Ribonuclease U_4 from *Ustilago sphaerogena*. Purification and Physical Properties†

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ABSTRACT: Several related ribonucleases designated RNases U_{4A}, U_{4B}, and U_{4C}, and collectively designated RNase U_4, were identified in the medium of the fungus *Ustilago sphaerogena*. An efficient procedure was developed for their purification from large volumes of culture medium. Using a ribonuclease assay slightly modified from that described by Kenney and Dekker (*Biochemistry 10*, 4962 (1971)) the highest specific activity observed for RNase U_{4A} was 47,500, for RNase U_{4B} 53,400, and for RNase U_{4C} 47,100 units/mg of protein. On disc gel electrophoresis at pH 9.5 RNases U_{4A} and U_{4B} appeared nearly homogeneous. The RNase U_{4C} preparation displayed a major component accounting for about two-thirds of the material stained by Amido Black; the remaining material was distributed in two bands of higher mobility. Each preparation appeared homogeneous with respect to sedimentation velocity; assuming a partial specific volume of 0.700, the $s_{20,w}$ of RNase U_{4A} is 2.83, of RNase U_{4B} 2.76, and of RNase U_{4C} 2.62. Molecular weights of 29,500, 26,900, and 26,000 were obtained for RNases U_{4A}, U_{4B}, and U_{4C} by equilibrium ultracentrifugation. Filtration through Sephadex G-100 yielded apparent molecular weights in the range of 20,000–23,000. Carbohydrate was not detected in 220-μg samples of RNase U_{4A} and U_{4B} assayed by the phenol–sulfuric acid method (DuBois *et al.*, *Anal. Chem. 28*, 350 (1956)). The RNase U_{4C} preparation displayed a carbohydrate content of 3 μg of sucrose equivalents/100 μg of Lowry-positive material. It is concluded from their behavior in purification, in disc gel electrophoresis, in ultracentrifugation, and in gel filtration, and from their ultraviolet absorption properties, that ribonucleases U_{4A}, U_{4B}, and U_{4C} are structurally related proteins. Although no evidence was obtained for heterogeneity within the entities designated RNase U_{4A} and RNase U_{4B}, it is surmised that RNase U_{4C} may contain at least two very similar components having ribonuclease activity, one or more of which could be a glycoprotein. Catalytic properties of the U_4 ribonucleases are described in an accompanying paper (Blank, A., and Dekker, C. A., *Biochemistry 11*, 3962 (1972)).

The smut fungus *Ustilago sphaerogena* is a rich source of ribonucleases of practical utility and biochemical interest. Ribonuclease U_1 [EC 2.7.7.26 ribonucleate guaninenucleotide-2′-transferase (cyclizing)], a guanylyl-specific enzyme very similar to ribonuclease T_1, is readily purified in milligram quantity from the culture medium of cells grown on RNA (Kenney and Dekker, 1971). Like RNase T_1, U_1 has been used as a fundamental tool in determination of nucleotide sequences in RNA (J. Abelson, personal communication, and Pinkerton *et al.*, 1971). Present in very small quantity in the extracellular medium of *U. sphaerogena* are ribonucleases U_2 and U_3 (Arima *et al.*, 1968a). These enzymes, under certain conditions, will cleave predominantly adjacent to adenylyl and guanylyl residues (Arima *et al.*, 1968b; Uchida *et al.*, 1970; Rushizky *et al.*, 1970); employed in conjunction with RNase T_1, RNase U_2 has expedited determination of nucleotide sequences in RNA (*e.g.*, Adams *et al.*, 1969; Cory and Marcker, 1970).

While the present study was in progress, a brief report of a fourth ribonuclease from *U. sphaerogena* given the trivial name RNase U_4 was included in two papers by Arima *et al.* (1968a,b). The ribonucleases described here appear to correspond to the activity designated RNase U_4 by these authors, and their nomenclature has been adopted.

RNase U_4, like RNase U_1, is abundant in the medium of *U. sphaerogena* grown on RNA. We have found that ribonuclease U_4 is comprised of at least three separable components, designated RNases U_{4A}, U_{4B}, and U_{4C}. A rapid, efficient, reproducible procedure is reported here for the purification of these enzymes from large volumes of culture medium. Examination of physical properties of RNases U_{4A}, U_{4B}, and U_{4C} has led to the conclusion that they are structurally related proteins. Study of their enzymatic properties, described in an accompanying paper, has shown that they are catalytically related phosphotransferases, novel in that they degrade RNA in a primarily, if not exclusively, exonucleolytic manner.

Experimental Procedure

Materials. DEAE-cellulose (0.7 ± 0.1 mequiv/g) and CM-cellulose (0.7 ± 0.1 mequiv/g) were purchased from Bio-Rad Laboratories, DEAE-Sephadex A-50 (3.5 ± 0.5 mequiv/g) and CM-Sephadex C-50 (4.5 ± 0.5 mequiv/g) from Pharmacia Fine Chemical Corp.; Sephadex G-100 was purchased from Pharmacia. Crystallized bovine serum albumin and crystallized pancreatic ribonuclease A were obtained from Sigma Chemical Co., crystallized ovalbumin from Nutritional Biochemicals Corp. Poly(A) was from Miles Laboratories. Whenever possible, chemicals used were of reagent grade.

Assay Procedures. Ribonuclease activity was assayed by two procedures, each employing measurement of perchloric acid

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soluble nucleotides liberated from high molecular weight wheat germ RNA. Activity in assay 1, described by Kenney and Dekker (1971), was used as the standard measure of ribonuclease activity. Where purified RNase U_4 preparations devoid of RNase U_1 were assayed, a correction factor of 0.040[1] was added to the enzyme catalyzed increase in A_{260}, the sum being used to calculate ribonuclease activity. Thus corrected, assay 1 gave a linear response to added RNase U_4 up to an increase in A_{260} of at least 0.650. Ribonuclease assay 2 was employed as an adjunctive procedure for monitoring large numbers of samples, *e.g.*, fractions from columns. The incubation mixture was the same as for assay 1, halved in volume. A single 0.5-ml aliquot was withdrawn after 15-min incubation at 28° and added to perchloric acid as for assay 1. The A_{260} of the supernatant solution, minus that of a blank without enzyme, was used to calculate ribonuclease activity. One unit in this assay is defined as that quantity of enzyme catalyzing an increase in A_{260} of 0.500. Where purified RNase U_4 preparations devoid of RNase U_1 were assayed, a correction factor of 0.030[1] was added to the enzyme catalyzed increase in A_{260}, the sum being used to calculate ribonuclease activity. Thus corrected, assay 2 gave a linear response to added RNase U_4 up to an A_{260} of at least 1.000. (Though accurate correlation of activity determined in assay 2 to that measured in assay 1 was neither required nor attempted, ratios between 0.9 and 1.0 have been most frequently estimated for RNases U_{4A}, U_{4B}, and U_{4C}.) When dilution of preparations was required prior to assay, enzyme was mixed with 0.1% bovine serum albumin–0.10 M imidazole-HCl (pH 7.0); dilution into unbuffered serum albumin caused loss of RNase U_4 activity.

The qualitative assay described by Glitz and Dekker (1964) was used for detection of ribonuclease activity in fractions from chromatographic columns. As little as 50 units of RNase activity could be detected by allowing the mixture to stand, covered with Saran wrap (Dow Chemical Corp.), for several hours.

Depolymerization of poly(A) was assayed in a procedure differing from ribonuclease assay 2 in the substitution of 12.0 A_{260} units of poly(A) for RNA in the incubation mixture. Absorbancy at 260 nm of the perchloric acid supernatant solution was linear with added enzyme, within experimental error, up to at least 1.00. One unit in this assay is defined as that quantity of enzyme catalyzing an increase in A_{260} of 0.500.

Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard; specific activities given in terms of milligrams of protein are based on this measurement. Where samples containing either Tris or glycine buffer were assayed, protein was estimated from standard curves constructed with aliquots of serum albumin containing an identical amount of buffer. Protein was also estimated by measurement of absorbancy at 280 nm. Carbohydrate was measured by the phenol–sulfuric acid method of DuBois *et al.* (1956) using sucrose as a standard.

Purification of Ribonucleases U_{4A}, U_{4B}, and U_{4C}. GROWTH AND HARVESTING OF CELLS (STEP I). Cells were grown in 15-l. batches of RNA-containing medium at 20°, essentially as described by Kenney and Dekker (1971). At 65 hr after inoculation cells were removed from the culture medium by continuous flow centrifugation in a refrigerated Sharples electric centrifuge. Subsequent steps were performed at 2–4°.

DEAE-CELLULOSE TREATMENT (STEP II). Approximately 0.4 g of DEAE-cellulose (Cl^-) was added per l. of culture supernatant and the suspension was stirred for 1 hr. After most of the resin had settled, the turbid supernatant solution was decanted through glass wool and then filtered through Whatman No. 41 paper. The residual suspension containing most of the DEAE-cellulose was centrifuged 15 min at 14,600*g* and the resin was discarded. The entire volume was again treated with DEAE-cellulose as described; the resin was removed by filtration and discarded.

ADSORPTION TO CM-CELLULOSE (STEP III). For each liter of DEAE-cellulose supernatant was added approximately 0.8 g of CM-cellulose (Na^+). The well-stirred suspension was brought to pH 4.0 by the rapid addition of 6 N HCl and gently stirred for an additional 10 min. The resin containing bound ribonuclease U_4 was collected by filtration on Whatman No. 41 paper and resuspended in 0.05 M sodium acetate buffer (pH 4.8), using about 30 ml of buffer/g of CM-cellulose initially added; the pH of the suspension was brought to 4.8 with 6 N NaOH. Following 10–20 min of gentle stirring the CM-cellulose was again collected by filtration and thoroughly washed with further acetate buffer; the acetate washes were discarded. The resin was then suspended in 0.05 M Tris-HCl (pH 7.0), using about 20 ml of buffer/g of resin initially added; the pH of the suspension was brought to 7.4 with 6 N NaOH. After 20–30 min of gentle stirring the suspension was filtered and the resin was washed with further Tris buffer. The combined Tris filtrate and washes were concentrated to about 100 ml under 50 psi of N_2 in an Amicon ultrafiltration cell equipped with a UM-10 membrane (Amicon Corp.).

CHROMATOGRAPHY ON CM-SEPHADEX (STEP IV). The concentrated ribonuclease solution was dialyzed against 2 l. of acetate buffer.[2] The dialysate was acidified, if necessary, to pH 6.4–6.5 with 0.2 N acetic acid and chromatographed on CM-Sephadex as described in Figure 1.

RECHROMATOGRAPHY OF RIBONUCLEASE U_{4A} ON CM-SEPHADEX (STEP V_A). Ribonuclease activity eluting from CM-Sephadex (step IV) at *ca.* 0.16 M NaCl is designated RNase U_{4A} (Figure 1). Those RNase U_{4A} fractions having a specific activity[3] greater than 25,700 were pooled. The pooled fractions were dialyzed, and if necessary acidified as in step IV, and applied to a 1.0 × 21 cm CM-Sephadex column equilibrated with acetate buffer. The column was then washed with 2 l. of acetate buffer containing a linear 0.00–0.24 M NaCl gradient, yielding a single peak of ribonuclease activity. Fractions of about 8.5 ml were collected in tubes containing 0.05 ml of 0.8 M Tris-HCl–0.02 M EDTA (pH 9.0). Those fractions on the trailing edge of the peak having a specific activity[3] greater than 31,800 were pooled.

CHROMATOGRAPHY OF RIBONUCLEASE U_{4B} on DEAE-SEPHADEX (STEP V_B). The peak of ribonuclease activity eluting from CM-Sephadex (step IV) at *ca.* 0.09 M NaCl is designated RNase U_{4B} (Figure 1). Those RNase U_{4B} fractions having a specific activity[3] greater than 26,400 were pooled. The pooled fractions were dialyzed against 4 l. of

[1] When the increase in A_{260} catalyzed by ribonuclease U_{4A}, U_{4B}, or U_{4C} in assays 1 and 2 is plotted *vs.* enzyme concentration, straight lines are obtained which extrapolate to −0.040 and −0.030, respectively, on the y intercept. As an explanation for displacement of these curves from the origin it is suggested that the ribonuclease U_4 catalyzed, exonucleolytic degradation of RNA may be initiated with a slow step, *i.e.*, removal from the 5′ terminus of nucleoside diphosphate (Blank and Dekker, 1972).

[2] Buffers used for column chromatography and for the immediately preceding dialyses were 0.01 M sodium acetate (pH 6.5) or 0.01 M sodium glycinate–0.01 M NaCl (pH 8.5).

[3] Specific activity here refers to units of activity in assay 2 per A_{260} unit.

TABLE I: Purification of Ribonucleases U_{4A}, U_{4B}, and U_{4C}.[a]

Step	Vol (ml)	Total RNase Act. (Units $\times 10^{-6}$)	RNase Act. (Units/ml)	Protein (mg/ml)	A_{280}	Specific Activity: Units/mg	Specific Activity: Units/A_{280} Unit	Yield[b] (%)
			Ribonuclease U_{4A}					
I. Culture medium	23,100	19.4	838	0.356	11.3	2,350	74	100 (100)
II. DEAE-cellulose	23,100	15.9	690	0.348	11.0	1,980	63	82 (100)
III. CM-cellulose	672	4.77	7,100	0.386	0.580	18,400	12,200	25 (100)
IV. CM-Sephadex I	139	0.549	3,950	0.0955	0.117	41,400	33,800	2.9 (11.5)
V_A. CM-Sephadex II	59.5	0.168	2,820	0.0594	0.091	47,500	31,000	0.87 (3.5)
			Ribonuclease U_{4B}					
I–III. As above								
IV. CM-Sephadex I	63	0.704	11,200	0.282	0.356	39,600	31,400	3.6 (15)
V_B. DEAE-Sephadex I_B	96	0.595	6,200	0.116	0.152	53,400	40,900	3.1 (12.5)
			Ribonuclease U_{4C}					
I–III. As above								
IV. CM-Sephadex I	81	0.451	5,570	0.274	0.367	20,300	15,200	2.3 (9.5)
V_C. DEAE-Sephadex I_C	80	0.304	3,800	0.099	0.132	38,400	28,800	1.6 (6.4)
VI_C. DEAE-Sephadex II_C	47	0.112	2,370	0.050	0.069	47,100	34,400	0.58 (2.3)

[a] Ribonuclease activity was determined in assay 1. [b] Yields are given with respect to total ribonuclease activity in the culture medium. Values in parentheses are the estimated yields with respect to total RNase U_4 species as described in the Discussion.

glycine buffer[2] and chromatographed on DEAE-Sephadex as described in Figure 2.

CHROMATOGRAPHY AND RECHROMATOGRAPHY OF RIBONUCLEASE U_{4C} ON DEAE-SEPHADEX (STEPS V_C AND VI_C). The peak of ribonuclease activity eluting from CM-Sephadex (Step IV) at *ca.* 0.07 M NaCl is designated RNase U_{4C} (Figure 1). Those RNase U_{4C} fractions having a specific activity[3] greater than 11,700 were pooled. The combined fractions were dialyzed *vs.* 4 l. of glycine buffer and applied to a 1.2 × 16 cm DEAE-Sephadex column equilibrated with glycine buffer.[2] The column was washed with buffer until the A_{280} of the effluent was *ca.* 0.01 and then with a linear gradient of 0.0–0.12 M NaCl in 600 ml of buffer. Fractions of approximately 9 ml were collected at a flow rate of about 1 ml/min. Those fractions having a specific activity[3] greater than 26,500 were pooled, dialyzed *vs.* glycine buffer, and rechromatographed on DEAE-Sephadex as described in the legend to Figure 3.

Concentration of Purified Enzymes. Purified 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 dialyzed against glycine buffer;[2] each preparation was concentrated by adsorption to a 1.0 × 1.5 cm DEAE-Sephadex column equilibrated with the same buffer, followed by elution in a few milliliters of 0.01 M sodium glycinate–0.11 M NaCl (pH 8.5). Unless indicated otherwise, these preparations were used for the studies described below. Purified ribonucleases U_{4A}, U_{4B}, and U_{4C} are stable to at least 1-years' storage at 4° in 0.01 M sodium glycinate–0.11 M NaCl–10^{-4} M EDTA (pH 8.5).

Sedimentation Coefficient. Sedimentation coefficients were determined using a Beckman Model E analytical ultracentrifuge equipped with absorption optics. Ribonuclease solutions were extensively dialyzed *vs.* 0.01 M sodium glycinate–0.11 M NaCl–10^{-4} M EDTA (pH 8.5) prior to centrifugation at 52,000 rpm at 7°. RNase U_{4A} was examined at protein concentrations of *ca.* 0.015, 0.03, and 0.05%, RNase U_{4B} at *ca.* 0.015, 0.03, and 0.06%, and RNase U_{4C} at *ca.* 0.02, 0.025, and 0.03%.

Molecular Weight. Molecular weights were determined by the high-speed equilibrium method of Yphantis (1964) in a Beckman Model E analytical ultracentrifuge. Samples were centrifuged 22–28 hr at 29,500 or 33,450 rpm at 7°. Solutions examined were those employed for determination of sedimentation coefficients.

Ultraviolet Absorption Properties. Ultraviolet absorption spectra were obtained using a Cary Model 14 recording spectrophotometer. Enzyme solutions contained 0.01 M potassium phosphate–0.01 M sodium glycinate–0.11 M NaCl–10^{-4} M EDTA (pH 6.9).

Results

Purification of Enzymes. Table I summarizes the purification of ribonucleases U_{4A}, U_{4B}, and U_{4C} from 23 l. of culture medium. Figure 1 illustrates resolution of RNase U_4 on CM-Sephadex into components U_{4A}, U_{4B}, and U_{4C} (step IV); chromatography on DEAE-Sephadex of RNase U_{4B} (step V_B) and RNase U_{4C} (step VI_C) is illustrated in Figures 2 and 3.

Disc Gel Electrophoresis. Ribonucleases U_{4A}, U_{4B}, and U_{4C} were subjected to discontinuous gel electrophoresis in 7% polyacrylamide at pH 9.5. On visual inspection of gels (Figure 4), the major component of the RNase U_{4A} and RNase U_{4B} preparations appeared to contain about 90 and 95%, respectively, of the material stained by Amido Black. (The three faint bands of higher mobility were absent from a ribonuclease U_{4A} preparation (44,800 units/mg of protein) purified as in Table I but substituting for step V_A elution from a 2.0 × 19 cm DEAE-Sephadex column with 1 l. of glycine buffer[2] containing a linear 0.0–0.20 M NaCl gradient.) The slowest component of the RNase U_{4C} preparation accounted for about two-thirds of the material stained by the dye, and

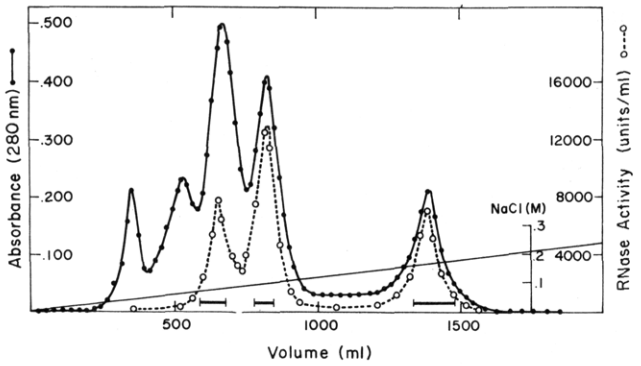

FIGURE 1: Resolution of ribonuclease U_4 on CM-Sephadex at pH 6.5 (step IV). Dialyzed ribonuclease U_4 was applied to a 2.0 × 20 cm CM-Sephadex column equilibrated with acetate buffer.[2] The column was washed with buffer until the A_{280} of the effluent was *ca.* 0.01 and then with 2 l. of buffer containing a linear 0.0–0.24 M NaCl gradient. Fractions of approximately 10 ml were collected at a flow rate of about 1 ml/min in receiving tubes containing 0.05 ml of 0.8 M Tris-HCl–0.02 M EDTA (pH 9.0). Ribonuclease activities eluting at *ca.* 0.16, 0.09, and 0.07 M NaCl are designated RNase U_{4A}, U_{4B}, and U_{4C}, respectively. Activity was measured in assay 2. Fractions were pooled as indicated by bars.

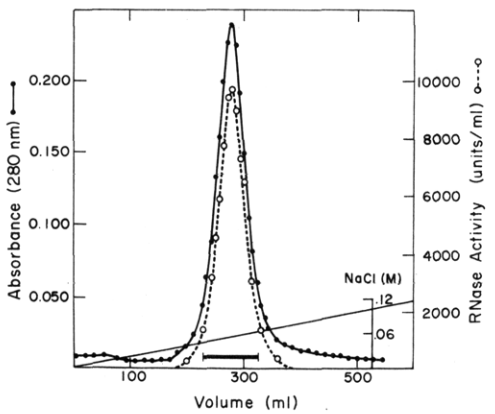

FIGURE 2: Chromatography of ribonuclease U_{4B} on DEAE-Sephadex at pH 8.5 (step V_B). Dialyzed ribonuclease U_{4B} was applied to a 1.0 × 21 cm DEAE-Sephadex column equilibrated with glycine buffer.[2] The column was washed with buffer until the A_{280} of the effluent was *ca.* 0.01 and then with a linear gradient of 0.0–0.12 M NaCl in 600 ml of buffer. Fractions of approximately 7.5 ml were collected at a flow rate of about 1 ml/min. Those fractions having a specific activity[3] greater than 33,300 were pooled, as indicated by the bar.

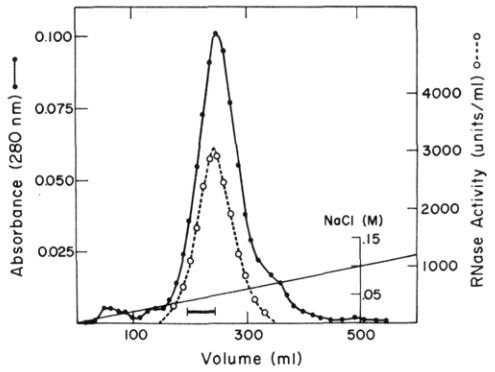

FIGURE 3: Rechromatography of ribonuclease U_{4C} on DEAE-Sephadex at pH 8.5 (step VI_C). Dialyzed ribonuclease U_{4C} was applied to a 1.2 × 20.5 cm DEAE-Sephadex column equilibrated with glycine buffer.[2] Activity was eluted with a linear gradient of 0.0–0.12 M NaCl in 600 ml of buffer. Fractions of approximately 6 ml were collected at a flow rate of about 1.5 ml/min. Those fractions having a specific activity[3] greater than 30,100 were pooled, as indicated by the bar.

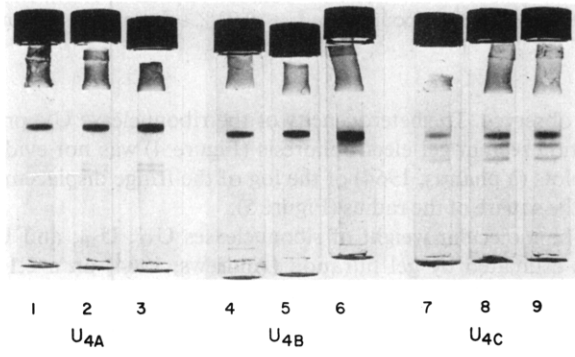

FIGURE 4: Discontinuous gel electrophoresis of ribonucleases U_{4A}, U_{4B}, and U_{4C}. Electrophoresis in 7% polyacrylamide (pH 9.5) was carried out at 4° at 2.5 mA/column (Williams and Reisfeld, 1964). Bromophenol Blue tracking dye, visible at the bottom of several gels, was allowed to run approximately 4.5 cm into the 0.5 cm diameter running gels. Gels 1, 2, and 3 contain *ca.* 15, 25, and 50 μg, respectively, of ribonuclease U_{4A}; gels 4, 5, and 6 *ca.* 10, 22.5, and 55 μg, respectively, of ribonuclease U_{4B}; gels 7, 8, and 9 *ca.* 10, 15, and 25 μg of ribonuclease U_{4C}. Gels were stained overnight in 0.5% Amido Black (K & K Laboratories) in 7% acetic acid and destained in 7% acetic acid.

migrated with the same mobility relative to Bromophenol Blue as RNase U_{4B}. On coelectrophoresis, the major components of the RNase U_{4B} and RNase U_{4C} preparations migrated together, while that of the RNase U_{4A} preparation moved more slowly in a separate band.

Sedimentation Coefficient. The absorption patterns of all samples examined showed a single sedimenting boundary yielding linear plots of log x *vs.* time, such as that shown in Figure 5. At the protein concentrations employed, dependence of sedimentation coefficient on concentration was not detected. Assuming a partial specific volume of 0.700[4] the calculated $s_{20,w}$ for RNase U_{4A} is 2.85, that for RNase U_{4B} is 2.76, and that for RNase U_{4C} 2.62. An additional determination of the sedimentation coefficient of independently isolated ribonuclease U_{4A} (44,800 units/mg of protein) yielded an $s_{20,w}$ = 2.81. (Approximately 0.06, 0.02, and 0.01% solutions in 0.3 M NaCl–0.01 M sodium acetate–0.02 M sodium glycinate (pH 8.6) were centrifuged at 52,000 or 59,780 rpm at 8°; dependence of sedimentation coefficient on concentration was not observed).

Molecular Weight. Assuming a partial specific volume of 0.700, the molecular weight of RNase U_{4A} was found, by equilibrium ultracentrifugation, to be 29,200, that of RNase U_{4B} 26,900, and that of RNase U_{4C}, 26,000. An additional determination of the molecular weight of independently isolated ribonuclease U_{4A} (44,800 units/mg of protein) yielded a value of 29,800. (Solutions of the enzyme (0.06 and 0.04%) in 0.3 M NaCl–0.01 M sodium acetate–0.02 M sodium glycinate (pH 8.6) were centrifuged 23 hr at 29,500 rpm at 8°.) Dependence of molecular weight on concentration was

[4] A $\bar{v}$ of 0.700 was assumed for the RNase U_4 species since values for other ribonucleases, *e.g.*, RNase A (Rothen, 1940; Harrington and Schellman, 1956; Schachman, 1957), RNase T_1 (Ui and Tarutani, 1961), and RNase U_1 (Kenney and Dekker, 1971), are rather low, varying from *ca.* 0.685 to 0.717. Choice of $\bar{v}$ = 0.75 would yield molecular weights 20% higher than those reported.

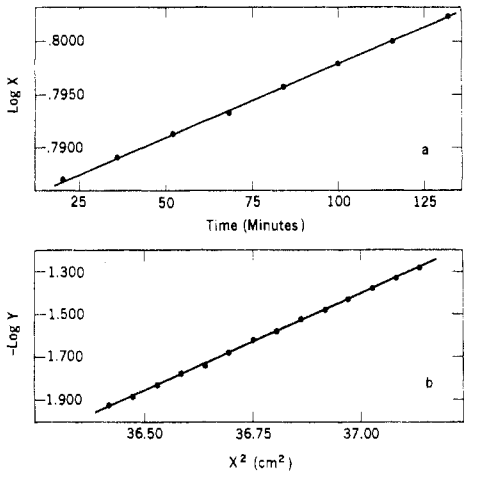

FIGURE 5: Ultracentrifugation of ribonuclease U_{4C}. (a) Sedimentation coefficients were determined from plots of log x *vs.* time where x is the distance in centimeters from the boundary to the axis of rotation and time is expressed in minutes after reaching speed (Schachman, 1957). (b) Molecular weights were determined from plots of $-\log y$ *vs.* x^2, where y is the fringe displacement in centimeters and x is the distance from the axis of rotation (Yphantis, 1964). Representative plots for ribonuclease U_{4C} (0.03% solution) are shown.

not observed. The heterogeneity of the ribonuclease U_{4C} preparation seen in gel electrophoresis (Figure 4) was not evident in plots (Yphantis, 1964) of the log of the fringe displacement *vs.* the square of the radius (Figure 5).

The molecular weight of ribonucleases U_{4A}, U_{4B}, and U_{4C} was estimated by gel filtration (Andrews, 1964) on a 2.1 × 81 cm Sephadex G-100 column equilibrated with 0.01 M sodium glycinate–0.10 M NaCl (pH 8.5). Each enzyme eluted as a single symmetrical peak of ribonuclease activity; elution volumes were plotted on a calibration curve established with ribonuclease A, ovalbumin, and bovine serum albumin, yielding apparent molecular weights of 20,700 ± 800 for RNase U_{4A}, 21,300 ± 900 for RNase U_{4B}, and 22,100 ± 900 for RNase U_{4C}.

Ultraviolet Absorption Properties. The ultraviolet (uv) absorption spectra of the ribonuclease U_{4A}, U_{4B}, and U_{4C} preparations are very similar and typical of protein, showing maxima near 279 nm, minima near 253 nm, A_{280}/A_{260} *ca.* 1.9 and A_{max}/A_{min} *ca.* 2.3.

Carbohydrate Content. Carbohydrate was not detected in 220-μg samples of ribonuclease U_{4A} and U_{4B}. However, since the smallest quantity of sucrose reliably measured in the assay is about 2 μg, a carbohydrate content less than *ca.* 1 μg of sucrose equiv/100 μg of Lowry-positive material cannot be excluded. Although the ribonuclease U_{4C} preparation was found to contain 3 μg of sucrose equiv /100 μg of Lowry-positive material, its electrophoretic inhomogeneity precludes identification of the active component(s) as glycoprotein. The material measured in the phenol–sulfuric acid assay may have been present as a nonglycoprotein contaminant; it is noteworthy in this regard that the uv absorption spectrum of the preparation does not indicate presence of nucleic acid.

Discussion

The existence of several ribonucleases in the *Ustilago* culture medium complicates determination of the yield and purification of any single RNase. The yield of ribonucleases U_{4A}, U_{4B}, and U_{4C} can be approximated however if it is assumed that nearly all the RNase U_4 in the medium is bound to CM-cellulose at step III of the purification, thus permitting estimate of the total RNase U_4 activity. This assumption is a reasonable one since (a) the preceding steps I and II involve only centrifugation and treatment with DEAE-cellulose under mild conditions where significant loss of RNase U_4 does not occur,[5] (b) recovery of activity in step III is virtually quantitative, and (c) only about 5% of the total poly(A) depolymerizing activity of the medium fails to bind to CM-cellulose in step III, signifying complete or nearly complete adsorption of RNase U_4. Assuming further that all of the activity bound to CM-cellulose is RNase U_4 and correcting for the small amount of residual unbound activity it is estimated that the final yield of RNase U_{4A} was 3.5% of the total ribonuclease U_4 of the culture, RNase U_{4B} 12.5% and RNase U_{4C} 2.3%. The overall yield of RNase U_4 is then a modest one of about 18%, reflecting the sacrifice of RNase U_{4A}, U_{4B}, and U_{4C} activity accepted for purification at steps IV, V_A, V_C, and VI_C.

The procedure presented approaches a minimal one for large-scale purification of ribonucleases U_{4A}, U_{4B}, and U_{4C}. The DEAE-cellulose step (II) is required to remove, by adsorption, viscous material which renders filtration of resin and column chromatography inconvenient. (The resin also removes some RNase U_1 from the medium; adsorption of RNase U_1 to DEAE-cellulose by repeated batchwise treatment of crude medium, or by a combination of batchwise and column chromatography, is the branch point in purification of RNases U_1 and U_4 [Kenney and Dekker, 1971]).

Adsorption of ribonuclease U_4 to CM-cellulose at pH 4 (step III) serves three essential functions: concentration of the enzyme from the unwieldy volume of culture medium, separation of ribonuclease U_4 from the large quantity of RNA degradation products remaining in the medium, and fractionation of ribonuclease U_4 and remaining ribonuclease U_1. As shown in Table I, step III effects a 34-fold reduction in volume of the RNase U_4 preparation. Whereas the DEAE-cellulose treated medium (step II) has an A_{280}/A_{260} of 0.39, indicative of the large quantity of purine and pyrimidine derivatives, the CM-cellulose pH 7.4 wash (step III, Table I) has an A_{280}/A_{260} of 1.52, characteristic of protein. Whereas the activity failing to bind to CM-cellulose displayed a low ratio (0.07) of units in the poly(A) depolymerization assay to units in ribonuclease assay 1, the enzymes in the CM-cellulose pH 7.4 wash displayed a ratio of 2.0. Highly purified RNase U_1 has a ratio less than 0.01 (Kenney, 1967) whereas RNase U_4 preparations display a ratio of 2.0 to 2.4. Despite the fact that RNase U_4 is unstable at pH 4 where binding to CM-cellulose occurs, recovery of ribonuclease activity in step III is virtually quantitative. In order to obtain complete recovery it is essential that the RNase preparation be equilibrated with resin prior to addition of acid, and that the pH be lowered quickly; under these conditions binding to the polyanionic resin occurs rapidly, protecting the enzyme from inactivation.

Chromatography on CM-Sephadex at pH 6.5 (step IV; Figure 1) is the only procedure known to resolve the entities designated ribonucleases U_{4A}, U_{4B}, and U_{4C}; reproducibility

[5] Passage of crude medium through a column of DEAE-cellulose results in adsorption of enzyme having a low ratio of poly(A) depolymerizing activity to ribonuclease activity characteristic of the guanylyl-specific RNase U_1.

is excellent.[6] Rechromatography of RNases U_{4A}, U_{4B}, and U_{4C} yields, in each case, a single peak of ribonuclease activity eluting at or very near the salt concentration required for elution in the initial chromatography.

It is concluded from their behavior in purification, in disc gel electrophoresis, in ultracentrifugation and in gel filtration, and from their ultraviolet absorption properties, that the U_4 ribonucleases are structurally similar. Although no evidence has been obtained for heterogeneity of RNase U_{4A} or U_{4B}, it is surmised that RNase U_{4C} may contain at least two very similar components having ribonuclease activity. This possibility was first suspected when the RNase U_{4C} preparation, having a specific activity comparable to that of RNases U_{4A} and U_{4B}, displayed in gel electrophoresis three components the major one of which contained only about two-thirds of the material stained by Amido Black. Failure to detect on ultracentrifugation the extensive heterogeneity displayed in gel electrophoresis suggests that the RNase U_{4C} preparation contains molecular species of similar size and shape. This conclusion is consistent with the hypothesis that RNase U_{4C} contains at least two similar but distinct components having ribonuclease activity. Although its significance is uncertain, the finding of carbohydrate in the RNase U_{4C} preparation could be connected with the proposed existence of multiple ribonucleases. Perhaps at least one of the putative U_{4C} RNases is a glycoprotein; presence of, or variation in, a small charged carbohydrate moiety might account for differences in electrophoretic mobility of the proposed enzymes without introducing heterogeneity detectable in ultracentrifugation.

The small differences between ribonuclease U_{4A}, U_{4B}, and U_{4C} in physical and catalytic (Blank and Dekker, 1972) properties may reflect differences in primary structure rather than variation in conformation or ligand binding of a single protein. Since each purified preparation "rechromatographs true," the U_4 ribonucleases are not in equilibrium under the conditions of CM-Sephadex chromatography at pH 6.5. Early purification studies strongly suggest that RNases U_{4A} and U_{4B} do not equilibrate under the conditions of DEAE-Sephadex chromatography at pH 8.5; the gel filtration experiments, performed under similar conditions, likewise suggest that equilibration does not take place. The relative mobility and proportion of RNases U_{4A}, U_{4B}, and U_{4C} appear the same after disc gel coelectrophoresis with or without 10^{-4} M EDTA; this finding, together with the results of ultracentrifugation, performed in EDTA, suggests that differing kinds or amount of bound metal ions probably do not determine the variation in physical behavior of the U_4 ribonucleases. Equilibrium ultracentrifugation indicates that RNase U_{4A} is larger than RNases U_{4B} and U_{4C}. We believe that the observed difference is significant and that RNase U_{4A} probably does have a more extensive primary structure than the latter two. RNases U_{4B} and U_{4C} may also differ in amino acid composition or perhaps in carbohydrate content.

The U_4 ribonucleases, having molecular weights of approximately 26,000–29,500, are about two and one-half times larger than the guanylyl-specific ribonucleases with their molecular weights of *ca.* 11,000 (Barnard, 1969; Uchida and Egami, 1971). And, the RNase U_4 species may be somewhat smaller than the nonspecific endonuclease T_2, the protein portion of which is reported to have a molecular weight of 36,200 determined from amino acid composition (Uchida, 1966). The U_4 RNases with their novel catalytic properties (Blank and Dekker, 1972) conform to the general division of microbial ribonucleases (Barnard, 1969) into two classes, the first comprised of very small proteins with a high degree of base specificity, the second of proteins of 20,000–40,000 molecular weight displaying relatively little base preference. The molecular weights of the U_4 ribonucleases estimated by filtration through Sephadex G-100 (*ca.* 20,000–23,000) are lower than those found by ultracentrifugation. A similar observation has been made for calf adenosine deaminase by Wolfenden *et al.* (1968). As discussed by Siegel and Monty (1966), elution volume from Sephadex is probably more closely related to Stokes radius than to molecular weight. It is also possible that the apparent retardation of the ribonuclease U_4 species reflects secondary adsorption phenomena, such as hydrophobic interaction, or interaction of binding sites on the enzymes with the dextran resin.

Because ribonuclease U_4 activity binds to CM-Sephadex at pH 6.5 and to DEAE-cellulose, albeit very weakly, at pH 7.6, it was surmised that the isoelectric point of RNases U_{4A}, U_{4B}, and U_{4C} might lie near pH 7; indeed that of RNase U_{4B}, determined by isoelectric focusing, is 7.4 (W. K. Holloman and C. A. Dekker, unpublished results).

Additional evidence for the structural relatedness of ribonucleases U_{4A}, U_{4B}, and U_{4C} is presented in the following paper (Blank and Dekker, 1972) which describes the similarity of their catalytic properties.

[6] The relative amounts of RNase U_{4A}, U_{4B}, and U_{4C} have varied in different preparations, either RNase U_{4A} or U_{4B} constituting the major peak of activity on CM-Sephadex. In some preparations, peaks of activity eluting from CM-Sephadex after RNase U_{4A} were observed; whether these activities are physically or catalytically related to those designated RNases U_{4A}, U_{4B}, and U_{4C} is not known.

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

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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

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