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Ribonuclease U_1. Physical and Chemical Characterization of the Purified Enzyme*

William C. Kenney† and Charles A. Dekker‡

ABSTRACT: An improved procedure is described for the formation and purification of an extracellular ribonuclease (RNase U_1) from *Ustilago sphaerogena*. Conditions favorable for growth of the organism and induction of the enzyme by RNA have been established, permitting isolation of suitable amounts of RNase U_1 for characterization of its physical and chemical properties. DEAE-cellulose and ammonium sulfate were used to concentrate the enzyme from the extracellular medium. Acetone fractionation was essential to remove an unidentified viscous component of the extracellular medium which interfered with other means of purification. Repetition of DEAE-cellulose chromatography and ammonium sulfate precipitation gave a preparation which was found to be homogeneous in discontinuous gel electrophoresis and in ultracentrifugation studies; yield, 40 mg of enzyme from 40 l. of culture medium. At pH 7.2 and μ 0.01 this ribonuclease has an ultraviolet absorption maximum at 277 mμ and a minimum at 251 mμ. The A_{280}:A_{260} ratio is 2.5 and a 0.1% solution has an absorbance of 1.71 at 280 mμ. The specific activity of RNase U_1 is 300,000 units/mg of anhydrous protein. The amino acid composition of RNase U_1 is Lys_3, His_2, Arg_2, Asp_{15}, Thr_9, Ser_{13}, Glu_6, Pro_4, Gly_{15}, Ala_5, half-Cys_4, Val_6, Ile_2, Leu_1, Tyr_{12}, and Phe_4 for a total of 103 residues. The amide content of this enzyme is 12 ± 1 and the nitrogen content is 16.2%. The molecular weight is 10,998 based on the above amino acid composition. The amino-terminal residue is half-cystine. This enzyme has a sedimentation coefficient of 1.9 S. From sedimentation equilibrium data a molecular weight of 11,100 was calculated. RNase U_1 has many properties in common with RNase T_1 of *Aspergillus oryzae*. Both enzymes are stable to heat and acid conditions and are inactivated by iodoacetic acid at pH 5.5 in a similar manner. Moreover, the specific activity, specificity, mode of action, and molecular weight of these two enzymes are very similar.

In the search for highly specific ribonucleases, several genera of fungi have been examined (Sato and Egami, 1957; Tanaka, 1961; Glitz and Dekker, 1964a,b; Rushizky *et al.*, 1964; Tatarskaya *et al.*, 1964). In this paper, we are concerned with an RNA-induced extracellular RNase (EC 2.7.7.26, ribonucleate guaninenucleotido-2′-transferase (cyclizing), *Ustilago sphaerogena*) which was shown by Glitz and Dekker (1964b) to be specific for guanylyl residues in RNA. In keeping with the nomenclature of similar RNases from related organisms, this enzyme from *U. sphaerogena* is referred to as RNase U_1.[1] To produce adequate quantities of this enzyme for determination of structure and active site, we have reexamined the conditions for optimum growth, induction, and purification on a large scale. The method described, which makes extensive use of DEAE-cellulose for concentration of the enzyme (Uchida, 1965) and fractionation, permits the preparation of 40 mg of highly purified enzyme from 40 l. of culture medium. This has enabled us to study some of the physical and chemical properties of the enzyme, including the amino acid composition, and to compare them with the corresponding properties of RNase T_1.

Experimental Section

Materials. Yeast sodium ribonucleate was obtained from Schwarz BioResearch, Inc. DEAE-cellulose, a product of Bio-Rad Laboratories (Cellex D), had a capacity of 0.7 ± 0.1 mequiv/g. Bovine serum albumin (BSA)[2] and tris(hydroxymethyl)aminomethane were products of the Sigma Chemical Co. HCl was used to adjust the buffers to the desired pH. Na_2EDTA was obtained from Matheson Coleman & Bell. Iodoacetic acid (Eastman Organic Chemicals) was twice recrystallized before use. For dialysis, cellulose casing (Union Carbide Corp.), $^{18}/_{32}$-in. diameter, was used. Whenever possible, reagent grade chemicals were employed.

Methods

Assay for RNase Activity. The assay for RNase activity was performed by measuring the absorbance at 260 mμ of perchloric acid soluble products obtained from the digestion of high molecular weight RNA according to the method of

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[1] The subscript, 1, is used to denote the major extracellular RNase. Other nucleases from the extracellular medium have been observed with distinct chromatographic properties and specificities.

[2] Abbreviations used are: BSA, bovine serum albumin; CM-RNase U_1, carboxymethylated ribonuclease U_1.

Glitz and Dekker (1964a). The RNA was obtained from wheat germ as previously described (Glitz and Dekker, 1963). The rate of liberation of acid-soluble nucleotides was not linear during the initial period of incubation, presumably because the intranucleotide bonds cleaved did not immediately yield acid-soluble products. The rate of reaction from 3 to 20 min was linear, however, so the difference between the acid-soluble nucleotides liberated at 19.0 and 4.0 min was used as a measure of enzymatic activity. Negligible changes in A_{260} were observed when RNA was incubated in the absence of the enzyme. Blank readings of 0.05–0.08 indicated that the RNA was suitable as a substrate. The assay was linear up to changes in A_{260} of 0.8–0.9. BSA (0.1%) was used as a stabilizing agent when it was necessary to dilute RNase U_1 prior to the assay procedure. One unit of enzyme activity is defined as an increase in A_{260} of 0.250 in this assay. RNase activity was detected qualitatively by the procedure of Glitz and Dekker (1964a). Protein was measured by the method of Lowry *et al.* (1951), using BSA as a standard, and was used in the calculation of the specific activity of the RNase. Protein concentrations were approximated by measurement of the absorbance at 280 mμ.

Culture of the Organism. A modification of the culture medium described by Grimm and Allen (1954) was used in the growth of *U. sphaerogena*. The medium contained 0.08 μM cupric chloride, 0.64 μM manganous sulfate, 36 μM ferrous sulfate, 31 μM zinc chloride, 3.3 mM magnesium sulfate, 6.6 μM thiamine, 11.2 mM ammonium chloride, 0.48 mM citric acid, 29.3 mM sucrose, and 5 g/l. of RNA was added as an inducer and as a phosphate source. The pH of the medium was adjusted to 7.0 with KOH.

Sterile culture medium (40 l.) in a large carboy fitted with several inlets for aeration was inoculated with one liter of a 24-hr culture of *U. sphaerogena* grown on the above medium with the exception that P_i (1 g of K_2HPO_4) was substituted for RNA as the phosphate donor. Antifoam (Dow Chemical Corp., SAG-471), sterilized separately, was added at a level of 0.1 ml/l. of medium. Vigorous aeration—3 l./min per l. of medium—was required for the maximum production of enzyme.[3] In order to keep foaming at a minimum, a slight back-pressure was maintained on the culture medium, which was accomplished by restricting the outlet for the exhaust gases. Glass tubing of 4-mm i.d. was suitable for this purpose. The temperature of the culture medium was maintained at 24 ± 1°.

Growth was followed at A_{445} with appropriate dilutions such that the readings were between 0.1 and 0.2. RNase activity was followed during growth, and the cells were harvested after the activity had reached a maximum level—approximately 70 hr after inoculation.

Experiments to determine the extent of RNase production under various conditions were performed on 25–250 ml of culture medium in erlenmeyer flasks four to five time sthis volume, shaken at 280 rpm at 30°.

Purification of RNase U_1. Cells were removed from the medium by continuous-flow centrifugation using a steam-driven centrifuge (The Sharples Corp.) at 48,000 rpm and a flow rate of 0.5 l./min (step 1). All subsequent operations were performed at 4° unless otherwise noted. The extracellular medium was applied to a column (6.8 × 15 cm) of DEAE-cellulose, preequilibrated with 0.04 M Tris-HCl (pH 7.4) at a flow rate of 30 ml/min. The upper portion of the cellulose was stirred occasionally to decrease the possibility of channeling and to improve the flow rate. The cellulose was then washed with 0.04 M Tris buffer to remove the residual medium. To the contents of the column was added 2 M NaCl to a final concentration of 0.3 M NaCl (assuming 50% of the volume was occupied by resin). The cellulose was stirred into a slurry and allowed to repack in the column before elution with 0.3 M NaCl in 0.04 M Tris-HCl buffer (pH 7.4) to remove RNase U_1 (step 2). The column effluent was brought to 100% saturation with respect to ammonium sulfate by the slow addition with stirring, of 69.7 g of $(NH_4)_2SO_4$/100 ml of solution. The pH was adjusted to 3.6 with 0.5 M H_2SO_4 and the suspension was stirred slowly for 18 hr. The precipitate was collected by centrifugation for 30 min at 10,000*g* and dissolved in a minimum volume of H_2O (step 3). The following fractionation step was performed at −6°. Acetone was added dropwise to the crude enzyme solution until a concentration of 55% (v/v) had been reached. The suspension was stirred for 30 min, and then centrifuged at 10,000*g* for 10 min. The supernatant was then brought to 75% (v/v) concentration with respect to acetone, stirred for an additional 30 min, and centrifuged at 10,000*g* for 10 min. The 75% acetone precipitate was dissolved in H_2O to a concentration of about 1 mg of protein/ml (step 4) and dialyzed against two 3-l. volumes of distilled water for 8 hr.

The dialyzed 75% acetone fraction was applied to a column of DEAE-cellulose (1.3 × 16 cm) preequilibrated with 0.04 M Tris-HCl buffer (pH 7.4). The column was washed with 100 ml of the same buffer and the effluent discarded. The enzyme was eluted using 600 ml of Tris buffer incorporating a linear gradient of 0.0–0.3 M NaCl. Fractions from the column were examined using the qualitative assay for RNase activity and those fractions giving a positive test were pooled (step 5) and subjected to an additional ammonium sulfate fractionation.

The pooled fractions were brought to 70% saturation with respect to $(NH_4)_2SO_4$ by the slow addition with stirring, of 43.6 g of salt/100 ml of solution, stirred for 2 hr, and centrifuged for 10 min at 10,000*g*. Ammonium sulfate (26.1 g/100 ml of original effluent) was then added to bring the concentration to 100%. The pH was adjusted to 3.6 with 0.5 M H_2SO_4, and the suspension was stirred for 18 hr to complete the precipitation. The precipitate was isolated by centrifugation at 10,000*g* for 15 min and dissolved in H_2O (step 6). Dialysis and DEAE-cellulose chromatography were then repeated as described above (step 7). The active RNase fractions of constant specific activity (units/A_{280}) were pooled and extensively dialyzed for 24 hr against four 4-l. portions of distilled H_2O. The dialyzed material was lyophilized and stored at −10°. An aliquot of the lyophilized enzyme was dried *in vacuo* at 115° to a constant weight for determination of the water content (Light and Smith, 1963).

Discontinuous Electrophoresis of RNase U_1. Disc gel electrophoresis in 15% polyacrylamide gel was carried out according to the procedure of Williams and Reisfeld (1964). Homogeneity was examined at pH 7.5 and pH 9.5 using 50, 100, and 200 μg of protein. The gels were analyzed for protein by staining with 0.55% Amido-Schwarz in 7% acetic acid. Destaining was accomplished with 7% acetic acid.

Determination of the Molecular Weight. The molecular weight of RNase U_1 was estimated by sedimentation equilibrium experiments in the Beckman Model E analytical ultracentrifuge. In one set of experiments, the molecular weight was

[3] Two inlets were provided for aeration. Each was constructed with Y-type connecting tubes such that there were eight open branches per inlet through which the air could pass into the medium; inside diameter of tubing, 6 mm.

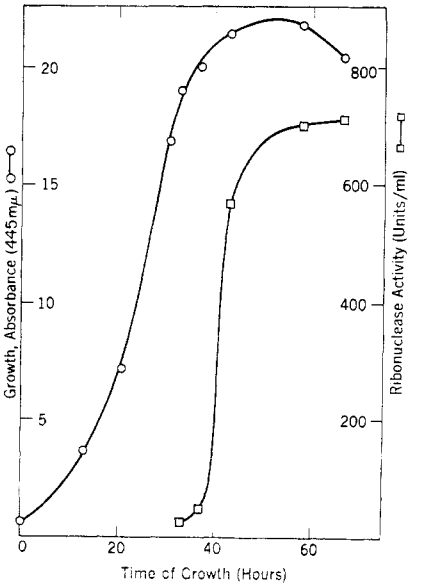

FIGURE 1: Production of RNase and cell growth. Aliquots from 40 l. of culture medium were removed at the designated times. Growth was measured from the turbidity at A_{445} and RNase activity was measured in the usual quantitative assay. The composition of the medium and growth conditions are detailed in Methods.

determined at low centrifugal forces according to the procedure of LaBar (1965). A cell centerpiece for observing three solution–solvent pairs simultaneously was used in the AN-D rotor. Three concentrations of protein were used, 0.02, 0.05, and 0.10% in 0.1 M potassium chloride–1×10^{-3} M potassium phosphate (pH 7.0). The enzyme solutions were dialyzed against this buffer for 6 hr prior to analysis and the dialysate was used as the solvent. The molecular weight was determined at 20,410 rpm at room temperature. After data had been obtained at this speed the rotor was accelerated to 59,780 rpm in order to determine the absolute value of the fringe number and to determine the molecular weight by the meniscus depletion method of Yphantis (1964). In a separate experiment, the molecular weight of a 0.5% enzyme solution was determined at high speeds using a computer program designed by Teller (1965). In these experiments, interference optics were used.

The sedimentation coefficient of RNase U_1 was determined at 59,780 rpm in a synthetic boundary cell at an enzyme concentration of 0.5% in the buffer described above. Schlieren optics were used in this experiment.

RNase U_1 Activity in the RNase T_1 Assay System. The RNase assay described by Takahashi (1965) was used in a comparison of RNase U_1 and the reported activity of RNase T_1. This assay is also based on the measurement of acid-soluble hydrolysis products of RNA. The reaction mixture contained 0.1 ml of enzyme solution, 0.25 ml of 0.2 M Tris-HCl buffer (pH 7.5), 0.1 ml of 2×10^{-2} M EDTA, and 0.3 ml of distilled water. After the reaction mixture had reached 37°, 0.25 ml of RNA (1.2%) was added to start the reaction. After 15 min the reaction was stopped by the addition of 0.25 ml of 0.75% uranyl acetate in 25% perchloric acid. The suspension was centrifuged at 10,000*g* and 0.2 ml of the supernatant was diluted with 5.0 ml of distilled water. The A_{260} was read and compared with a blank without enzyme. The amount of enzyme that causes an increase in absorbance at 260 mμ of 1.00 under the assay conditions is defined as one enzyme unit. Various parameters of the two assay systems were investigated in order to determine which variables caused the greatest differences.

TABLE I: Effect of Various Parameters on Formation of RNase U_1.[a]

Temp of Growth (°C)	Aeration (l./min)	RNase Act. (Units/ml)	% of RNase Bound to DEAE
30	60	400	57
30	90	640	60
24	120	930	74
Growth Medium			
Grimm and Allen (1954)			11
Same after fivefold dilution			68
Modified Medium			65
Same after threefold dilution			60
Recycle effluent of above			4

[a] The effects of temperature and aeration were determined on 40 l. of modified culture medium as described in Methods. Aeration was measured using a calibrated flowmeter. In these experiments, the RNase bound represents the difference between the units applied to the column and those units which could be eluted in the absence of NaCl in step 1 of the purification procedure. The effect of the composition of the medium was determined on 250 ml of culture medium grown at 30°. Columns (1.1 × 2 cm) of DEAE-cellulose, previously equilibrated with 0.04 M Tris-HCl (pH 7.4) were used in the determination of the per cent of RNase bound. The modified medium is that which is described in the text. RNase activity was measured by the usual quantitative assay.

Inactivation with Iodoacetic Acid. RNase U_1 (0.91 mg) was dissolved in 0.4 ml of 0.02 M sodium acetate buffer (pH 5.5) and to this was added 0.4 ml of 1% sodium iodoacetate (pH 5.5). The reaction mixture was incubated at 37° and aliquots were removed at several intervals in order to determine the time course of inhibition. Aliquots were diluted 200-fold into 0.1% BSA and stored at 4° until assayed in the usual quantitative RNase procedure. After 8 hr, mercaptoethanol was added to react with excess iodoacetate and the protein solution was extensively dialyzed against water. Samples were analyzed for glycolic acid according to the procedure of Takahashi *et al.* (1967).

Amino Acid Analysis. Lyophilized RNase U_1 was hydrolyzed in 6 N HCl in evacuated, sealed ampoules at 110° for 24–100 hr according to the procedure of Moore and Stein (1963). A crystal of phenol was added to the hydrolysis tubes in order to minimize the decomposition of tyrosine. The hydrolysates were analyzed on a Beckman-Spinco 120B amino acid analyzer equipped with an expanded absorbance scale.

The cystine content of RNase U_1 was determined on protein which had been oxidized with performic acid (Moore, 1963). Glitz and Dekker (1964a) previously determined the absence of cysteine in this enzyme by titration with *p*-chloromercuribenzoate.

The tyrosine and tryptophan content of RNase U_1 was determined by the spectrophotometric method of Beaven and Holiday (1952). The amide nitrogen content was determined according to the procedure of Hirs *et al.* (1954). A modification of the microkjeldahl method described by Kabat and

TABLE II: Purification of RNase U_1.[a]

Step	Vol (ml)	Total RNase Act. (Units $\times 10^{-6}$)	Total Protein (mg)[b]	Total A_{280}	$A_{280}:A_{260}$	Sp Act. (Units/mg of Protein)	Sp Act. (Units/A_{280})	Yield (%)
(1) Extracellular-medium	34,000	30.8	8900	270,000	0.50	3,460	115	100
(2) DEAE I	1,900	19.4	1100	13,700	0.66	17,600	1,420	63 (100)
(3) Ammonium sulfate I	309	20.6	1370	7,090	0.98	15,400	3,000	67
(4) Acetone	105	20.5	345	1,240	0.99	63,300	16,100	65
(5) DEAE II	178	21.2	142	167	1.57	148,000	124,000	69
(6) Ammonium sulfate II	17.3	15.1	95	98.6	1.79	159,000	153,000	49
(7) DEAE III	51.5	11.2	70.1[c]	65.0	2.64	160,000	172,000	36 (56)

[a] The purification of RNase from 40 l. of growth medium is summarized. The quantitative RNase assay was used in the measurement of activity and protein was estimated by the procedure of Lowry *et al.* (1951). A_{280} and $A_{280}:A_{260}$ were corrected for light scattering by the method of Bonhoeffer and Schachman (1960) in step 7. The values in parentheses represent the overall yield of RNase U_1, assuming that the material that did not bind to DEAE (step 2) was another RNase. [b] BSA equivalent. [c] The actual amount of purified protein is 0.6 times this value since RNase U_1 gives an anomalous color yield in the Lowry protein assay (see Results).

Mayer (1961) in conjunction with the Conway microdiffusion technique was used to determine the total nitrogen content of RNase U_1. The ninhydrin assay of Moore and Stein (1954) was used for the analysis for ammonia in the experiments designed to measure amide and nitrogen content.

Amino-Terminal Residue. The amino-terminal aminoacyl residue of RNase U_1 was analyzed according to the procedure of Stark and Smyth (1963) and Stark (1964).

Results

Culture of the Organism. The growth of the organism and formation of RNase in a 40-l. carboy is outlined in Figure 1. In a typical experiment turbidity reached a maximum at 40–45 hr and decreased slightly thereafter. RNase activity first appeared in the extracellular medium when growth approached the stationary phase and was maximal 20 hr later. Forced aeration of less than 3 l./min per l. of culture medium resulted in diminished enzyme production (Table I). The effect of temperature on enzyme production is given in the same table. When the organism was grown at 24°, 70–80% of the RNase was bound to DEAE-cellulose in the initial purification step. When the organism was grown at 30°, 60–70% of the RNase activity was bound to the cellulose. The failure to bind all activity was not due to saturation of the cellulose since a constant enzyme level appeared in the column effluent throughout the passage of the crude extract through the column. This observation as well as evidence from subsequent purification steps indicates a differential synthesis of at least two RNases. The lower temperature was chosen in order to obtain maximum levels of RNase U_1 in the growth medium.

Several modifications of the medium were made to determine conditions for promoting absorption of RNase to DEAE-cellulose. Use of the medium described by Grimm and Allen (1954) resulted in only slight binding of the enzyme to the DEAE-cellulose (Table I); however, when this medium was diluted fivefold following growth and cell removal, 60–70% of the total RNase activity was bound. Concentrations of salts in the growth medium were therefore reduced to the quantities given in Methods with no decrease in levels of growth of the organism or in RNase formation. A sucrose concentration of 1% and an RNA concentration of 0.5% were suitable for RNase formation.

Purification of RNase U_1. Results of purification of RNase U_1 from the extracellular medium of *U. sphaerogena* after induction with RNA are summarized in Table II. Levels of RNase U_1 approaching 3 mg/l. were generally obtained, corresponding to 1% or more of the extracellular protein. Initial purification steps fulfilled two functions—concentration of the enzyme fraction and removal of the large quantity of degraded RNA, derived from the inducer, which interfered with many conventional procedures of purification. The initial DEAE-cellulose not only concentrated the enzyme solution 18-fold, but also removed 95% of the material absorbing at 280 mμ. After the extracellular medium had passed through the column, the contents of the column were brought to 0.3 M in NaCl by the addition of 2 M NaCl and stirred. This was found to be necessary to prevent channeling due to contraction in the cellulose. An unidentified viscous component was also bound to the cellulose and eluted with 0.3 M NaCl. If the cellulose was eluted with 0.3 M NaCl without previously being brought to 0.3 M by this mixing procedure the solvent front was very viscous resulting in anomalous flow rates, or complete stoppage. The subsequent ammonium sulfate step was used to further concentrate the enzyme solution. The ammonium sulfate precipitate was collected by centrifugation and filtration.

In one experiment, the 0.3 M NaCl effluent was brought to 70% saturation with respect to ammonium sulfate, centri-

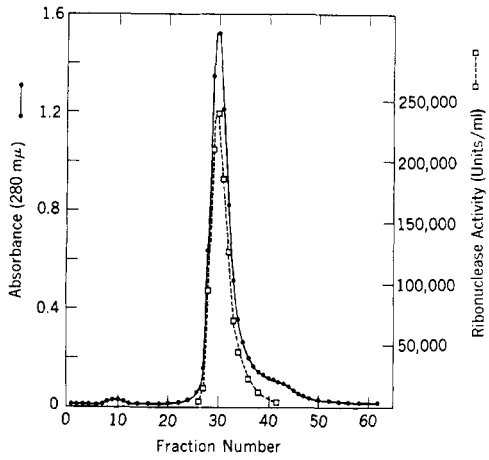

FIGURE 2: Final DEAE-cellulose chromatography of RNase U_1. RNase U_1 from the previous ammonium sulfate step was dialyzed, brought to 0.04 M in Tris-HCl (pH 7.4), and applied to a column of DEAE-cellulose of dimensions 1.3 cm diameter by 16 cm equilibrated with the above buffer. After washing with 100 ml of buffer, the enzyme was eluted using 600 ml of 0.04 M Tris-HCl buffer (pH 7.4), incorporating a linear gradient of 0.0–0.3 M NaCl. The flow rate of the column was 9.1-ml/12 min fraction. The protein concentration was estimated from the absorbance at 280 mμ while RNase activity was determined by the usual quantitative assay. Those fractions (28–32) with constant specific activity (units/A_{280} unit) were pooled, dialyzed, and lyophilized.

fuged, and the supernatant brought to 100% in ammonium sulfate and pH 3.6 as described in Methods. Although a twofold purification was obtained over that given in Table II, this step was generally not included in the purification procedure because of the large volume of enzyme solution still present at this stage.

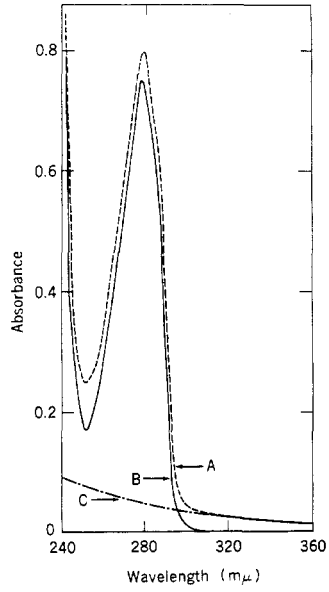

FIGURE 3: Ultraviolet absorption spectrum of RNase U_1. Lyophilized RNase U_1 with a specific activity of 160,000 units/mg (BSA) was dissolved in 0.01 M potassium chloride–1×10^{-4} M potassium phosphate (pH 7.2) at a concentration of 0.50 mg/ml. The spectrum was obtained using a Cary Model 14 recording spectrophotometer. Curve A, spectrum observed. Curve B, spectrum obtained after correcting for light scattering according to the method of Bonhoeffer and Schachman (1960). Curve C, calculated curve for light scattering.

TABLE III: RNase U_1 Activity in the RNase T_1 Assay System.[a]

Assay System	Sp Act. (Units/mg)	% of Control
Glitz and Dekker (1964a)		
Complete	292,000	100
−BSA	302,000	103
+0.002 M EDTA	284,000	97
−Imidazole and + Tris-HCl (0.05 M, pH 7.4)	158,000	54
Precipitation with 0.25% Uranyl acetate in 25% perchloric acid	117,000	40
Incubation at 37°	748,000	260
Takahashi (1965)		
Complete + RNase U_1	13,000[b]	
Reported for RNase T_1	14,000[b]	

[a] The effect of varying several parameters of the assay system has been investigated. The usual quantitative assay system for the measurement of RNase activity was used with the exception of those changes indicated in this Table. The protein concentration was estimated from the absorbance at 280 mμ after correcting for light scattering. [b] Units of RNase activity were calculated according to the procedure of Uchida and Egami (1966).

The solution obtained by dissolving the 100% ammonium sulfate fraction was extremely viscous, but the component producing this viscosity could be readily removed in the subsequent acetone fractionation step. Salt was necessary to complete the precipitation of the viscous component. Consequently, no attempt was made to remove the residual $(NH_4)_2SO_4$ from the previous step. The viscous component was precipitated at an acetone concentration of 35–40%, whereupon the viscosity of the supernatant decreased to the level expected for an aqueous acetone solution. It was then possible to purify the enzyme by repetition of ammonium sulfate fractionation and DEAE-cellulose chromatography. The final DEAE-cellulose profile is given in Figure 2. A nearly symmetrical peak containing all of the RNase activity was obtained. Although the specific activity was 160,000 units/mg of protein (BSA equiv) after the second ammonium sulfate step, it was necessary to repeat the DEAE-cellulose chromatography in order to remove residual ultraviolet-absorbing material which still contaminated the preparation. A standard enzyme solution was then made to determine the physical and chemical characteristics of the enzyme. In the Lowry assay for protein content, RNase U_1 gave 1.75 times the color value obtained from an equivalent weight of BSA. For convenience and economy, however, BSA was generally used as a standard in the measurement of protein concentrations, *e.g.*, see Table II. The moisture content of the lyophilized enzyme after equilibration with the atmosphere at room temperature was 8.35%. Thus, the specific activity is 300,000 units/mg of anhydrous RNase U_1. No higher specific activity has been obtained after further attempts at purification. This same solution was used to obtain the ultraviolet absorption spectrum of the enzyme shown in Figure 3. After correction for light scattering (Bonhoffer and Schachman, 1960) the RNase spectrum has a maximum at 277 mμ, an extinction

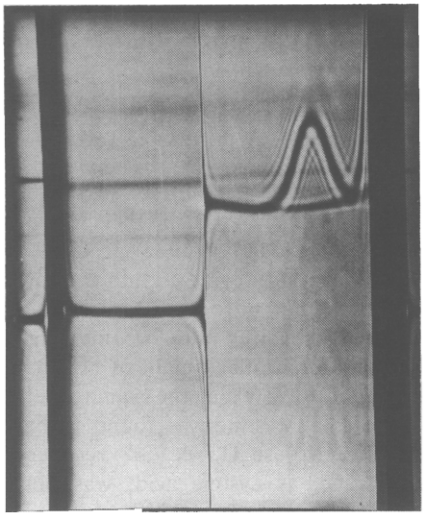

FIGURE 4: Sedimentation velocity pattern of RNase U_1. The boundary represents the pattern obtained 24 min after reaching a speed of 59,780 rpm. RNase U_1 of specific activity 160,000 units/mg (BSA) was dissolved in 0.1 M potassium chloride–0.001 M potassium phosphate (pH 7.2) at a concentration of 0.50 mg/ml. A synthetic boundary cell was used in these investigations.

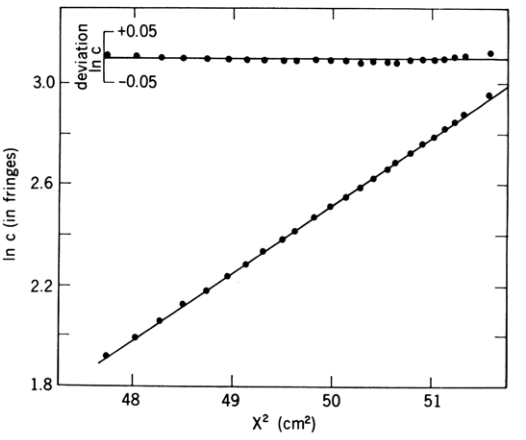

FIGURE 6: Sedimentation equilibrium of RNase U_1. RNase U_1 (160,000 units/mg) was dissolved in 0.1 M potassium chloride–0.005 M potassium phosphate buffer (pH 7.0) at a concentration of 0.5%. The solution was dialyzed against this buffer for 6 hr. Sedimentation was performed in a 12-mm double-sector cell at 17,980 rpm for 17 hr at 20.5°. The ordinate shows the logarithm of the protein concentration (ln c), and the abscissa, x^2, where x is the distance in centimeters from the center of rotation to the point at which the protein concentration is measured. The deviations of ln c from the least-squares slope were evaluated with the computer program of Teller (1965).

coefficient ($\epsilon_{280}^{0.1\%}$) of 1.71, and an unusually high A_{280}:A_{260} ratio of 2.5 which is useful as a measure of purification.

Comparison of the Activity of RNase U_1 with the Reported Value of RNase T_1. The results of a comparison of the quantitative assay described by Glitz and Dekker (1964a) with the assay procedure of Takahashi (1965) using RNase U_1 are given in Table III. RNase U_1 appears to have a specific activity comparable to RNase T_1. The reported specific activity for RNase T_1 is 14,000 units/mg (Uchida and Egami, 1966). Using the definition of "unit" and the method of protein determination which Uchida and Egami (1966) described in the calculation of the specific activity of RNase T_1, a value of 13,000 units/mg is obtained for RNase U_1.

As indicated in Table III, several factors influence the rate of hydrolysis of RNA by RNase U_1, *e.g.*, the nature and/or pH of the buffer solution, the precipitant used, and the temperature of incubation of the reaction mixture. Of these, the last factor, *i.e.*, temperature, influences the results obtained to the greatest extent, and between 28 and 37° the activity increases an average of 18% per degree.

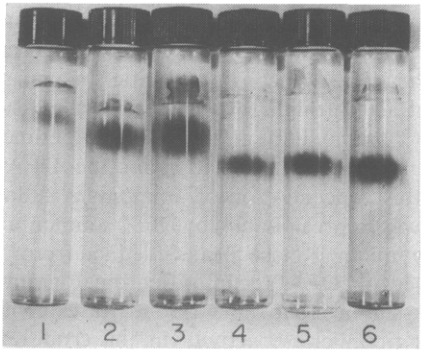

FIGURE 5: Discontinuous gel electrophoresis of RNase U_1. Electrophoresis was carried out at pH 7.5 and 9.5 according to the procedure of Williams and Reisfeld (1964), at three levels of protein concentrations. Gels 1, 2, and 3: run at pH 7.5. Gels 4, 5, and 6: run at pH 9.5. Gels 1 and 4 contain 50 μg of protein, 2 and 5 contain 100 μg, 3 and 6 contain 200 μg.

Homogeneity of RNase U_1. The sedimentation velocity pattern for RNase U_1 showed only a single symmetrical boundary as seen in Figure 4. A corrected value for the sedimentation coefficient, $s_{20,w}$ of 1.91 S, was observed for this preparation. This value is somewhat higher than that previously reported for this enzyme (Glitz and Dekker, 1964a); however, the earlier preparation was not completely homogeneous, as ascertained from the absorption spectrum and starch gel electrophoresis.

Under all conditions examined, discontinuous gel electrophoresis yielded only one heavy band of material as seen in Figure 5.

Sedimentation equilibrium experiments also indicate homogeneity of the enzyme preparation; a linear plot of ln c *vs.* x^2 is obtained (Figure 6).

Molecular Weight of RNase U_1. The observed molecular weights are summarized in Table IV. After extrapolation

TABLE IV: Molecular Weight of RNase U_1.[a]

Method Used	Concn (%)	Mol Wt (Wt Av)
LaBar (1965)	0.05	11,100 ± 300
LaBar (1965)	0.10	11,800 ± 400
Yphantis (1964)	0.02	11,200 ± 300
Yphantis (1964)	0.05	11,300 ± 300
Yphantis (1964)	0.10	11,500 ± 300
Teller (1965)	0.50	11,700 ± 300
Extrapolated value, Yphantis		11,100 ± 300

[a] Lyophilized RNase with a specific activity of 160,000 units/mg (BSA) was dissolved in 0.1 M potassium chloride–0.001 M potassium phosphate buffer (pH 7.2) and dialyzed against this solution for 6 hr. Sedimentation equilibrium experiments were performed as described in Methods.

TABLE V: Amino Acid Composition of RNase U_1.[a]

Amino Acid	g of Amino Acid Residue/ 100 g of Protein	Calcd No. of Residues	No. of Residues, Nearest Integer	RNase T_1 (Takahashi, 1965)
Lysine	3.54	3.04	3	1
Histidine	2.42	1.95	2	3
Arginine	2.81	1.98	2	1
Aspartic acid	15.59	14.9	15	15
Threonine[c]	8.24	8.98	9	6
Serine[c]	10.41	13.2	13	15
Glutamic acid	7.06	6.02	6	9
Proline	3.52	4.0	4	4
Glycine	8.00	15.4	15	12
Alanine	3.20	4.96	5	7
Half-cystine[b]	3.54	3.82	4	4
Valine[d]	5.23	5.81	6	8
Isoleucine	2.04	1.99	2	2
Leucine	1.09	1.05	1	3
Tyrosine[c]	17.91	12.1	12	9
Phenylalanine	5.25	3.92	4	4
Tryptophan			0	1
Amide[e]			11[f]	12[f]
Total	99.85		103	104

[a] The values given are for anhydrous RNase U_1, correcting for a moisture content of 8.35%. Tryptophan was determined by the method of Beaven and Holiday (1952) and found to be zero (less than 0.01) residues per molecule of RNase U_1. [b] The value of half-cystine was determined from the cysteic acid composition. [c] Extrapolated value, correcting for hydrolytic destruction. [d] 100-hr hydrolysis value used based on the slow release of this amino acid. [e] The amide nitrogen content was determined according to the method of Hirs *et al.* (1954). [f] Not included in the total.

to infinite dilution a value of 11,100 ± 300 was obtained in the Yphantis sedimentation equilibrium experiments. The value for the partial specific volume, $\bar{v}$, of RNase U_1 was obtained from a summation of the partial specific volumes of the amino acid residues in the protein. This value was then modified by comparison of the partial specific volume of RNase A determined from the amino acid composition (Schachman, 1957) with that determined by pycnometry (Harrington and Schellman, 1956). In the calculations, a value of 0.678 cm^3/g has, therefore, been used as the partial specific volume of RNase U_1.

An approximate molecular weight may be calculated from the sedimentation coefficient, 1.91 S, if we assume that the diffusion coefficient of RNase U_1 is comparable to that of RNase T_1, namely, 12.0×10^{-7} cm^2/sec (Ui and Tarutani, 1961). A value for the molecular weight of 11,300 ± 300 is then obtained.

The molecular weight determined from the amino acid composition is 10,998.

Inactivation by Iodoacetic Acid. After eight hours of incubation of RNase U_1 with iodoacetate at pH 5.5, greater than 96% of the enzyme had been inactivated, whereas RNase activity decreased by 10% in controls containing no iodoacetate. Analysis of the CM-RNase U_1 for glycolic acid gave 0.8–0.9 mole/mole of protein under a number of test conditions. These included analysis of the high molecular weight fraction from Sephadex G-25 purification of CM-RNase U_1 and analysis of the material present in the effluent after passage of an alkaline hydrolysate of CM-RNase U_1 through Dowex 50 (H^+).

Amino Acid Analysis. The amino acid composition of RNase U_1 is given in Table V. Using the spectrophotometric method of Beaven and Holiday (1952), RNase U_1 was found to contain 12 residues of tyrosine and no tryptophan. RNase U_1 contains 16.2% total nitrogen and 1.5 ± 0.1% amide nitrogen by weight. The latter value is equivalent to 12 amide residues, based on a molecular weight of 11,000.

Amino-Terminal Residue. Using the cyanate method of Stark and Smyth (1963), half-cystine was found to be the amino-terminal residue of RNase U_1. A 73% recovery of amino-terminal half-cystine, as cysteic acid, was obtained when corrected for the recovery of cysteic acid hydantoin carried through the same fractionation procedure. Glitz and Dekker (1964a) have previously established that all the half-cystine residues are present in disulfide-bond form, thus eliminating the possibility of amino-terminal cysteine.

Discussion

Purification of RNase U_1. The removal of highly degraded RNA, present in 1000-fold weight excess over RNase U_1 in the crude extract, was mandatory in the initial stages of purification. This was readily accomplished by passage of the crude extract through a column of DEAE-cellulose. A viscous material which was eluted from the column in the enzyme fraction became more apparent upon further concentration of the enzyme solution; however, this substance could be removed by precipitation with acetone. Once these steps were accomplished, RNase U_1 could be purified by a combination of DEAE-cellulose chromatography and ammonium sulfate fractionation (Table II).

The presence of more than one ribonuclease in the culture medium of *U. sphaerogena* has been reported by Arima *et al.* (1968a). In addition to RNase U_1, two enzymes having preferential specificity for purine nucleotide residues in RNA and a fourth with no base specificity were found (Arima *et al.*, 1968b). This last fraction has been further resolved into at least three ribonuclease components (Blank *et al.*, 1971). Consequently, six or more ribonucleases have been isolated from culture media of *U. sphaerogena*. These findings suggest explanations for several observations made during the purification of RNase U_1. Although a portion of the ribonuclease in the culture medium fails to bind to DEAE-cellulose in the initial stages of purification (Tables I and II), the ribonuclease activity in the effluent is constant during the passage of 40 l. of crude extract through the column. While RNase U_1 is soluble in 70% ammonium sulfate, in some experiments RNase active material was precipitated from the DEAE effluent (step 2) at this concentration of ammonium sulfate and on dissolving, was reprecipitable by 70% ammonium sulfate. Occasionally minor RNase peaks distinct from RNase U_1 were observed on DEAE-cellulose chromatography (step 5, Table II). Assuming that other ribonucleases comprise 37% of the total ribonuclease activity in the crude extract, the overall yield of RNase U_1 is 56% for the preparation outlined in Table II.

Homogeneity of RNase U_1. Several experiments indicate that the RNase U_1 preparation is homogeneous. In discontinuous electrophoresis of a highly purified enzyme prepara-

tion only one protein band is observed (Figure 5). The specific activity both with respect to Lowry protein and absorbance at 280 mμ remains constant during further attempts at purification and no higher levels of specific activity have been attained. The material in the ribonuclease preparation behaves as a single component during sedimentation velocity experiments in the analytical ultracentrifuge. The value obtained for the sedimentation coefficient, 1.91 S, is somewhat higher than that observed for RNase T_1 (1.62 S, Ui and Tarutani, 1961), as well as the value previously reported for RNase U_1 (1.6 S, Glitz and Dekker, 1964a); however, the value observed is consistent with molecular weight estimations (see Results).

Amino Acid Composition of RNase U_1. The amino acid composition of RNase U_1 is very similar to the reported composition of RNase T_1 (Table V). Like RNase T_1, RNase U_1 is an acidic protein as determined by its content of acidic and basic amino acids. The number of hydroxyamino acids in these two enzymes is also quite similar. Those residues which are generally implicated in the tertiary structures of proteins, proline and half-cystine, are present in identical amounts in both proteins. The number of aromatic amino acids is similar although RNase T_1 contains one residue of tryptophan which is absent in RNase U_1. Considerable agreement exists in the other amino acids as well. The total number of amino acid residues in RNase U_1 (103) is also very similar to that found in RNase T_1 (104).

The amino-terminal group of RNase U_1 was found to be half-cystine, which is the second residue from the amino terminus of RNase T_1 (Takahashi, 1965). RNase U_1 as isolated may be missing one residue at the amino terminus, which would be consistent with the shorter chain (one residue) as calculated from amino acid analysis.

Egami and coworkers have independently purified and analyzed RNase U_1. Their preliminary results (Uchida and Egami, 1971) also show 103 total residues but indicate one more aspartic acid residue and one less threonine residue than reported here. Since this variation could result from a 5 to 10% analytical error, it appears highly likely that the same protein is being examined in both laboratories.

Inactivation with Iodoacetic Acid. Further evidence for the similarity of RNase U_1 and RNase T_1 arises from the inactivation of these enzymes with iodoacetate. RNase U_1 was found to be inactivated under essentially the same conditions as described for RNase T_1, in which an active glutamyl residue was implicated (Takahashi *et al.*, 1967). After carboxymethylation of RNase U_1, glycolic acid was detected in the protein at a level of nearly 1 mole/mole of protein. Furthermore, glycolic acid was liberated from the modified protein upon hydrolysis for 18 hr at 20° with 0.1 N NaOH. Since no methionine is found in RNase U_1 and no carboxymethylhistidine is detected in an acid hydrolysate of CM-RNase U_1, this evidence supports the probability of an active carboxylate group in RNase U_1. The amino acid sequence will have to be determined before the reactive component of RNase U_1 can be located in the primary structure of this protein.

Comparison of RNase U_1 with RNase T_1. When expressed in the same units, RNase U_1 and RNase T_1 appear to have similar if not identical specific activities (Table III).

The ribonuclease assay described by Glitz and Dekker (1964a) is approximately six times more sensitive than that used by Takahashi (1965). At 37°, the sensitivity is about 15-fold greater. This is due to the enhanced activity using imidazole buffer, the different dilutions made prior to reading the absorbance at 260 mμ, and the greater effectiveness of uranyl acetate–perchloric acid than perchloric acid alone as a precipitating agent. Due to the latter, high molecular weight RNA should be used in the Glitz and Dekker ribonuclease assay.

Molecular Weight of RNase U_1. A molecular weight of 11,100 ± 300, calculated from sedimentation equilibrium experiments, was obtained for RNase U_1 after extrapolation of the observed values (Table IV) to infinite dilution. A molecular weight of 10,998 was obtained from the amino acid composition. This is very similar to the molecular weight of RNase T_1, 11,085 (Takahashi, 1965).

Conclusion

RNase U_1 obtained from *U. sphaerogena* appears to be quite similar to other fungal ribonucleases, especially RNase T_1 from *A. oryzae*. RNase U_1 and RNase T_1 have similar specific activity, molecular weight, specificity, and mode of action. Furthermore, the two enzymes react similarly with iodoacetate and have recently been shown to be immunologically related (Uchida, 1970). They also respond similarily towards various methods of protein fractionation. Moreover, both organisms give rise to more than one extracellular ribonuclease. It will be of interest to compare the amino acid sequence of RNase U_1 with the known sequence of RNase T_1 (Takahashi, 1965). RNase U_1 should be readily susceptible to sequence analysis because of the three lysine and two arginine residues present in its structure.

The purification procedure described herein should be generally useful for obtaining large quantities of guanylyl specific RNases from microorganisms for biochemical investigations. The structural requirements responsible for the unique specificity of these RNases may then be established.

This enzyme, like RNase T_1, should be useful in the elucidation of primary sequences in ribonucleic acids. Other ribouncleases of limited specificity are present in the culture medium of *U. sphaerogena* (Arima *et al.*, 1968a). Thus a number of specific ribonucleases, as well as nonspecific ones, may be obtained from the extracellular medium of this microorganism.

U. sphaerogena is a parasitic microorganism and as such would require mechanisms by which polymeric substances could become available for growth. The function of these ribonucleases may be to scavenge phosphate or a carbon or nitrogen source from an extracellular environment. A highly specific ribonuclease, *e.g.*, RNase U_1, may be formed in order to produce, possibly in conjunction with other enzymes, fragments which can be taken up by the cell directly, or which are more readily attacked by other less specific enzymes.

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Determination of Nucleotide Sequences at Promoter Regions by the Use of Dinucleotides*

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ABSTRACT: The nucleotide sequences at the promoter regions and the initiating sequences of RNAs synthesized *in vitro* with T4 or T5 DNAs as templates have been determined by using dinucleotides to initiate RNA synthesis. This method is based on the observations that only dinucleotides that are complementary to the nucleotide sequences at promoter regions, in accordance with Watson–Crick base pairing, can serve as initiators and are incorporated into the 5′ ends of RNA chains. Initiation with dinucleotides was measured by the stimulation of the overall rate of RNA synthesis at low substrate concentrations and by the competition of dinucleotides with [γ-^{32}P]ATP and [γ-^{32}P]GTP for incorporation into the 5′ ends of RNA chains. The initiating dinucleotide sequence of RNA synthesized in the presence of σ factor consists primarily of ApU when either T4 or T5 DNA is used as a template, whereas with core polymerase and T5 DNA as template, the initiating dinucleotide sequence is primarily GpA. The present studies suggest that σ factor recognizes the nucleotide sequence of d(Pu←T←A) at the promotor region of either T4 or T5 DNA, and core polymerase recognizes the sequence d(T←C←T), or a cluster of pyrimidines, in the DNA template. The possible heterogeneity of core polymerase is also discussed.

The regulation of gene expression by positive control elements has now been demonstrated in several microbial systems (Burgess *et al.*, 1969; Travers and Burgess, 1969; Bautz *et al.*, 1969; Losick and Sonenshein, 1969; Travers, 1970). It has been suggested that positive control factors, such as the *E. coli* σ factor, direct the core polymerase to bind to specific promoter sites on the DNA template where RNA chains are initiated. This specificity is reflected in a higher degree of asymmetric transcription in the presence of σ factor (Bautz *et al.*, 1969; Goff and Minkley, 1969; Sugiura *et al.*, 1970).

It is believed that each positive control factor would allow recognition of a unique nucleotide sequence at a specific promoter site or sites on the DNA template. Thus, it is of interest to develop a rapid and reliable method to determine the 5′-terminal nucleotide sequence of *in vitro* synthesized RNA and

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