Pinkerton, T. C., Paddock, G., and Abelson, J. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1218.
Rothen, A. (1940), *J. Gen. Phys. 24*, 203.
Rushizky, G. W., Mozeka, J. H., Rogerson, D. L., and Sober, H. A. (1970), *Biochemistry 9*, 4966.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112*, 346.
Uchida, T. (1966), *J. Biochem. (Tokyo) 60*, 115.
Uchida, T., Arima, T., and Egami, F. (1970), *J. Biochem. (Tokyo) 67*, 91.
Uchida, T., and Egami, F. (1971), *Enzymes 4*, 205.
Ui, N., and Tarutani, O. (1961), *J. Biochem. (Tokyo) 49*, 9.
Williams, D. E., and Reisfeld, R. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 373.
Wolfenden, R., Tomozawa, Y., and Bamman, B. (1968), *Biochemistry 7*, 3965.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Ribonuclease U_4. Novel Phosphotransferases Catalyzing Exonucleolytic Degradation of Ribonucleic Acid†

A. Blank and Charles A. Dekker*

ABSTRACT: Several structurally related ribonucleases designated RNases U_{4A}, U_{4B}, and U_{4C} have been isolated from the culture medium of the fungus *Ustilago sphaerogena;* their purification and some physical properties are described in the preceding paper (Blank, A., and Dekker, C. A., *Biochemistry 11*, 3956 (1972)). Their mode of action on RNA is described here. The major products of exhaustive digestion of wheat germ ribosomal RNA by ribonucleases U_{4A}, U_{4B}, or U_{4C} are mononucleotides. The 3′ isomers account for 95% of the A_{260} of an exhaustive RNase U_{4B} digest; the remaining, high molecular weight material can be depolymerized by the enzyme upon dialysis of product mononucleotide from the limit digest. Presence of 2′:3′-cyclic mononucleotides in partial RNase U_{4A}, U_{4B}, and U_{4C} digests of RNA was definitively established. Chromatography of partial digests on Sephadex G-25 yielded only mononucleotide and material excluded by the gel; the apparent absence of oligonucleotide was confirmed by chromatography of digests on DEAE-cellulose in 7 M urea. On degradation by RNase U_{4A}, U_{4B}, or U_{4C} of RNA bearing ^{32}P in the 5′-terminal phosphate, 50% of the radioactivity was rendered acid-soluble concomitant with hydrolysis of 10–15% of the material absorbing at 260 nm. Radioactivity was released as nucleoside diphosphate. RNases U_{4A}, U_{4B}, and U_{4C} have little or no hydrolytic activity toward denatured DNA, bis(*p*-nitrophenyl) phosphate, or *p*-nitrophenyl phosphate. Hydrolysis of RNA is not inhibited by EDTA nor significantly inhibited by several phosphate compounds. The RNA depolymerizing activities of RNases U_{4A}, U_{4B}, and U_{4C} display similar but not identical dependence on pH, with optima lying between 6 and 7, and similar but not identical response to a variety of buffers. It is concluded that the U_4 ribonucleases are novel phosphotransferases catalyzing nonprocessive, exonucleolytic degradation of RNA from its 5′ terminus.

Representatives of two major groups of enzymes, phosphodiesterases and phosphotransferases, catalyze net hydrolysis of 3′,5′-phosphodiester bonds in RNA. Cleavage of internucleotide linkages by RNA phosphotransferases is distinguished by the formation of obligatory intermediates having nucleoside 2′:3′-cyclic phosphate termini, arising from participation of the 2′-OH in intramolecular attack on the adjacent phosphodiester bond. The intranucleotide linkages thus formed may be hydrolyzed to the 3′- or perhaps rarely to the 2′-phosphate derivatives. In contrast, cleavage of RNA by phosphodiesterases is characterized by direct hydrolysis of 3′,5′-diester bonds. The catalytic action of ribonucleases has been discussed recently by Barnard (1969), Richards and Wyckoff (1971), and Uchida and Egami (1971).

The RNA phosphotransferases, or cyclizing ribonucleases, include enzymes having specificity for the base adjacent to the bond cleaved, as well as those having little or no base preference. Among the former are bovine pancreatic ribonuclease (EC 2.7.7.16) which cleaves adjacent to cytidylyl and uridylyl residues (Markham and Smith, 1952), ribonucleases T_1 and U_1 (EC 2.7.7.26) from *Aspergillus oryzae* and *Ustilago sphaerogena*, which cleave adjacent to guanylyl residues (Sato-Asano, 1959; Glitz and Dekker, 1964), and ribonucleases U_2 and U_3 from *U. sphaerogena* which under restricted conditions cleave predominantly adjacent to adenylyl and guanylyl residues (Arima *et al.*, 1968; Uchida *et al.*, 1970; Rushizky *et al.*, 1970). Included among the nonspecific cyclizing ribonucleases is RNase T_2 from *A. oryzae* (EC 2.7.7.17) which hydrolyzes high molecular weight yeast RNA entirely to 3′-mononucleotides (Rushizky and Sober, 1963).

The catalytic activity of cyclizing ribonucleases appears to be independent of divalent cations; pH optima for individual

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received March 6, 1972.* Supported in part by a grant (GB-4018) from the National Science Foundation, departmental grants (USPHS TI GM 31, USPHS AM 8845, and USPHS AM 10109), University of California Cancer Research Funds, and an American Cancer Society Postdoctoral Fellowship (PF-616) to A. B. Submitted in part by A. B. in partial fulfillment of the requirements for the Ph.D. degree in comparative biochemistry at the University of California, Berkeley. A preliminary report of this work was given at the 62nd annual meeting of the American Society of Biological Chemists, San Francisco, Calif., June 1971.

enzymes for the depolymerization reaction may vary over the range, pH 4–9 (Barnard, 1969; Uchida and Egami, 1971). The known RNA phosphotransferases are endonucleases. Although, as Barnard (1969) has remarked, phosphotransferase activity might in principle be specific for the terminal internucleotide bond in RNA such restriction has been observed only among phosphodiesterases. Evidence will be presented here showing that several related ribonucleases from *U. sphaerogena*, designated RNases U_{4A}, U_{4B}, and U_{4C}, and collectively designated RNase U_4 (Blank and Dekker, 1972), are novel phosphotransferases catalyzing exonucleolytic degradation of RNA.

Experimental Procedure

Ribonucleases U_{4A} (47,500 units/mg of protein), U_{4B} (53,400 units/mg of protein), and U_{4C} (47,100 units/mg of protein) were purified from the culture medium of *U. sphaerogena* as described in the preceding paper (Blank and Dekker, 1972). In addition to those described therein, the following materials and procedures were employed. Ribonuclease U_1 (160,000 units/mg of protein) was purified by Dr. W. C. Kenney (Kenney and Dekker, 1971). Whole snake venom (*Crotalus adamanteus*) was obtained from Sigma, snake venom (*C. adamanteus*) phosphodiesterase and *Escherichia coli* alkaline phosphatase from Worthington. Polynucleotide kinase (fraction VI of Richardson, 1965) was the generous gift of Dr. Stuart Linn.

High molecular weight wheat germ RNA, used throughout this study, was prepared by Dr. Kenney and Mr. Owen Griffith essentially as described by Singh and Lane (1964); its concentration was estimated spectrophotometrically using the $E_{260}^{1\%}$ of 220 given by these authors. To prepare RNA radioactively labeled at its 5′ terminus, RNA (12 mg/ml) was incubated 27 hr at 4° with 2 μg/ml of alkaline phosphatase in 0.1 M Tris-HCl (pH 8). The mixture was extracted with phenol and then with ether; RNA was precipitated from the aqueous phase with 3 M NaCl. The redissolved precipitate was incubated with polynucleotide kinase and [γ-^{32}P]ATP, essentially as described by Richardson (1965); incorporation of ^{32}P into acid-insoluble material was shown to reach a limit. The radioactive RNA (2.3×10^5 cpm/mg) was separated from unreacted ATP by passage through a 1.0×48 cm Sephadex G-50 column equilibrated with 0.1 M ammonium formate buffer (pH 7.9). Radioactivity was measured in Bray's (1960) solution in a Nuclear-Chicago liquid scintillation counter.

Salmon sperm DNA was purchased from Calbiochem, *p*-nitrophenyl phosphate from Sigma; bis(*p*-nitrophenyl) phosphate was prepared in this laboratory. [γ-^{32}P]ATP was the kind gift of Dr. Stuart Linn. 2′(3′)-Mononucleotides and 5′-mononucleotides were obtained from Calbiochem; 3′-mononucleotides, dinucleoside monophosphates, and 2′:3′-cyclic uridylic acid were prepared in this laboratory. Chemicals used were of reagent grade. Sephadex G-25 (fine) and G-50 were purchased from Pharmacia, DEAE-cellulose (DE-52) from Whatman, Dowex 1-X8, 200–400 mesh, from Dow Chemical Co.

Deoxyribonuclease, phosphodiesterase, and phosphomonoesterase activities of ribonuclease U_4 preparations were measured using salmon sperm DNA incubated 10 min at 100° and rapidly cooled in ice–water, bis(*p*-nitrophenyl) phosphate, and *p*-nitrophenyl phosphate, respectively, as substrates. Each activity was measured at pH 5.0 in sodium acetate buffer, at pH 6.5 in sodium phosphate buffer, and at pH 8.0 in Tris-HCl buffer. Incubation mixtures contained 7.7×10^{-3} M substrate, 0.1% bovine serum albumin, 0.1 M buffer, and either 13.5 units of ribonuclease U_{4A}, 28 units of ribonuclease U_{4B}, or 11 units of ribonuclease U_{4C} in 0.75 ml. (DNA concentration is given with respect to phosphorus [8.1%].) For reference mixtures containing RNA (7×10^{-3} M), $^1/_{100}$th as much enzyme was used. After 20- to 28-hr incubation at 28° measurement of hydrolysis products was carried out as follows. One-half milliliter of each mixture containing RNA was removed to 1 ml of ice-cold 10% perchloric acid; following 10-min standing at 0°, 2 ml of H_2O was added and the suspension was centrifuged 10 min at 14,000*g* at 0°. The A_{260} of the supernatant solution was used to estimate the concentration of acid-soluble products, assuming that absorption was due entirely to mononucleotides and employing a weighted $\epsilon_{260} = 10.7 \times 10^3$ reflecting the base composition of the RNA (Glitz and Dekker, 1963). DNA hydrolysis products were estimated in the same way using an analogous, weighted $\epsilon_{260} = 10.2 \times 10^3$ (Chargaff *et al.*, 1951). Mixtures containing bis(*p*-nitrophenyl) phosphate were diluted with 0.75 ml of 1 N NH_4OH and the A_{400} was measured; an ϵ_{400} of 17.5×10^3 for the *p*-nitrophenolate ion (Bessey *et al.*, 1946) was used to estimate the amount of substrate hydrolyzed. Mixtures containing *p*-nitrophenyl phosphate were diluted with 2.5 ml of 1 N NH_4OH and the amount of substrate hydrolyzed was determined as for those containing phosphodiesterase substrate.

Descending chromatography on Whatman No. 3MM paper was carried out for 16–18 hr in solvent 1 [isopropyl alcohol–water, 7:3, v/v, with 0.35 ml of concentrated NH_4OH/l. of air space (Markham and Smith, 1952)]. Two-dimensional, ascending chromatography was carried out on 21×21 squares of Whatman No. 1 paper, 3–3.5 hr in the first dimension in solvent 2 (isobutyric acid–0.5 N NH_4OH, 5:3, v/v, pH 3.7) and 3–3.5 hr in the second dimension in solvent 3 (saturated $NH_4(SO_4)_2$–isopropyl alcohol–0.1 M sodium citrate, pH 6.0, 40:1:9, v/v) (Arima *et al.*, 1968; Wyatt, 1955). Electrophoresis was carried out for 1–1.5 hr on Whatman No. 3MM paper, 56 cm in length, at 35 V/cm in solvent 4 [0.25 M ammonium acetate, 0.005% EDTA, pH 3.6 (Bielski and Young, 1963)]. Chromatograms and electrophoretograms were viewed under an ultraviolet light having maximum emission at 254 nm.

Results

Enzymatic Activity toward Phosphate Esters. Ribonucleases U_{4A}, U_{4B}, and U_{4C} were tested for hydrolytic activity toward denatured DNA, bis(*p*-nitrophenyl) phosphate, and *p*-nitrophenyl phosphate. As indicated in Table I, the ribonuclease U_{4A} preparation displayed measurable phosphodiesterase activity at pH 5.0 although the average rate of hydrolysis of bis(*p*-nitrophenyl) phosphate was *ca.* 2000 times slower than that of RNA. Phosphodiesterase activity was not observed in the other RNase preparations nor was phosphomonoesterase activity detected.

As is reported in Table I, 0.6 to 1.1% of the substrate was rendered acid soluble on incubation of DNA with the RNase U_4 preparations. However, it is probable that the observed hydrolysis products were derived from RNA contaminating the DNA preparation; the substrate had not been treated with ribonuclease by the supplier and was hydrolyzed to the extent of 1.5% on 17-hr incubation at pH 7 with 3 or 13 μg per ml of five-times-crystallized pancreatic RNase.

pH Dependence of Activity. The depolymerizing activity of ribonucleases U_{4A}, U_{4B}, and U_{4C} was measured in 0.04 M

TABLE I: Hydrolytic Activity of Ribonucleases U_{4A}, U_{4B}, and U_{4C} toward Several Phosphate Esters.[a]

pH	Ester	Av Rate of Hydrolysis (nmoles/hr/unit of enzyme)		
		U_{4A}	U_{4B}	U_{4C}
8.0	RNA	1.8×10^{2}	1.8×10^{2}	1.8×10^{2}
	DNA	1.1×10^{-1}	0.7×10^{-1}	1.5×10^{-1}
	Bis(*p*-nitrophenyl) phosphate	$\leq 2.9 \times 10^{-3}$	$\leq 1.4 \times 10^{-3}$	$\leq 3.7 \times 10^{-3}$
	p-Nitrophenyl phosphate	$\leq 6.8 \times 10^{-3}$	$\leq 3.3 \times 10^{-3}$	$\leq 8.6 \times 10^{-3}$
6.5	RNA	2.0×10^{2}	1.8×10^{2}	1.9×10^{2}
	DNA	1.4×10^{-1}	0.7×10^{-1}	1.9×10^{-1}
	Bis(*p*-nitrophenyl) phosphate	$\leq 2.9 \times 10^{-3}$	$\leq 1.4 \times 10^{-3}$	$\leq 3.7 \times 10^{-3}$
	p-Nitrophenyl phosphate	$\leq 7.0 \times 10^{-3}$	$\leq 3.4 \times 10^{-3}$	$\leq 8.8 \times 10^{-3}$
5.0	RNA	0.7×10^{2}	1.1×10^{2}	0.7×10^{2}
	DNA	1.9×10^{-1}	0.9×10^{-1}	2.4×10^{-1}
	Bis(*p*-nitrophenyl) phosphate	3.2×10^{-2}	$\leq 1.4 \times 10^{-3}$	$\leq 3.6 \times 10^{-3}$
	p-Nitrophenyl phosphate	$\leq 6.7 \times 10^{-3}$	$\leq 3.2 \times 10^{-3}$	$\leq 8.4 \times 10^{-3}$

[a] Following extended incubation of enzyme with ester, hydrolysis products were measured spectrophotometrically as described in Experimental Procedure. Where changes in optical density due to enzyme-catalyzed hydrolysis were not indicated, a value of 0.010 was assumed in order to calculate an upper limit for the rate of hydrolysis.

sodium phosphate buffer in the pH range 5–8. Results are illustrated in Figure 1.

Effect of Several Buffers on Activity. The depolymerizing activity of ribonucleases U_{4A}, U_{4B}, and U_{4C} was measured in a variety of buffers at pH 6.0 and 7.0. Results are given in Table II.

Products of Exhaustive Digestion of RNA. RNA (1.7 mg) was incubated 18 hr at 30° in 0.30 ml of 0.10 M sodium citrate buffer (pH 7.0), containing either 778 units of RNase U_{4A}, 1630 units of RNase U_{4B}, or 1968 units of RNase U_{4C}. To 0.15 ml of the corresponding digest was added 389 units of RNase U_{4A}, 762 units of RNase U_{4B}, or 984 units of RNase U_{4C}; incubation was continued an additional 12 hr. Digests were stored frozen.

Aliquots of the 30-hr ribonuclease U_{4A}, U_{4B}, and U_{4C} digests containing degradation products of 65–80 μg of RNA were chromatographed in solvent 1. As illustrated in Figure 2, nearly all the detectable material in the digests traveled

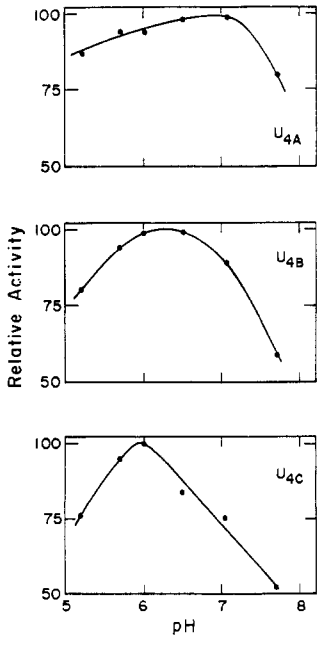

FIGURE 1: Dependence of ribonuclease activity on pH in 0.04 M sodium phosphate buffer. Activity was measured in assay 2 (Blank and Dekker, 1972) substituting sodium phosphate buffer at the indicated pH for imidazole-HCl. Enzyme was diluted as necessary into 0.04 M sodium phosphate–0.1% bovine serum albumin (pH 7.1) prior to assay of 10 μl.

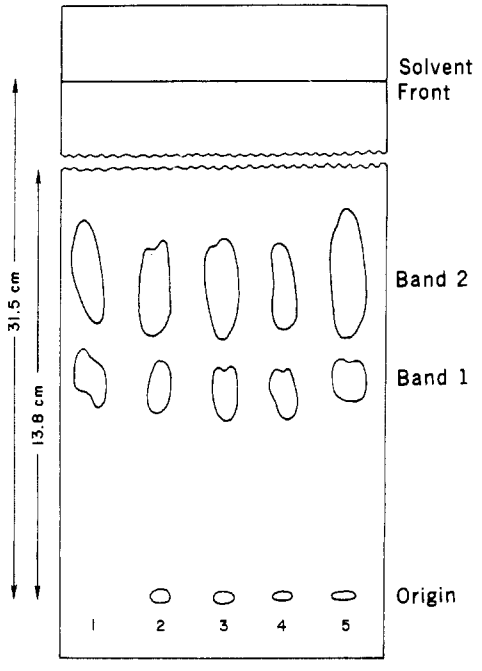

FIGURE 2: Paper chromatography of the products of extended digestion of RNA. RNA was incubated 30 hr with ribonuclease U_{4A}, U_{4B}, or U_{4C}, as described in the text and chromatographed in solvent 1. Resolved samples are: 1, a mixture of 2′(3′)-Gp, -Up, -Cp, -Ap; 2, ribonuclease U_{4A} digest; 3, ribonuclease U_{4B} digest; 4, ribonuclease U_{4C} digest; 5, 2′(3′)-mononucleotides plus ribonuclease U_{4B} digest. Band 1 contains Gp, band 2 Up, Cp, and Ap (Markham and Smith, 1952; Reddi, 1958).

TABLE II: Effect of Several Buffers on Ribonuclease Activity.[a]

Buffer	pH	Relative Activity			
		U_{4A}	U_{4B}	U_{4C}	U_1
Imidazole (Cl^-)	7	100	100	100	100
EDTA (Na^+)	7	86	70	68	45
Bicarbonate (NaCl)	7	41	27	28	10
Tris (Cl^-)	7	85	59	61	49
Phosphate (Na^+)	7	85	59	64	2
Pyrophosphate (NaCl)	7	54	53	42	<1
β-Glycerolphosphate (NaCl)	7	73	70	62	10
Citrate (Na^+)	7	75	71	62	
Imidazole (Cl^-)	6	100	100	100	100
EDTA (Na^+)	6	73	84	88	65
Bicarbonate (NaCl)	6	77	114	114	112
Phosphate (Na^+)	6	74	98	95	24
Pyrophosphate (NaCl)	6	55	56	60	
β-Glycerolphosphate (NaCl)	6	71	70	78	30
Citrate (Na^+)	6	68	70	96	28
Acetate (Na^+)	6	58	67	114	

[a] Ribonuclease activity was determined in assay 2 (Blank and Dekker, 1972) substituting the indicated buffers (0.04 M) for imidazole; inorganic ions introduced with each buffer are given in parentheses. Enzyme was diluted into 0.1 M imidazole-HCl–0.1% bovine serum albumin (pH 7.0), and 10 μl of diluted enzyme was assayed; incubation mixtures with the alternate buffers contained therefore 1.3×10^{-3} M imidazole. Activity of ribonuclease U_1 was determined in 0.04 M buffer by a procedure similar to ribonuclease assay 1 (Blank and Dekker, 1972) except that aliquots were withdrawn at 0 and 15 min after mixing of enzyme and substrate (data from Glitz and Dekker, 1964). Activity is expressed relative to that found in imidazole buffer.

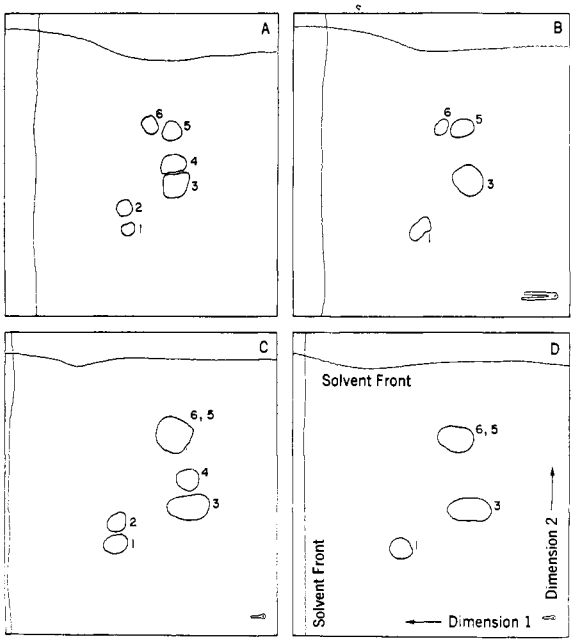

FIGURE 3: Paper chromatography of the products of extended digestion of RNA. RNA was incubated 30 hr with ribonuclease U_{4B} and subjected to two-dimensional chromatography as described in the text. Chromatograms were spotted with (a) a mixture of 2′(3′)-mononucleotides, (b) ribonuclease U_{4B} digest, (c) ribonuclease U_{4B} digest plus 2′(3′)-mononucleotides, (d) ribonuclease U_{4B} digest plus 3′-Ap and 3′-Gp. The numbered spots are: 1, 3′-AMP; 2, 2′-AMP; 3, 3′-GMP; 4, 2′-GMP; 5, 2′- and 3′-UMP; 6, 2′- and 3′-CMP (Arima *et al.*, 1968; Wyatt, 1955).

with the mobility of 2′(3′)-mononucleotides; a very small amount remained in a tight, discrete spot at the origin. Ultraviolet-absorbing material did not appear in the area between Gp and the origin; this region would be expected to contain dinucleotides having guanylyl residues and trinucleotides.[1]

Aliquots of the 30-hr ribonuclease U_{4A}, U_{4B}, and U_{4C} digests containing degradation products of 55–65 μg of RNA were subjected to two-dimensional paper chromatography. As shown for the ribonuclease U_{4B} digest (Figure 3b), all detectable uv-absorbing material traveled with the mobility of 2′(3′)-mononucleotide standards (Figure 3a) and cochromatographed with these standards (Figure 3c), or remained at the origin; similar results were observed for the ribonuclease U_{4A} and U_{4C} digests. The small amount of material at the origin displayed very slight mobility in the first dimension. Ultraviolet-absorbing material having low mobility in both dimensions was not detected at about 45° from the origin; this region would be expected to contain small oligonucleotides (Arima *et al.*, 1968). Chromatograms of the 30-hr ribonuclease U_{4A}, U_{4B}, and U_{4C} digests displayed a single spot in the Ap region corresponding to the 3′ isomer and a single spot in the Gp region corresponding to the 3′ isomer. Comparable single spots were observed on cochromatography of the ribonuclease U_{4B} digest with authentic 3′-Ap and 3′-Gp (Figure 3d), in contrast to the double spots seen on cochromatography with the mixed 2′(3′) isomers (Figure 3c).

Mononucleotides appearing in RNase U_{4A}, U_{4B}, or U_{4C} digests were treated with snake venom 5′-nucleotidase in the following manner. Each 30-hr digest was chromatographed in solvent 1 (see Figure 1) and the partially resolved mononucleotides (bands 1 and 2) were eluted in H_2O and lyophilized. An aliquot of each eluate estimated spectrophotometrically to contain 0.1 μmole of nucleotide[2] was incubated 16 hr at 36° with 10 μmoles of sodium glycinate (pH 8.5), 10 μmoles of $MgCl_2$, and 20 μg of whole snake venom in a volume of 1 ml. A mixture of *ca.* 0.03 μmole each of 5′-AMP, -CMP, -GMP, and UMP, and an alkaline digest of RNA containing 0.1 μmole of nucleotide were treated in the same manner. Following incubation, aliquots of the digests containing 0.02 μmole of phosphate were assayed for P_i by the procedure of Ames and Dubin (1960). Mononucleotide derived from alkali- or ribonuclease U_4 treated RNA yielded no more than the trace of P_i found on incubation without venom; the 5′-mononucleotide mixture, on the other hand, gave 76% of the theoretical amount of inorganic phosphate under the same conditions.

Mononucleotides appearing in a ribonuclease U_{4B} digest were fractionated on Dowex 1; as shown in Figure 4, each nucleotide chromatographed as the 3′ isomer.

[1] Resolution in this solvent system depends upon several factors. All guanine compounds have lower mobility than do the corresponding derivatives of the other bases; all cyclic phosphates have greater mobility than the corresponding noncyclic derivatives; mononucleotides move faster than dinucleotides which in turn move faster than trinucleotides.

[2] Total micromoles was calculated using the nucleotide composition of high molecular weight wheat germ RNA given by Glitz and Dekker (1963).

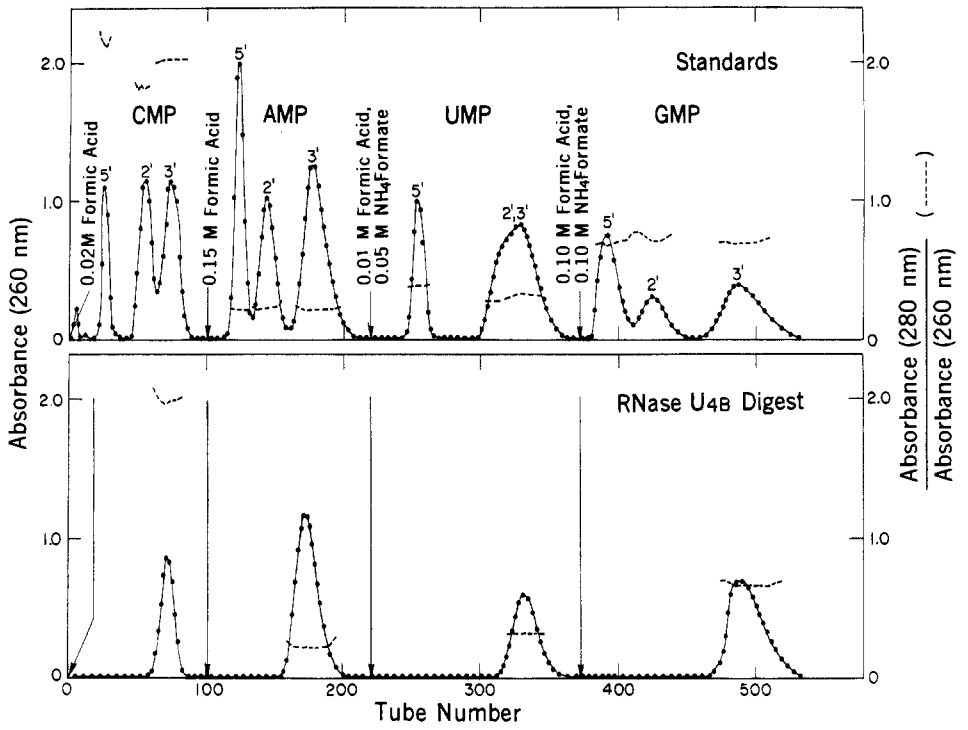

FIGURE 4: Resolution of mononucleotides by anion-exchange chromatography on Dowex 1. RNA (17 mg) was incubated 5 hr at 28° with 5400 units of ribonuclease U_{4B} in 3 ml of 0.03 M imidazole-HCl–0.1% bovine serum albumin (pH 7.0). The digest was applied to a 0.8 × 11 cm Dowex 1-X8 column, 200–400 mesh, equilibrated with 0.02 M formic acid. The column was developed as shown (Cohn and Volkin, 1951); 5-ml fractions were collected at a flow rate of 1 ml/min.

Aliquots of the 18- and 30-hr ribonuclease U_{4B} digests were chromatographed on a 1.5 × 6.8 cm Sephadex G-25 column. (Procedures are the same as those described later in Figure 6.) In both cases, 95–96% of the A_{260} eluted from the column as mononucleotide; the remaining 5%, having an A_{280}/A_{260} of 0.50, emerged as a well-defined peak at the elution position of undigested RNA. Evidently the products of exhaustive digestion include, in addition to mononucleotide, a core of relatively high molecular weight material largely resistant to enzymatic degradation.

To determine whether the material resistant to degradation might result from product inhibition of the enzyme, RNA was digested with ribonuclease U_{4B} for 30 hr as described earlier except that the last 12 hr of incubation was carried out, without the additional aliquot of enzyme, in dialysis tubing immersed in a 17-fold volume of buffer. At the conclusion of digestion 7% of the total A_{260} units of the digest remained in the dialysis tubing; the A_{260} of the dialyzed sample (A_{280}/A_{260} = 0.56) was 11% higher than that of the dialysis medium (A_{280}/A_{260} = 0.51). Chromatography on Sephadex G-25 of the dialyzed digest yielded a profile essentially unchanged from previous ones, about 95% of the A_{260} eluting as a bifurcated peak in the mononucleotide region and the remaining 5% eluting at the RNA position; however, the core material was found to comprise less than 0.3% of the total A_{260} of the digest when corrections were made for the 17-fold greater amount of mononucleotide outside the dialysis sac and the absorbancy due to the enzyme (0.030 A_{260} unit).

Products of Partial Digestion. RNA (2.8 mg) was incubated with 4–5 units of ribonuclease U_{4A}, U_{4B}, or U_{4C} for 4 hr at 28° in 0.25 ml of 0.1 M imidazole-HCl (pH 7.0). On chromatography of the partial digests in solvent 1 (Figure 5), five bands were visible, their positions and relative intensities appearing the same among the three digests. By comparison with previous chromatograms (Figure 2), with the data of Markham and Smith (1952) and Reddi (1958), and with the observed behavior of standards, the following tentative assignments were made. Band 1 contains Gp, band 2 Up, Cp, and Ap, band 3 G>p, band 4 U>p, and band 5 C>p and/or A>p. The identity of the substances present in each of the five bands was established in the following experiments.

RNA (14 mg) was partially digested with ribonuclease U_{4B} as described above and chromatographed in solvent 1. The material in each of the five bands was eluted in H_2O, lyophilized, redissolved in 0.2 to 1 ml of H_2O, and brought to pH 8–9 with a drop of dilute NH_4OH.

BANDS 1 AND 2. Bands 1 and 2 occupied, in the chromatograms of partial digests, positions comparable to those in chromatograms of exhaustive digests (Figure 2), band 1 having the mobility of authentic 2′(3′)-Gp and band 2 the mobility of 2′(3′)-Up, -Cp, and -Ap. Electrophoresis of band 1 eluate in solvent 4 yielded a major spot with the mobility of 2′(3′)-Gp. Electrophoresis of band 2 eluate yielded three spots having the mobilities of authentic 2′(3′)-Cp, -Ap, and -Up, respectively.

BANDS 3, 4, AND 5. Aliquots of eluate containing 0.1–0.2 A_{260} units were spotted on Whatman No. 3MM paper (previously washed in solvent 1 and thoroughly dried) and subjected to electrophoresis in solvent 4. Each eluate yielded a single spot traveling, for bands 3, 4, and 5, very slightly ahead of 2′(3′)-Gp, -Up, and -Cp, respectively.

To 90 μl of each eluate containing 0.4–0.6 A_{260} unit was added 10 μl of 1 N HCl. After 5 hr at room temperature the eluates were neutralized with 1 N NaOH and 30 μl of each was again subjected to electrophoresis. Each eluate yielded a single spot having, for bands 3, 4, and 5, the mobility of 2′-(3′)-Gp, -Up, and -Cp, respectively.

Each acid-treated, neutralized eluate was chromatographed

in solvent 1 along with the corresponding untreated material. Whereas each untreated sample migrated to a position comparable to that in the original chromatogram (Figure 5), the acid-treated eluates displayed diminished mobilities, that of band 3 having the mobility of 2′(3′)-Gp and that of bands 4 and 5 the mobility of 2′(3)-Up and -Cp.

To 30 μl of each eluate were added 3.0 μmoles of NH_3–NH_4Cl buffer (pH 7.7) and 2 μg of pancreatic ribonuclease. After 18-hr incubation at 30° the mixtures were chromatographed, together with control samples incubated without enzyme, in solvent 1. Whereas each control eluate, as well as the ribonuclease-treated eluate of band 3 migrated to a position comparable to that in the original chromatogram (Figure 5), the ribonuclease-treated eluates of bands 4 and 5 migrated with the mobility of 2′(3′)-Up and -Cp.

Mode of Degradation of RNA. Gel filtration through Sephadex G-25 was used to distinguish the products of endonucleolytic and exonucleolytic cleavage of RNA; the method was adapted from the procedure of Birnboim (1966). Undigested RNA held 5 min at 100° eluted from the short, 1.5 × 6.8 cm G-25 column in a single peak, slightly after the void volume; that some degradation of the RNA occurred during heat treatment is indicated by the residual uv-absorbing material eluting at higher volumes (Figure 6b). As shown in Figure 6a, a mixture of 2′(3′)-mononucleotides arising from alkaline digestion of RNA emerged well separated from the polymer. Splitting of the monomer peak reflects partial resolution of the four major 2′(3′)-mononucleotides.[3]

When RNA is cleaved by ribonuclease U_1, an endonuclease (Glitz and Dekker, 1964), the products of partial digestion (Figure 6b) chromatograph as an increasingly broad peak in the RNA region with progressive accumulation of heterodisperse material retarded by the gel, reflecting formation by ribonuclease U_1 of oligonucleotides of decreasing average molecular weight. When RNA is degraded by snake venom phosphodiesterase, an exonuclease (Razzell and Khorana, 1959), the partial digestion products chromatograph as two discrete peaks, one of diminishing size at the position of undigested RNA and a second of increasing size in the mononucleotide region (Figure 6c).[4] At the stages of digestion examined, material of intermediate elution volume does not accumulate, reflecting removal by the enzyme of terminal mononucleotide residues. When RNA is degraded by ribonuclease U_{4B} (Figure 6d), the partial digestion products display a chromatographic pattern fundamentally similar to that found for venom phosphodiesterase. There is a gradually diminishing peak in the RNA position and a second, increasingly large peak in the mononucleotide region; the monomer peak is split, with maxima at the positions found in the elution pattern of 2′(3′)-mononucleotides. At the stages of digestion examined products having an elution volume intermediate between RNA and its monomer do not accumulate. Results similar to those illustrated for ribonuclease U_{4B} (Figure 6d) were obtained on Sephadex G-25 chromatography of partial ribonuclease U_{4A} and U_{4C} digests. The apparent absence of oligonucleotides was confirmed by chromatography of the partial digestion products on DEAE-cellulose, illustrated in Figure 7.

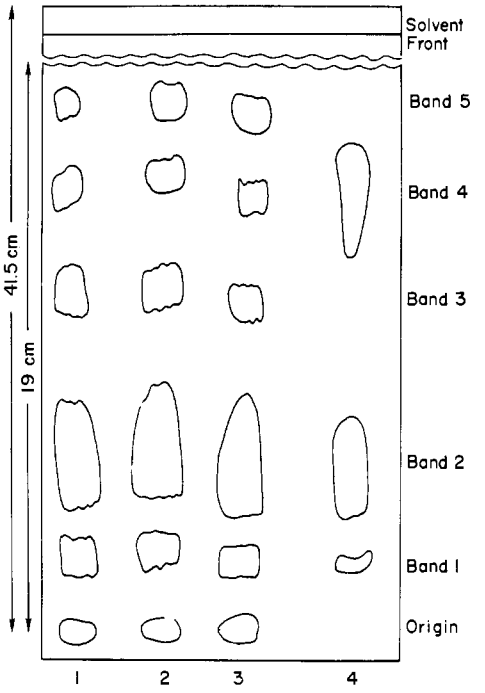

FIGURE 5: Paper chromatography of partial digests of RNA. RNA was incubated with ribonuclease U_{4A}, U_{4B}, or U_{4C} as described in the text, and the partial degradation products of 570 μg of RNA were chromatographed in solvent 1. The resolved samples are: 1, ribonuclease U_{4A} digest; 2, ribonuclease U_{4B} digest; 3, ribonuclease U_{4C} digest; 4, 2′(3′)-mononucleotides and 2′:3′-cyclic uridylic acid. Band 1 contains Gp; band 2 Up, Cp, and Ap; band 4 U>p.

Ribonuclease U_{4A}, U_{4B}, or U_{4C} was incubated with RNA bearing ^{32}P in its 5′-terminal phosphate. As shown in Figure 8 for ribonuclease U_{4B}, 50% of the radioactivity was rendered acid soluble when 13% of the uv-absorbing material was hydrolyzed; during every time interval examined, *e.g.*, 0–2 or 2–4 min, the proportion of acid-precipitable label solubilized exceeded the proportion of acid-precipitable absorbancy solubilized. Similar results were obtained for ribonucleases U_{4A} and U_{4C}.

Exhaustive ribonuclease U_{4A}, U_{4B}, and U_{4C} digests of [^{32}P]RNA were subjected to electrophoresis in solvent 4. Four spots, given by Cp, Ap, Gp, and Up, respectively, were visible in each digest. Lanes containing radioactivity were cut into 0.5- or 1.0-cm strips and the strips were counted in Bray's solution (Bray, 1960) in a Nuclear-Chicago liquid scintillation counter. Four radioactive spots having the mobilities expected for pCp, pAp, pGp, and pUp (Bielski and Young, 1963) were detected in each digest. The slowest and fastest of these spots migrated with the mobility of authentic pCp and pUp, respectively.

Discussion

That RNases U_{4A}, U_{4B}, and U_{4C} display little or no hydrolytic activity toward denatured DNA and bis(*p*-nitrophenyl) phosphate (Table I) indicates that, using the terminology recommended by Barnard (1969), these enzymes are

[3] Purine ribonucleotides migrate more slowly than pyrimidine ribonucleotides, due, presumably, to enhanced hydrophobic interaction of the purine derivatives with the resin. Similar behavior has been observed for the corresponding deoxy compounds (Hohn and Schaller, 1967).

[4] The monomers arising from treatment of RNA with crude snake venom phosphodiesterase chromatographed as a broad peak with its maximum lying between the two maxima given by 2′(3′)-mononucleotides. In accord with this observation, we have found that a mixture of 5′-AMP, -CMP, -GMP, and -UMP [containing nucleotides in the molar proportions found in high molecular weight wheat germ RNA (Glitz and Dekker, 1963)] is less readily resolved on Sephadex G-25 than a comparable mixture of either the 3′ or the 2′(3′) isomers.

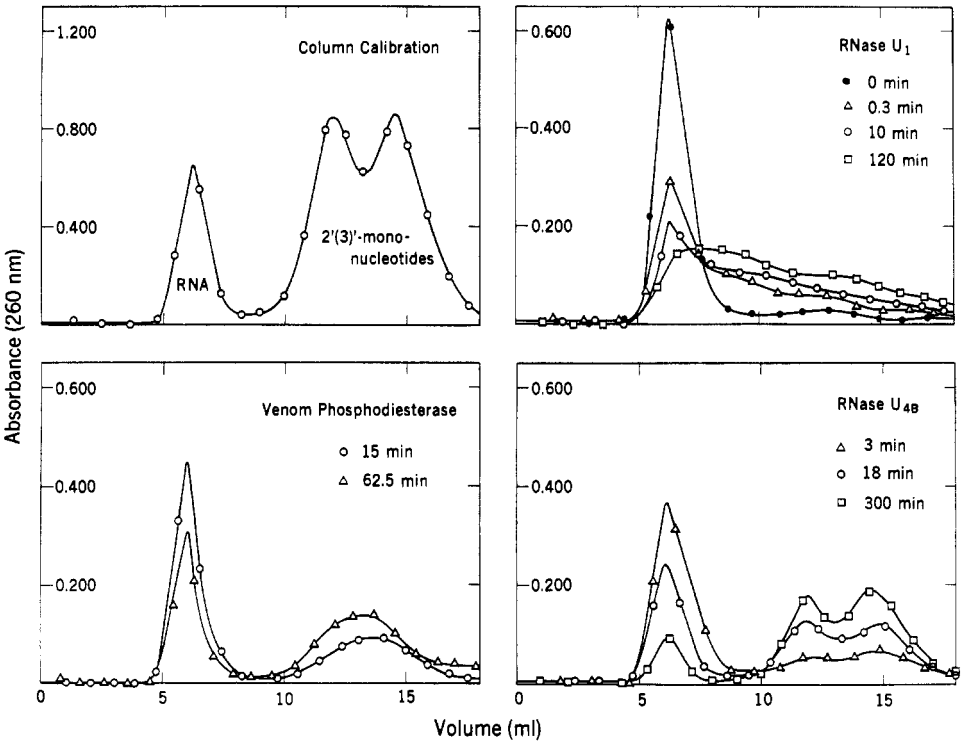

FIGURE 6: Chromatography on Sephadex G-25 of partial enzymatic digests of RNA. Following incubation at 28° for the times indicated, 1 ml aliquots of digests were removed, held 5 min at 100°, chilled rapidly in ice–water, and frozen. The samples were subsequently applied to a 1.5 × 6.8 G-25 column equilibrated with 0.015 M sodium citrate–0.15 M NaCl (pH 7.0). The column was washed with 25 ml of the same buffer; fractions of *ca.* 1 ml were collected at a flow rate of 1 ml/min. Following determination of the A_{260} of individual fractions, eluate volumes were determined by careful measurement of the volume of appropriately pooled fractions. (a) RNA (50 μg) and RNA (150 μg) digested 18 hr at 37° in 0.32 N KOH and neutralized with $HClO_4$ were dissolved in 1 ml of 0.025 M sodium citrate buffer (pH 7.0). The mixture was held 5 min at 100°, cooled in ice–water, frozen, and subsequently chromatographed. (b) Digests contained 50 μg/ml of RNA and 13 units/ml of ribonuclease U_1 in 0.023 M Tris-HCl (pH 7.6). (c) Digests contained 100 μg/ml of RNA and 500 μg/ml of snake venom phosphodiesterase preparation in 0.025 M sodium glycinate buffer (pH 8.8). For these digests, 0.5-ml aliquots were withdrawn to 0.5 ml of 0.25 M sodium phosphate buffer (pH 7.0); the 1.0-ml samples were placed in a boiling-water bath and treated as described above. (d) Digests contained 50 μg/ml of RNA and 9 units/ml of ribonuclease U_{4B} in 0.025 M sodium citrate buffer (pH 7.0).

ribonucleases rather than nucleases or phosphodiesterases. The slight but real hydrolysis of bis(*p*-nitrophenyl) phosphate displayed by the RNase U_{4A} preparation at pH 5 could reflect contamination with an acid phosphodiesterase. None of the preparations bears intrinsic or contaminating phosphomonoesterase activity as judged by their inability to hydrolyze *p*-nitrophenyl phosphate.

As shown in Table II, the individual U_4 enzymes do not, in general, vary greatly in their depolymerizing activity in a number of commonly used buffers. Imidazole buffer, with few exceptions, permitted the maximum rates of hydrolysis observed for RNases U_4 and U_1, possibly reflecting synergism of buffer and enzyme in catalysis. Interestingly bicarbonate buffer at pH 7 was uniformly inhibitory for RNases U_4 and U_1, yet allowed the highest rates of hydrolysis observed at pH 6 for RNases U_{4B}, U_{4C}, and U_1. Complete retention of RNase U_4 activity in EDTA buffer reflects the independence of metal ions typical of the RNA phosphotransferases (Barnard, 1969). Of interest is the relative insensitivity of the U_4 ribonucleases to inorganic and organic phosphate buffers which might be expected to compete with substrate for binding;[5] in comparison, inhibition of RNase U_1 activity by these buffers is severe. In sodium phosphate buffer the pH optimum of RNases U_{4A}, U_{4B}, and U_{4C} for depolymerization of RNA lies between 6 and 7. The curves in Figure 1, which may reflect the pH dependence of charge and conformation of the enzymes and/or the polyvalent, macromolecular substrate (Wolfe *et al.*, 1968), are rather broad. The data of Figure 1 and Table II demonstrate that activity of the U_4 ribonucleases does not respond identically to alterations in pH and buffer, supporting the conclusion that RNases U_{4A}, U_{4B}, and U_{4C} are catalytically distinguishable. Although individual differences in response are for the most part small, these differences, as a group, show patterns attesting to their significance.

The major products of exhaustive digestion of high molecular weight wheat germ RNA by RNase U_{4B} are 3′-Ap, -Cp, -Gp, and -Up, the mononucleotide fraction comprising about 95% of the A_{260} of a limit RNase U_{4B} digest; comparable results were obtained for RNases U_{4A} and U_{4C}. A very small proportion of the material visible on paper chromatograms of exhaustive ribonuclease U_{4A}, U_{4B}, and U_{4C} digests of RNA was immobile or nearly immobile (Figures 2 and 3) in three solvent systems; it is presumably this material, comprising the remaining 5% of the A_{260} of a limit RNase U_{4B} digest, which was excluded from Sephadex G-25. An estimate of its minimum molecular weight based on the exclusion limit of G-25 for polysaccharides would be 5000, equivalent to a polymer of 15 residues. The material excluded by Sephadex G-25 is reduced to 0.3%

[5] Inhibition by phosphate might conceivably be masked by the 10^{-3} M imidazole present in the assay mixtures. However, sodium phosphate was not inhibitory when tested in the absence of imidazole.

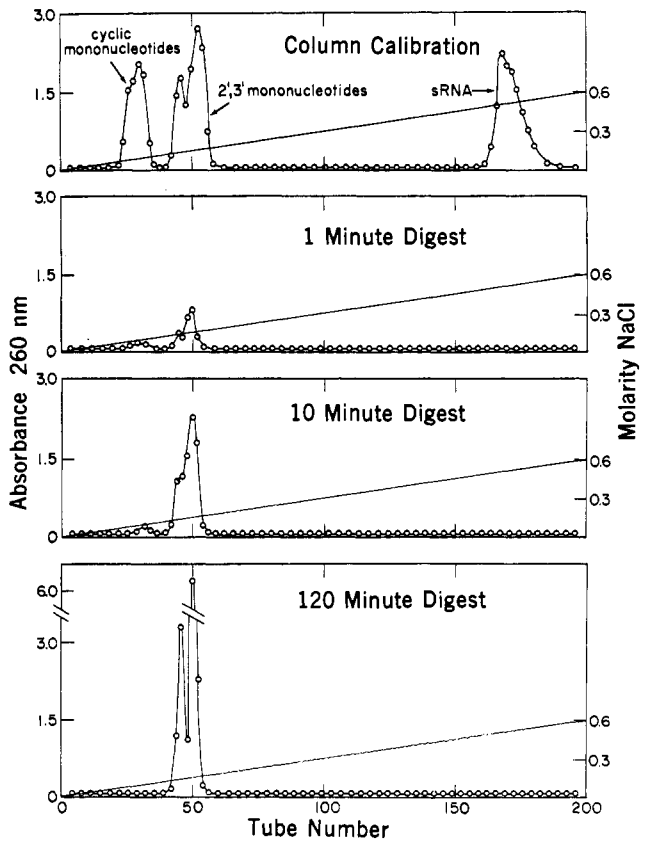

FIGURE 7: Chromatography of partial digests of RNA on DEAE-cellulose. RNA (5 mg) was digested at 28° with 930 units of ribonuclease U_{4B} in 1 ml of 0.04 M imidazole-HCl (pH 7.0). After 1-, 10-, or 120-min incubation, samples were held 5 min at 100° and applied to a 1.3 × 31 cm DEAE-cellulose column equilibrated with 0.02 M Tris-HCl (pH 7.5)–7 M urea. The column was washed with 1 l. of the same solution containing a linear, 0–0.6 M NaCl gradient; 5-ml fractions were collected. The column was calibrated with a mixture of A>p, C>p, G>p, U>p; a mixture of Ap, Cp, Gp, Up; and sRNA. Not shown are the positions of oligonucleotides which elute according to increasing chain length in the region between mononucleotide and sRNA (Tomlinson and Tener, 1963). For the three ribonuclease U_{4B} digests shown, *ca.* 12, 32, and 55% of the A_{260} units applied to the column was recovered as mononucleotide.

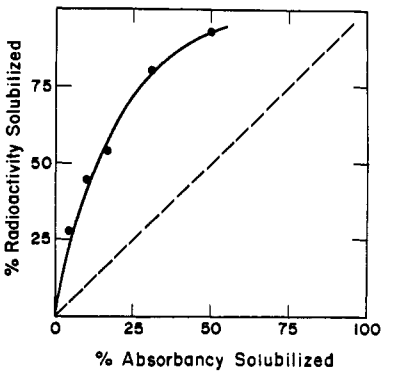

FIGURE 8: Ribonuclease U_{4B} catalyzed digestion of RNA radioactively labeled in the 5'-terminal phosphate. [^{32}P]RNA (0.74 mg) was incubated with 7 units of ribonuclease U_{4B} at 28° in 3.3 ml of 0.04 M imidazole-HCl–0.1% bovine serum albumin (pH 7.0). At intervals up to 15 min elapsed time, 0.5-ml aliquots were withdrawn to 1.0 ml of 5% perchloric acid, held 10 min at 0°, and centrifuged 10 min at 14,600*g* at 0°. The A_{260} of the supernatant solution was measured; radioactivity of a 100-μl aliquot of supernatant was determined in Bray's solution (Bray, 1960) in a Nuclear-Chicago liquid scintillation counter; values were corrected for a blank incubation mixture containing no enzyme. "Per cent absorbancy solubilized" is given as per cent of acid-precipitable A_{260}, *i.e.*, per cent of the difference between the A_{260} of a solution of alkali hydrolyzed [^{32}P]RNA (0.11 mg) in 1.5 ml of 3.3% perchloric acid and the A_{260} observed for the perchloric acid supernatant of the blank incubation mixture containing no enzyme. "Per cent radioactivity solubilized" is given as per cent of acid-precipitable label, *i.e.*, per cent of the difference between the radioactivity in 0.033 ml of incubation mixture and that in 100 μl of perchloric acid supernatant from the blank incubation mixture containing no enzyme. For the blank mixture, 2.1% of the absorbancy and 6.6% of the radioactivity were acid soluble; these values were essentially unchanged throughout incubation.

of the total A_{260} of a limit RNase U_{4B} digest when digestion is carried out in dialysis tubing immersed in a 17-fold volume of buffer, strongly suggesting that product inhibition is at least partially responsible for the resistance of the excludable fraction to depolymerization. It is possible that the residual (0.3%) excludable material observed after dialysis includes, or consists of, DNA, present as a contaminant of the RNA (Glitz and Dekker, 1963). The Sephadex G-25 profile of exhaustive RNase U_{4B} digests and paper chromatograms of exhaustive RNase U_{4A}, U_{4B}, and U_{4C} digests of RNA indicate absence of oligonucleotides.

Chromatography on Sephadex G-25 of the products of partial digestion of RNA (Figure 6) strongly suggests that ribonucleases U_{4A}, U_{4B}, and U_{4C} act exonucleolytically. Failure to detect oligonucleotide on paper chromatography of partial digests (Figure 5), and on DEAE-cellulose chromatography of partial ribonuclease U_{4B} digests (Figure 7), is consistent with this hypothesis. The early release of nucleoside 3',5'-diphosphate from the 5' terminus of RNA chains relative to the release of mononucleotide from the remainder of the RNA (Figure 8) is consistent with exonucleolytic attack, and indicates that depolymerization proceeds in the 5' → 3' direction. The data show that the ribonuclease U_{4B} catalyzed degradation of RNA is not processive, *i.e.*, the enzyme does not tend to degrade a single chain to completion before attacking any other chain (Nossal and Singer, 1968). The results do not rule out occasional endonucleolytic attack on RNA.

The presence of 2': 3'-cyclic Gp, 2': 3'-cyclic Up, and 2': 3'-cyclic Cp in a partial RNase U_{4B} digest was firmly established; the presence of the same compounds in the comparable RNase U_{4A} and U_{4C} digests is seen in Figure 5. Demonstration of the cleavage of synthetic U>p and C>p by RNase U_{4A} (P. Tosukhowong and C. A. Dekker, unpublished results) and of the failure of RNase U_{4B} to hydrolyze the dinucleotide 2'-*O*-methylguanylylguanylic acid (A. Blank and J. Abelson, unpublished data) strengthens the conclusion that cyclic nucleotides are intermediates in the RNase U_4 catalyzed degradation of RNA. Absence of A>p from the RNase U_4 digests probably reflects its rapid cleavage under the incubation conditions. A similar absence of A>p was observed on partial digestion of RNA with ribonuclease M, a T_2-like endonuclease degrading commercial yeast RNA to 3'-mononucleotides (Irie, 1967). Furthermore, A>p accumulates to a lesser extent than other nucleoside cyclic phosphates in RNase T_2 digests of RNA (Uchida and Egami, 1971).

On the basis of evidence presented here it is concluded that the U_4 ribonucleases are novel phosphotransferases degrading RNA exonucleolytically from its 5' terminus. It has been suggested previously by Egami and his coworkers that "RNase U_4 seems to be an exonuclease because the products

of exhaustive digestion [of RNA] were four mononucleotides and no oligonucleotides appeared. . ." (Arima *et al.*, 1968). That digestion products having 2′:3′-cyclic termini were not detected in this early study apparently led Uchida and Egami (1971) to misclassify ribonuclease U_4 as an enzyme catalyzing direct hydrolysis of phosphodiester bonds in RNA. In fact, intermediates in the ribonuclease U_4 catalyzed digestion of RNA include the 2′:3-cyclic mononucleotides sequentially cleaved from chain termini and polynucleotides having new 5′-hydroxyl ends; unlike the cyclizing endonucleases (Barnard, 1969), the U_4 ribonucleases do not form, in detectable amount, high molecular weight intermediates having new cyclic phosphate termini.

The U_4 ribonucleases constitute a second and new group of known exoribonucleases. Members of the first group, isolated from several sources (*e.g.*, Singer and Tolbert, 1965; Lazarus and Sporn, 1967), are catalytically very different, degrading RNA to 5′-mononucleotides by direct, Mg^{2+}-dependent hydrolysis; these enzymes, interestingly, do not attack DNA. Other well-known enzymes hydrolyzing RNA exonucleolytically, *e.g.*, venom exonuclease (Laskowski, 1971) and spleen exonuclease (Bernardi and Bernardi, 1971), are nucleases (Barnard, 1969), attacking DNA as well as RNA.

Preliminary experiments with oligonucleotides of known sequence indicate that the ribonuclease U_{4B} catalyzed degradation of oligomers of chain length three to six is mainly exonucleolytic, proceeding from the 5′ terminus, and that occasional endonucleolytic cleavage occurs (A. Blank and J. Abelson, unpublished data). Apparently, specificity of ribonuclease U_{4B} for the terminal internucleotide bond of short oligomers may be less strict than for that of RNA, depending, perhaps, on both chain length and base sequence of the oligonucleotide. Although the underlying mechanisms may differ, such relaxation of a restrictive mode of action with decreasing chain length is reminiscent of the exonucleolytic action of polynucleotide phosphorylase, which depolymerizes polynucleotides processively until some minimum chain length is reached after which phosphorolysis becomes random (Chou and Singer, 1970). The results of oligonucleotide digestion clearly illustrate the potential utility of ribonuclease U_4 for determination of base sequence.

The almost complete (>95%) digestion by the U_4 ribonucleases of high molecular weight (ribosomal) wheat germ RNA with its 1.7% content of 2′-*O*-methyl groups (Lane, 1965) is not fully understood. If the nucleoside 2′:3′-cyclic phosphate is indeed an obligate intermediate in exonucleolytic degradation, one would expect stepwise depolymerization to terminate at residues bearing a 2′-*O*-methyl group. The observed results could be explained in any one of the following ways. (1) The RNA population is heterogeneous with the 2′-*O*-methyl groups restricted to a small proportion of molecules. (2) The 2′-O-methylated nucleotides are localized near the 3′ termini of all molecules. (3) The RNA is contaminated with a trace amount of an endonuclease(s). (4) The ribonuclease U_4 preparations have trace endonuclease activity toward RNA as the result of impurities or as an inherent property of the enzymes. We favor alternative (3) or (4) at this time.

Acknowledgment

We acknowledge with gratitude the generous contribution of Dr. William K. Holloman who performed the chromatography of ribonuclease U_{4B} digests on Dowex 1 and on DEAE-cellulose, and carried out pilot experiments in preparation and degradation of radioactive RNA. We thank Mr. Pichit Tosukhowong for helpful discussions, and Drs. W. C. Kenney and A. S. Delk for useful criticism of the manuscript.

References

Ames, B. N., and Dubin, T. D. (1960), *J. Biol. Chem. 235*, 769.

Arima, T., Uchida, T., and Egami, F. (1968), *Biochem. J. 106*, 609.

Barnard, E. A. (1969), *Annu. Rev. Biochem. 38*, 677.

Bernardi, A., and Bernardi, G. (1971), *Enzymes 4*, 329.

Bessey, O. A., Lowry, O. H., and Brock, M. J. (1946), *J. Biol. Chem. 164*, 321.

Bielski, R. L., and Young, R. E. (1963), *Anal. Biochem. 6*, 54.

Birnboim, H. (1966), *Biochim. Biophys. Acta 119*, 198.

Blank, A., and Dekker, C. A. (1972), *Biochemistry 11*, 3956.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Chargaff, E., Lipshitz, R., Green, C., and Hodes, M. E. (1951), *J. Biol. Chem. 192*, 223.

Chou, J. Y., and Singer, M. F. (1970), *J. Biol. Chem. 245*, 995.

Cohn, W. E., and Volkin, E. (1951), *Nature (London) 167*, 483.

Glitz, D. G., and Dekker, C. A. (1963), *Biochemistry 2*, 1185.

Glitz, D. G., and Dekker, C. A. (1964), *Biochemistry 3*, 1391, 1399.

Hohn, Th., and Schaller, H. (1967), *Biochim. Biophys. Acta 138*, 466.

Irie, M. (1967), *J. Biochem. (Tokyo) 62*, 509.

Kenney, W. C., and Dekker, C. A. (1971), *Biochemistry 10*, 4962.

Lane, B. G. (1965), *Biochemistry 4*, 212.

Laskowski, M. (1971), *Enzymes 4*, 313.

Lazarus, H. M., and Sporn, M. B. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1386.

Markham, R., and Smith, J. D. (1952), *Biochem. J. 52*, 558.

Nossal, N. G., and Singer, M. F. (1968), *J. Biol. Chem. 243*, 913.

Razzell, W. E., and Khorana, H. G. (1959), *J. Biol. Chem. 234*, 2214.

Reddi, K. K. (1958), *Biochim. Biophys. Acta 28*, 386.

Richards, F. M., and Wyckoff, H. W. (1971), *Enzymes 4*, 647.

Richardson, C. C. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 158.

Rushizky, G. W., Mozejka, J. H., Rogerson, A. L., and Sober, H. A. (1970), *Biochemistry 9*, 4966.

Rushizky, G. W., and Sober, H. A. (1963), *J. Biol. Chem. 238*, 371.

Sato-Asano, K. (1959), *J. Biochem. (Tokyo) 46*, 31.

Singer, M. F., and Tolbert, G. (1965), *Biochemistry 4*, 1319.

Singh, H., and Lane, B. G. (1964), *Can. J. Biochem. 42*, 1011.

Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.

Uchida, T., Arima, T., and Egami, F. (1970), *J. Biochem. (Tokyo) 67*, 91.

Uchida, T., and Egami, F. (1971), *Enzymes 4*, 205.

Wolfe, F. H., Oikawa, K., and Kay, C. M. (1968), *Can. J. Biochem. 46*, 643.

Wyatt, G. R. (1955), *Nucl. Acids 1*, 243.