 5'-AMP and resistant oligonucleotide; no adenosine was detected.

The degradation of oligonucleotides of the general structure  $(Ap)_nX$ , where X is cytidine or uridine (Table VI), confirms the conclusion that hydrolysis is 3' to 5', since 5'-XMP is always a product. The data in Table VI are also relevant to the possible preference of the enzyme for specific bases. Thus, as X is varied, no very large differences in rate of hydrolysis of  $-ApX$  phosphodiester bonds appear. Similar results have also been obtained with  $(Ap)_5G$ .

It is of interest that  $(Ap)_7$ , which is phosphorylated at the 3'-terminus, is hydrolyzed by RNase II at the same rate as the dephosphorylated heptamer. This observation is unexpected, for snake venom phosphodiesterase and polynucleotide phosphorylase act very slowly on oligonucleotides bearing a terminal 3'-phosphate group (31). The 3',5'-nucleoside diphosphate was identified as a product of the hydrolysis of oligonucleotide phosphorylated at the 3' chain end with the phosphorylated oligonucleotide  $(Ap)_5G^*p$ . The oligonucleotide (0.174  $\mu$ mole as oligonucleotide) was incubated with 0.75 unit of enzyme for 5 hours at 37°. When the digest was chromatographed with Solvent 5, all of the radioactivity was either at the origin or in the area just ahead of the origin. The latter area was eluted and subjected to electrophoresis in 0.05 M ammonium formate buffer, pH 3.5. The major radioactive spot, migrating just in front of GDP, was eluted and concentrated. The spectrum of the nucleotide agreed with that of 5'-GMP. Total phosphate determination gave 1.9 phosphates per guanosine residue.

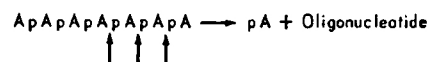
TABLE IV

*Hydrolysis of  $(pA)_5$  in presence of poly A*

Duplicate reaction mixtures, containing 2.15 mM poly A or 0.135 mM  $^{14}C$ -(pA)<sub>5</sub> (or both), 0.1 M  $NH_4HCO_3$  (pH 7.5), 1.5 mM  $MgCl_2$ , 0.1 mg of BSA, and 0.4 unit of enzyme in a volume of 0.2 ml, were incubated for 45 min at 37°. The reaction was started by addition of enzyme. Hydrolysis of poly A was determined by the absorption at 259  $m\mu$  of the acid-soluble product. Hydrolysis of  $(pA)_5$  was determined by chromatography of the entire reaction mixture on DEAE-cellulose paper with 0.5 M  $NH_4HCO_3$ , and counting of the appropriate areas of the paper in the toluene scintillation solution. With the pentamer alone, 2.7  $m\mu$ moles of  $(pA)_5$  (as oligonucleotide) were hydrolyzed, yielding (in millimicromoles of oligonucleotide) 0.5  $(pA)_4$ , 2.3  $(pA)_3$ , 0.0  $(pA)_2$ , and 5.4 AMP.

Substrate	AMP from	
	Poly A	$(pA)_5$
	<i>m\mu</i> moles	
Poly A	56	
$^{14}C$ -(pA) <sub>5</sub>		5.4
Poly A + $^{14}C$ -(pA) <sub>5</sub>	53	0.6

From 3'-OH chain end



From 5'-OH chain end

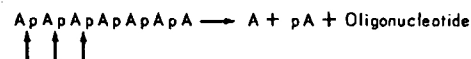
FIG. 1. Products expected from the hydrolysis of  $(Ap)_6A$ 

TABLE V

*Products of hydrolysis of  $(Ap)_6A$  and  $(Ap)_7$  by RNase II*

In Experiment 1,  $(Ap)_6A$  or  $(Ap)_7$  (0.052  $\mu$ mole of oligonucleotide) was incubated at 37° in a volume of 0.1 ml with 0.1 M ammonium bicarbonate buffer (pH 7.5), 1.5 mM  $MgCl_2$ , 5  $\mu$ g of BSA, and 1 unit of enzyme for 135 min. Each reaction was set up in duplicate. The reactions were stopped by heating for 5 min at 100°, and the  $NH_4HCO_3$  was removed by repeated lyophilization. The relative quantities of the mono- and oligonucleotides were determined by chromatography of one of each pair on DEAE cellulose paper with 0.5 M  $NH_4HCO_3$  for 5 hours. The spots were eluted with 0.4 M KOH for 18 hours at 37°, and the absorbance at 259  $m\mu$  was determined after correction for an appropriate paper blank. The other duplicate was chromatographed with Solvent 2, and the area corresponding to marker adenosine was eluted with 0.01 M HCl. In Experiment 2, the reaction mixtures were the same as described for Experiment 1 except that they contained 0.6 unit of enzyme and were incubated for 30 min at 37°.

Substrate	Products <sup>a</sup>					
	$(Ap)_6A$ , $(Ap)_7A$ , $(Ap)_7^*p$	$(Ap)_4A$	$(Ap)_3A$	$(Ap)_2A$	pAp + AMP	Adenosine
	<i>% total absorbance</i>					
Experiment 1						
$(Ap)_6A$	15	3	28	11	44	0
$(Ap)_7$	20	1	24	13	42	
Experiment 2						
$(Ap)_6A$	54	4	13	5	25	
$(Ap)_7$	58	1	12	5	25	

<sup>a</sup> Expressed as ratio of absorbance eluted from spot to total eluted absorbance.

<sup>b</sup> These oligonucleotides were not separated by this chromatography.

TABLE VI

*Digestion of  $(Ap)_5X$* 

Reaction mixtures (0.1 ml), containing 3.8  $A_{260}$  units of oligonucleotide (580  $\mu$ M oligonucleotide), 5 mM Tris buffer (pH 7.8), 1.5 mM  $MgCl_2$ , 0.1 M KCl, 5  $\mu$ g of BSA and 0.6 unit of enzyme, were incubated for 60 min at 37°, heated for 2 min at 100°, and chromatographed in Solvent 4. The products were quantitatively eluted with 0.01 M HCl, and the concentrations were determined from the absorption at the wave length of maximum absorbance, corrected for an appropriate paper blank.

Substrate	Total $A_{260}$ recovered	AMP	UMP or CMP	Terminal nucleotide hydrolyzed
		<i>m\mu</i> moles		%
$(Ap)_5A$	3.7	14.9		
$(Ap)_5U$	3.8	32.6	31.0	53
$(Ap)_5C$	3.9	28.2	21.6	37

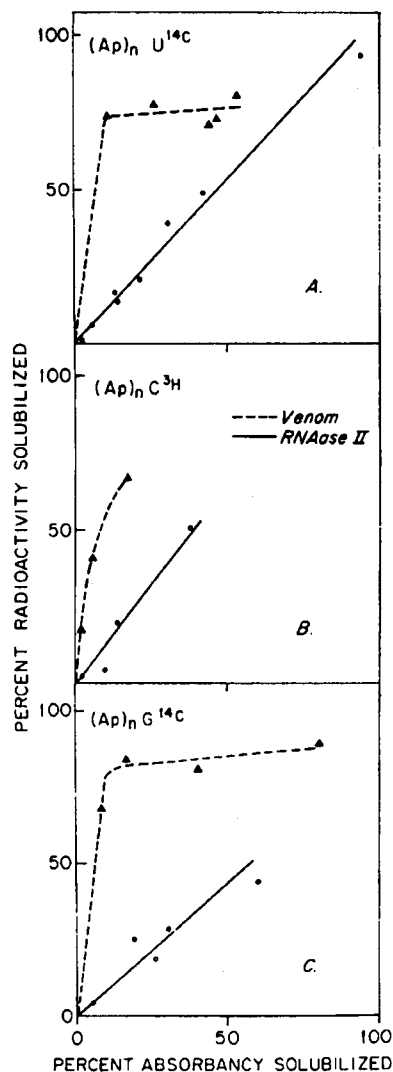


FIG. 2. Hydrolysis of  $(Ap)_nU^*$ ,  $(Ap)_nC^*$ , and  $(Ap)_nG^*$ . *A*,  $(Ap)_nU^*$ . Reaction mixtures (0.1 ml), containing (per ml) 0.1 M  $NH_4HCO_3$  buffer (pH 7.5), 1.5 mM  $MgCl_2$ , 0.05 mg of BSA, and either 1.02 mM polymer with 1.0 unit of RNase II ( $\bullet$ — $\bullet$ ) or 0.76 mM polymer and 3 units of enzyme ( $\circ$ — $\circ$ ), were incubated for periods from 5 to 120 min at 37°. The reaction was stopped by adding perchloric acid. Aliquots of the supernatant solutions were diluted with 0.05 M potassium phosphate buffer, pH 7.0, and read at 257  $m\mu$  or counted in Bray's solution. Venom phosphodiesterase reaction mixtures (0.1 ml), containing (per ml) 0.135 M  $NH_4HCO_3$  buffer (pH 9.0), 1.02 mM polymer, and 0.45 unit of enzyme, were incubated for 5 to 180 min and treated in the same manner. *B*,  $(Ap)_nC^*$ . Reaction mixtures (0.1 ml) for RNase II contained (per ml) 2.96 mM polymer, 0.1 M Tris-HCl (pH 7.5), 0.1 M KCl, 1.5 mM  $MgCl_2$ , and 4 units of enzyme and were treated as in *A*. Venom phosphodiesterase reaction mixtures had (per ml) 1.01 mM polymer, 12 mM Tris-HCl (pH 7.5), 1 mM  $MgCl_2$ , and 0.19 unit of enzyme. Aliquots (0.1-ml) were removed at various times; perchloric acid was added, and the supernatant was analyzed as in *A*. *C*,  $(Ap)_nG^*$ . Reaction mixtures for RNase II contained (per ml) 2.6 mM polymer, 0.1 M Tris-HCl (pH 7.5), 0.1 M KCl, 1.5 mM  $MgCl_2$ , 0.5 mM EDTA, and 1.2 ( $\circ$ — $\circ$ ) or 1.6 ( $\bullet$ — $\bullet$ ) units of enzyme. Venom phosphodiesterase mixtures were as described for *B*, except that they contained 2.6 mM polymer and 0.23 unit of enzyme. Aliquots (0.1-ml) were taken at various times and analyzed as in *A*.

Neither this nucleotide nor pAp was dephosphorylated when 2.9  $\mu$ moles were incubated with enough snake venom 5'-nucleotidase (32) to dephosphorylate 230  $\mu$ moles of 5'-AMP. The nucleotide (2.9  $\mu$ moles) was quantitatively converted to guanosine when incubated with enough *E. coli* alkaline phosphatase to remove 500  $\mu$ moles of  $P_i$  from pAp.

#### Experiments with Polyribonucleotides

*Polymers Labeled at 3'-Hydroxyl End*—By using polynucleotides labeled specifically at one end of the chain, the extent of hydrolysis of the labeled end can be compared with the extent of hydrolysis of the molecule as a whole at any time during digestion. Thus, with polymers of the general structure  $(Ap)_nX$ , where X is radioactive, the degradation of the total chain can be estimated by measuring the conversion of ultraviolet-absorbing material to an acid-soluble form, while the hydrolysis of the 3'-hydroxyl end is estimated by measuring the release of radioactivity into an acid-soluble form. The results expected for an enzyme which degrades in the 3' to 5' direction are exemplified by those obtained with snake venom phosphodiesterase (Fig. 2) (33); most of the radioactivity is released before a large percentage of the ultraviolet-absorbing material is solubilized. Similar experiments with RNase II yield markedly different results (Fig. 2). Thus, when the polymer  $(Ap)_nU^*$  (containing 34% of  $(Ap)_nU^*p$ ) is hydrolyzed by RNase II, the percentage of ultraviolet-absorbing material and the percentage of radioactivity released at all times, up to 100% digestion, are identical. Similar results were obtained with completely dephosphorylated  $(Ap)_nU^*$ ,  $(Ap)_nC^*$ , and  $(Ap)_nG^*$  (Fig. 2). Results such as those shown in Fig. 2 might be expected if the enzyme hydrolyzed bonds of the type  $-ApX$  more slowly than bonds of the type  $-ApA$ . However, the experiments with oligonucleotides of the type  $(Ap)_3X$  (Table VI) show that the rate of hydrolysis of the 3'-terminal group does not depend markedly on the base in that position. In order to eliminate possible endonuclease contamination as an explanation for these findings, the products of digestion of  $(Ap)_nG^*$  were studied as a function of time by chromatography in Solvent 5 for 18 hours. At all times, radioactivity from the 3'-terminal guanylic acid residue was found only in mononucleotide or in polymeric material too large to migrate from the origin. No small radioactive oligonucleotides were detected. Thus this experiment, as well as others which are summarized under "Discussion," indicate that the results described in Fig. 2 cannot be attributed to endonuclease. An alternative interpretation of the data in Fig. 2 is that RNase II begins hydrolysis of a polyribonucleotide at the 3'-hydroxyl end and continues hydrolyzing that same chain until it is essentially completely degraded (processive degradation). In contrast, venom phosphodiesterase also begins hydrolysis by removing the 3'-hydroxyl-terminal moiety of a chain, but then releases that chain and initiates hydrolysis of some other molecule.

*Polymers Labeled at 5'-Hydroxyl End*—The products of the hydrolysis of  $(pA^*)_3(pA)_n$  (average chain length, 108) by RNase II were investigated as a function of time (Fig. 3). As expected for an exonuclease that degrades in the 3' to 5' direction and leaves a resistant oligonucleotide, no  $^{14}C$ -AMP was detected until the terminal stages of the digestion. Indeed, the entire substrate was degraded completely to acid-soluble products before  $^{14}C$ -AMP was found. The data in Fig. 3 also show that, when only 30% of the total polymer was degraded, significant amounts of

$^{14}\text{C}$  (23%) had accumulated in an oligonucleotide fraction containing mainly di- and trinucleotides. No radioactivity was detected between the origin and that fraction. This indicates that those chains that were degraded at all were degraded completely. When the radioactive oligonucleotide peaks from the last two time points were pooled, lyophilized repeatedly to

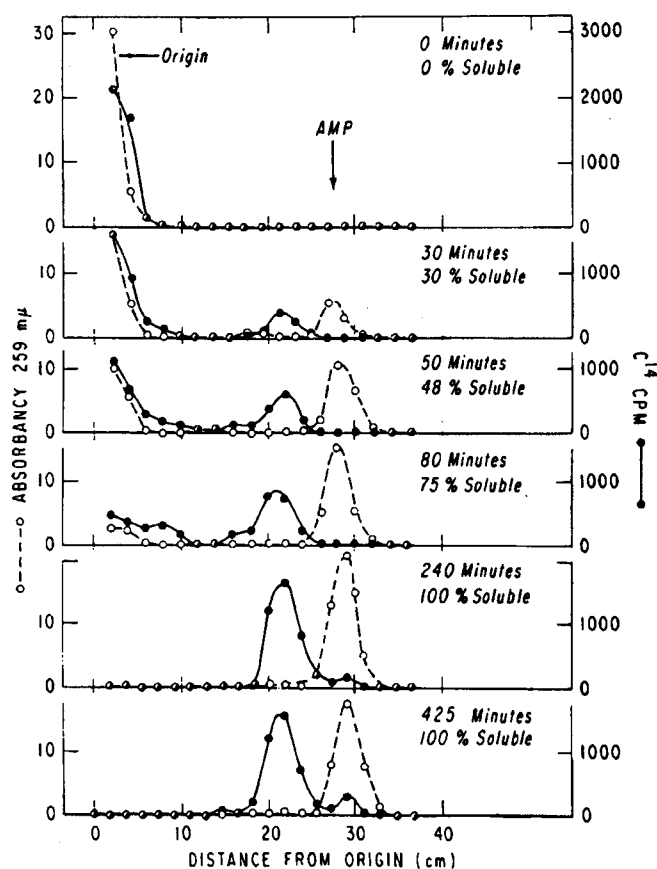


FIG. 3. Products of the hydrolysis of  $(\text{pA}^*)_2(\text{pA})_n$  by RNase II. For each time point, duplicate reaction mixtures, containing 2.75 mM  $(\text{pA}^*)_2(\text{pA})_n$ , 0.1 M  $\text{NH}_4\text{HCO}_3$  buffer (pH 7.5), 1.5 mM  $\text{MgCl}_2$ , 0.05 mg of BSA, and 3 units of enzyme in a total volume of 1 ml, were incubated for the indicated times. One reaction mixture was used to measure the distribution of radioactivity, and the other for the distribution of ultraviolet absorbance. Aliquots (50- $\mu\text{l}$ ) were removed to determine the percentage of material absorbing at 259 m $\mu$  which was acid-soluble; the remainder was lyophilized and chromatographed on DEAE-cellulose paper for 6 $\frac{1}{2}$  hours with 1 M  $\text{NH}_4\text{HCO}_3$ . Under these conditions  $(\text{pA})_2$  just leaves the origin. The position of AMP is shown in the figure. Each chromatogram was divided into 1.8-cm squares. One of each duplicate series from a given time point and a blank series for each paper were counted in the toluene scintillation solution. The second series from each time point and a series of blanks from each paper were eluted to determine the distribution of material absorbing at 259 m $\mu$ . The first four squares from the origin were eluted for 36 hours at 37° with 0.4 M KOH, and the other squares were eluted with 2 M  $\text{NH}_4\text{HCO}_3$  for 36 hours at 37°. In control experiments, 90 and 105% of the radioactivity and 105 and 104% of the absorbance were thus recovered from  $(\text{pA}^*)_2(\text{pA})_n$  and  $^{14}\text{C}$ - $(\text{pA})_2$ , respectively. No further material was eluted by 0.4 M KOH from the squares which had been eluted with 2 M  $\text{NH}_4\text{HCO}_3$ . The recovery of absorbance from successive time points was 40.4, 38.1, 41.7, 41.8, 48.5, and 43.2  $A_{259}$  units, and recovery of radioactivity was 4.3, 4.1, 4.1, 4.0, 4.4, and  $4.6 \times 10^3$  cpm, respectively.

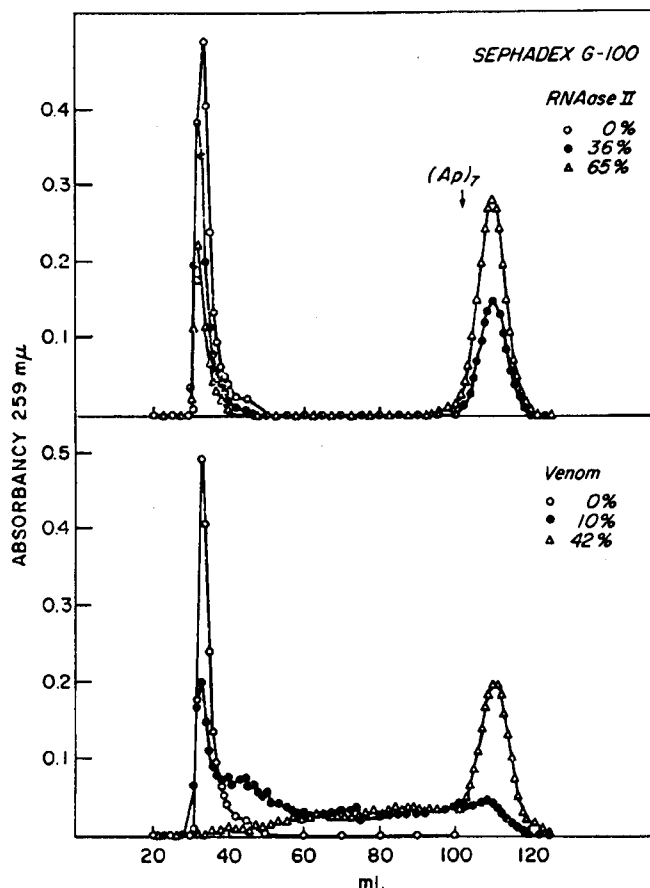


FIG. 4. Sephadex G-100 chromatography of the products of the degradation of poly A by RNase II and venom phosphodiesterase. The reaction mixtures described below were layered on a Sephadex G-100 column ( $2 \times 37$  cm) at 4°, and the column was developed with 0.1 M  $\text{NH}_4\text{HCO}_3$  (pH 8). The flow rate was approximately 6 ml per hour, and 1-ml fractions were collected. RNase II incubations contained 0.75 mM poly A (Fraction III (34)), 0.1 M Tris-HCl (pH 7.5), 0.1 M KCl, 1.5 mM  $\text{MgCl}_2$ , 0.02 mg of BSA, and enzyme in a total volume of 0.4 ml. The reaction mixtures were incubated in the chamber of a Beckman spectrophotometer (approximately 28°), and the degradation was followed by the hyperchromic shift at 259 m $\mu$  in a cell with a 1-mm path length. The amount of enzyme and times of incubation were: O, no enzyme, 0 min; ●, 1 unit, 60 min;  $\Delta$ , 2 units, 52 min. Venom phosphodiesterase reaction mixtures were incubated in a spectrophotometer cuvette as described above and contained 0.75 mM poly A (Fraction III (34)), 0.063 M Tris-HCl buffer (pH 9.0), and enzyme in a volume of 0.4 ml. The amount of enzyme and times were: O, no enzyme, 0 min; ●, 0.45 unit, 17 min;  $\Delta$ , 0.45 unit, 80 min. The position of the peak of  $(\text{Ap})_7$  is shown for reference.

remove  $\text{NH}_4\text{HCO}_3$ , and chromatographed on Whatman DE-81 paper with 0.5 M  $\text{NH}_4\text{HCO}_3$ , 4.0% of the radioactivity migrated with 5'-AMP, 23.9% with  $(\text{pA})_2$ , 66% with  $(\text{pA})_3$ , 5.8% with  $(\text{pA})_4$ , and 0.3% with  $(\text{pA})_5$ . No radioactivity was detected in compounds migrating more slowly than  $(\text{pA})_5$ .

**Size of Polymer Substrate during Hydrolysis**—The average size of the residual polymer substrate, during the course of digestion by RNase II or venom phosphodiesterase, was investigated by gel filtration on Sephadex G-100. Regardless of the extent of degradation, RNase II digests of poly A (Fig. 4) contained only poly A of the same size as the original substrate,

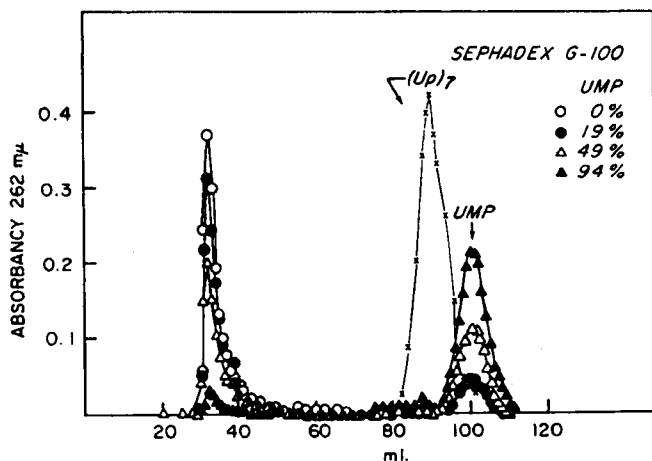


FIG. 5. Sephadex G-100 chromatography of RNase II digests of poly U. The incubations contained 2.9 mM poly U, 0.04 M Tris (pH 7.5), 0.04 M KCl, 0.5 mM MgCl<sub>2</sub>, 0.02 mg of BSA, and enzyme in a volume of 0.1 ml. Reaction mixtures were incubated at 37°. Cold H<sub>2</sub>O (0.3 ml) was added, and the mixture was layered on a Sephadex G-100 column as described in Fig. 4. O, no enzyme, 0 min; ●, 2 units, 15 min; △, 2 units, 50 min; ▲, 2 units, 75 min. The elution profile of (Up)<sub>7</sub> is given for reference.

TABLE VII  
Degradation of tRNA

In Experiment 1, the reaction mixture, containing 47 A<sub>260</sub> units of tRNA (Schwarz), 0.05 M Tris-HCl (pH 7.5), 0.1 M KCl, 1 mM MgCl<sub>2</sub>, and 36 units of RNase II in a volume of 0.6 ml, was incubated at 37°. Controls without enzyme or with neither enzyme nor tRNA were run simultaneously. At 0 and 240 min, duplicate 50-μl aliquots were removed, and the extent of hydrolysis was determined by the uranium acetate-perchloric acid method. No degradation occurred in the absence of enzyme. At the same times, 40-μl and 50-μl aliquots were taken to measure valine and serine acceptor activity, respectively (see "Experimental Procedure"). Results shown are the average of duplicate determinations. In Experiment 2, the conditions were the same as in Experiment 1 except that the RNase II digestion mixture contained 2 mM MgCl<sub>2</sub>, and 20-μl aliquots were used to determine acceptor activity. The conditions for Experiment 3 were like those for Experiment 1 except that 63 units of tRNA prepared by the method of Zubay (27) were used.

Experiment	tRNA degraded	Acceptor amino acid	Initial acceptor activity*	Loss of activity
	%			%
1	13.9	Serine	0.52	2
1	13.9	Valine	1.40	23
2	9.3	Phenylalanine	1.26	2
3	19	Valine	0.76	27

\* Nanomoles of amino acid accepted per μmole of tRNA.

AMP, and very short oligonucleotides in the shoulder of the mononucleotide peak. Similarly, digests of poly U (Fig. 5) had only the original substrate, UMP, and small amounts of very short oligonucleotides. These would be the expected products of processive degradation; no significant concentration of intermediate length polymers would accumulate. In contrast, venom phosphodiesterase digests of poly A do contain polynucleotides of intermediate length, as shown by the significant absorbance in the fractions between the original substrate and

AMP. A comparison of Figs. 4 and 5 shows that the fractionation on the gel under these conditions is not based solely on size, since UMP is eluted before AMP. However, within a homologous series, the volume required for elution decreases logarithmically with increasing chain length (35).

**Degradation of tRNA**—tRNA is a comparatively poor substrate for RNase II (2, 3), but it is degraded slowly by high levels of enzyme. It was of some interest to study the effect of this digestion on the ability of tRNA to accept amino acids, since degradation by two other exonucleolytic enzymes, namely, snake venom phosphodiesterase and polynucleotide phosphorylase, both of which degrade in the 3' to 5' direction, have yielded markedly different results. Thus, degradation with the venom enzyme quantitatively destroys amino acid acceptor activity when only 5% of the RNA has been degraded to soluble fragments (36). On the other hand, after degradation of 20% or more of a sample of tRNA by polynucleotide phosphorylase, the efficiency of the residual tRNA in accepting amino acids is the same as that of the starting material (37, 38). Table VII shows that digestion of tRNA by RNase II yields results similar to those obtained with polynucleotide phosphorylase. Even after more than 10% of the molecule has been degraded to acid-soluble material, acceptor activity remains. Furthermore, the data suggest that some species of tRNA are more susceptible to degradation by RNase II than others. For example, the same solution of tRNA lost 23% of its total valine acceptor activity, but only 2% of its serine acceptor activity, when 13.9% of the tRNA was digested.

#### DISCUSSION

Interpretation of these studies on the mechanism of action of RNase II is impossible unless contaminating endonuclease activity can be ruled out. An earlier report from this laboratory concluded that the action of highly purified RNase II on synthetic polyribonucleotides is primarily, if not exclusively, exonucleolytic (3). On the other hand, Spahr (1), using a much less purified enzyme preparation, reported significant endonucleolytic degradation of high molecular weight RNA from bacteriophage R-17. The experience in this laboratory since our earlier work has all tended to support our original conclusion. The additional evidence concerning the absence of significant endonuclease activity in our RNase II preparations will be summarized here.

Investigations of the products of the hydrolysis of oligonucleotides have given no indication of endonucleolytic cleavage even though rather high concentrations of enzyme were used. All of the products are consistent with an exonucleolytic attack from the 3'-hydroxyl end. The stoichiometry of the reaction products of the digestion of (Ap)<sub>6</sub>A (Table V) and (pA)<sub>5</sub> (legend, Table IV) show that one small oligonucleotide from the 5'-hydroxyl chain end and a corresponding number of mononucleotide units are formed from each molecule of heptamer or pentamer that is degraded.

Endonuclease activity would also have been detected when the products of polyribonucleotide digestion were studied by chromatography on paper or by gel filtration on Sephadex G-100. However, no oligonucleotides longer than tetramers moved from the origin when the products of the degradation of (Ap)<sub>n</sub>G\* and (pA\*)<sub>5</sub>(pA)<sub>n</sub> were chromatographed in Solvent 5 and on DEAE-cellulose paper, respectively. Under these conditions even nonamers have a finite mobility and would have been detected.



Similarly, Sephadex filtration of the products of poly A and poly U digestion showed no products migrating between the substrate and the small oligonucleotide derived from the 5'-hydroxyl chain end. Under these conditions, chains one-half (or less) the length of the original substrate could have been easily detected.

Recent studies by Bishop, Koch, and Levintow (39) with double stranded poliovirus RNA also suggest that highly purified RNase II is free of endonuclease. Thus, although the ineffectivity of the double stranded RNA is rapidly lost upon treatment with our Fraction VII of RNase II, the treated RNA sediments exactly like the control in 5 to 20% sucrose density gradient (Fig. 9 of Reference 39). A rapid lowering of the average size of the RNA chains would have been expected in the presence of endonuclease. It should be noted, however, that if an endonuclease hydrolyzed only one chain of the double helix, hydrogen bonding might hold the chains together, making a few such breaks per molecule undetectable by this method. The enzyme concentration in these experiments was approximately 100 times in excess of that necessary to hydrolyze completely a quantity of poly A equivalent to the viral RNA. It is of course possible that for some unknown reason the R-17 phage RNA used by Spahr is a better substrate for an endonuclease activity associated with RNase II than are the substrates we have used; on the other hand, the observed endonuclease activity in his experiments may have reflected the purity of the enzyme tested. In any case it is clear that the purified RNase II used in the present studies does not significantly hydrolyze the internal diester bonds of the synthetic polyribonucleotides used.

RNase II appears to hydrolyze both oligonucleotides and polyribonucleotides, beginning at the 3'-hydroxyl chain end. This is shown by the liberation of XMP from hexamers of the type  $(Ap)_6X$  (where X is cytidine, guanosine, or uridine), by the absence of adenosine in the products of the digestion of  $(Ap)_6A$ , and by the production of pAp and pGp upon hydrolysis of  $(Ap)_7$  and  $(Ap)_5Gp$ , respectively. Similarly, XMP is found at all stages during the digestion of polymers of the type  $(Ap)_nX$  (where X is uridine, cytidine, or guanosine), but  $^{14}C$ -AMP from a polymer labeled in the first 3 residues at the 5' hydroxyl chain end is found only during the terminal stages of the degradation.

While hydrolysis proceeds from the 3'-hydroxyl chain end, studies with polymers labeled at the 3'-terminal end show that the proportion of the terminal nucleotide and the proportion of the molecule as a whole which are acid-soluble are similar at all stages of the digestion (Fig. 2). This result contrasts sharply with that found for snake venom phosphodiesterase (Fig. 2) (33) and various *E. coli* DNA exonucleases (33, 40), in which most of the label from the 3'-terminal nucleotide residue is solubilized when less than 10% of the total molecule has been degraded. The results with RNase II are most easily interpreted as reflecting a processive degradation in which the enzyme repeatedly attacks a single polyribonucleotide chain, hydrolyzing it completely to 5'-mononucleotides and a small resistant oligonucleotide, before releasing that oligonucleotide and attaching to another chain. The products found during the digestion of the 5'-terminally labeled  $(pA^*)_3(pA)_n$  are also consistent with the processive mechanism (Fig. 3). Thus the proportion of the radioactivity from the 5' chain end which is found as oligonucleotide, and the proportion of the entire chain hydrolyzed to 5'-AMP, are similar throughout the course of the degradation. There is, in fact, a small lag in the liberation of

labeled oligonucleotide compared to AMP. This may reflect the preference of the enzyme for the longer chains, which have a higher ratio of  $^{12}C$ - to  $^{14}C$ -AMP. This lag was decreased when the substrate being hydrolyzed was more homogeneous with respect to size.<sup>4</sup> It should be pointed out that all experiments presented here were carried out at enzyme concentrations far below the substrate concentrations. Experiments with stoichiometric amounts of enzyme would be expected to yield results similar to those obtained with venom phosphodiesterase.

Independent evidence for the processive mechanism of degradation was obtained from the study of the size of the residual substrate during digestion, by gel filtration on Sephadex G-100 (Figs. 4 and 5). Even when 65% of the poly A was converted to AMP and resistant oligonucleotide, no polymers shorter than the original substrate were apparent. A small change in length of the portion of the poly A that was excluded by the gel would not have been detected, but a shortening of the bulk of the polymer would have been apparent under these conditions. The results obtained with the venom phosphodiesterase under the same conditions point out the ability of the Sephadex column to reveal polymers that are shorter than the initial substrate. The decrease in chain size observed, especially at the stage when only 10% of the polymer was degraded to AMP, is somewhat greater than that expected for simple exonucleolytic attack, and suggests that the venom preparation was probably contaminated with endonuclease. The contamination must have been quite small, however, since, when the same preparation was used for the studies with  $(Ap)_nU^*$ , most of the  $UMP$ - $^{14}C$  was liberated before 10% of the absorbance was solubilized (Fig. 2).

The effect of RNase II degradation on the capacity of tRNA to accept amino acids is also consistent with a processive attack. When 10% of the total tRNA was degraded, some of the molecules must not have lost even a single nucleotide, since the terminal -pCpCpA sequence must be intact for loading to occur (36). Consequently, other tRNA chains, which do lose their incorporation ability, are probably degraded much more than 10%.

Studies with short oligonucleotides showed that even with these comparatively poorly bound substrates the enzyme tends to attack in processive fashion. Thus, with compounds of the type  $(Ap)_6X$ , approximately equal quantities of AMP and XMP were produced at a time when up to 50% of the hexamer was still undegraded. Random attack would have resulted in a more rapid formation of XMP compared to AMP. Random attack would also have led to the accumulation, during the early stages of the digestion, of oligonucleotides 1 unit shorter than the substrate, as has been observed with snake venom phosphodiesterase (28, 41). In contrast, with RNase II, the oligonucleotide 1 unit shorter does not accumulate during any stage of the digestion of  $(pA)_5$  or  $(Ap)_6A$ . Rather, the most abundant oligonucleotide throughout the digestion is  $(pA)_3$  from chains phosphorylated at the 5'-hydroxyl end, such as  $(pA)_5$ , and  $(Ap)_3A$  from dephosphorylated chains, such as  $(Ap)_6A$  (Tables IV and V).<sup>4</sup>

The experiments reported here do not elucidate the molecular mechanism of processive degradation by RNase II. Under conditions in which  $(pA)_5$  does not accumulate as a product of the digestion of poly A, poly A strongly inhibits the hydrolysis of free  $(pA)_5$  (Table IV). This suggests that the  $(pA)_5$  unit formed

<sup>4</sup> Unpublished observations.

transitorily during the hydrolysis of poly A is not equivalent to free (pA)<sub>n</sub>. Thus, the polyribonucleotide behaves as if it were bound to the enzyme until it is degraded to an oligonucleotide of a size that no longer permits firm attachment. The interaction of polymer and enzyme may involve multiple sites or a large continuous site, thus assuring binding to the enzyme even after the terminal unit is released. The substrate might then shift position relative to the catalytic site of the enzyme, so that its new terminal residue could be hydrolyzed. For example, recent studies with lysozyme (42, 43) and various proteases (44-46) suggest that the active sites of these endodepolymerases are large enough to bind 6 or 7 monomer units simultaneously. Alternatively, the observed results may reflect the fact that the binding and diffusion constants of long polymers are such that there is a strong probability that released polymer molecules will bind again to the enzyme before they can move away. The reader is referred to the "Discussion" in the accompanying paper (34) for some further speculation on the mechanism of processive degradation.

The possibility that an exohydrolase might remove many units from one polymer molecule before beginning the degradation of another chain was previously considered by Bailey and French (47). Using specifically labeled amylase chains, they showed that sweet potato  $\beta$ -amylase removes between 3 and 4 maltose units during each period of association with the substrate. Some endopeptidases also hydrolyze a given protein molecule extensively before initiating the hydrolysis of another. However, in these latter cases, it appears that the hydrolysis of the first bond converts the protein to a form which is a better substrate than the original molecule (48, 49).

Studies of the products of the degradation of *E. coli* messenger RNA *in vivo* have led to the conclusion that both RNase II and polynucleotide phosphorylase may be involved (50, 51). It is therefore interesting that polynucleotide phosphorylase also appears to degrade by a processive attack on single polymer chains (34, 52). Preliminary studies suggest that an RNA exonuclease from Ehrlich ascites tumor cells may also function in this way (53). The mechanism may be advantageous because it allows the complete degradation of particular messenger RNA molecules without the accumulation of partial breakdown products which might interfere with protein synthesis.

*Acknowledgment*—The participation of Mr. G. P. C. Tolbert in the initial stages of this work is gratefully acknowledged.

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