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Studies on a Ribonuclease from *Ustilago sphaerogena*. I. Purification and Properties of the Enzyme*

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When grown on a medium in which the sole source of carbon is RNA, the smut fungus *Ustilago sphaerogena* produces an extracellular ribonuclease. This enzyme has been purified about 300-fold, and has a specific activity equivalent to that of other highly purified ribonucleases. The enzyme is essentially homogeneous upon ultracentrifugation and electrophoresis. The *Ustilago* nuclease is similar to ribonuclease T_1 in a number of characteristics including sedimentation coefficient (1.6 S), *p*H optimum, heat and acid stability, and lack of iodoacetate reactivity. Moreover, both the *Ustilago* and T_1 ribonucleases appear to be acidic proteins. Comparison of pancreatic ribonuclease with the *Ustilago* enzyme indicates differences in addition to the sedimentation coefficient and iodoacetate reaction cited: the *Ustilago* enzyme is not inhibited by polyvinyl sulfate, and shows a marked decrease in activity as the ionic strength is increased. The most striking properties of the *Ustilago* enzyme, not shared by either the T_1 or pancreatic ribonucleases, are a requirement for protective protein and a strong inhibition by added sodium or magnesium chloride when RNA is the substrate.

As a part of a search for new enzymes which could be of value in the determination of nucleotide sequences in ribonucleic acid, it was discovered that the smut fungus *Ustilago sphaerogena* produced an extracellular ribonuclease when grown on a medium containing RNA as the substrate. Studies of this enzyme have indicated a number of similarities to a second fungal ribonuclease, RNAase T_1 of *Aspergillus oryzae*, discovered by Sato and Egami (1957). In this report the purification as well as some of the physical and chemical properties of the *Ustilago* enzyme will be considered, while the enzymic activity and specificity of the nuclease will be examined in the following paper.

MATERIALS AND METHODS

Materials.—All chemicals used were of reagent grade unless otherwise specified. Imidazole (Aldrich Chemical Co.) and Tris (Sigma 121, primary standard grade tris[hydroxymethyl]aminomethane) buffers were adjusted to the desired *p*H using HCl. Pancreatic ribonuclease was purchased from either Armour or Sigma, while the sample of ribonuclease A was purchased from the Worthington Biochemical Corp. and rechromatographed by Dr. M. Irie of this laboratory. Crystalline bovine plasma albumin (Sigma or Armour) was used in all cases. Other proteins were obtained as follows: β-Lactoglobulin was purchased from the Nutritional Biochemicals Corp. Crystalline alkaline phosphomonoesterase of *Escherichia coli* was purchased from Worthington. The sample of ribonuclease T_1 was a product of Sankyo, Ltd., Tokyo, Japan, and was obtained through the courtesy of Drs. F. Egami and H. Fraenkel-Conrat. The previous history of the T_1 enzyme sample is not known.

Formation of the Enzyme.—Culture media containing RNA as a sole source of carbon were prepared using varying quantities of commercial yeast RNA (Schwarz, sodium salt), 0.05% ammonium chloride, and 0.01% yeast extract (Difco Laboratories). Twelve-ml portions contained in 2.5-cm culture tubes were sterilized in an autoclave for 15 minutes at 121°, inoculated with a drop of cell suspension, and incubated at 30° on a reciprocal shaker operated at 90 cycles/min. Growth was estimated by measurement of turbidity at 650 mμ using a Bausch and Lomb Spectronic 20 colorimeter.

Methylated albumin-kieselguhr chromatography was conducted as follows: Columns of 2.3 cm diameter by 2.8 cm length were poured using small sintered-glass funnels and 25 ml of methylated albumin-kieselguhr suspension, prepared according to the method described by Sueoka and Cheng (1962). The column was washed with 300 ml of 0.05 M NaH_2PO_4–0.05 M NaCl buffer before application of about 1.5 mg of RNA sample in a volume of 1 ml. Elution was begun immediately using 0.05 M NaH_2PO_4 buffer with a linear gradient of 0.05–0.95 M NaCl; total volume, 600 ml. Fractions of 10 ml were collected using a flow rate of 2.5 ml/min.

Purification of the Enzyme.—The growth medium employed for enzyme production consisted of 0.5% commercial yeast RNA (Schwarz, sodium salt), 0.05%

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TABLE I
PURIFICATION OF *Ustilago* NUCLEASE[a]

Step	Volume (ml)	RNAase Activity (units/ml)	Specific Activity (units/mg protein)	Yield (%)
(1) Crude medium	50,000	400	526	100
(2) Concentrate	525	28,820	1,900	76
(3) Sephadex G-25	2,450	4,280	5,700	52
(4) Ammonium sulfate I	100	112,600	24,750	55
(5) DEAE-cellulose	620	15,000	53,000	50
(6) Ammonium sulfate II	50	190,000	161,000	48
(7) Phenol extract	10	665,000	175,000	33

[a] The purification of nuclease from 50 liters of growth medium is summarized. Ribonuclease activity was measured in the usual quantitative assay, and protein by the procedure of Lowry *et al.* (1951).

ammonium chloride, and 0.01% yeast extract (Difco Laboratories). One-liter portions contained in 4-liter Erlenmeyer flasks were sterilized for 15 minutes at 121°, cooled to room temperature, and directly inoculated with a loop of *U. sphaerogena* from a malt extract–agar slant (Difco). The flasks were incubated for 72 hours at 30° while agitated on a reciprocal shaker operating at a rate of 90 cycles/min. The cells were removed by centrifugation at 1500 × *g* for 30 minutes and discarded. The supernatant (fraction 1) was concentrated about 100-fold using a rotary evaporator with a bath temperature of 40°. Any insoluble materials were removed by centrifugation and washed with a few ml of distilled water, and the washings were added to the supernatant (fraction 2).

All the following steps were carried out at 0–4° unless otherwise specified. Forty-ml portions of concentrated enzyme solution (fraction 2) were applied to a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) of dimensions 4.8 cm diameter by 35 cm length, and the protein was eluted with distilled water. The first 210 ml (including the volume of liquid eluted from the column as the concentrated enzyme solution was applied) was discarded, and the next 170 ml collected (fraction 3). The column was washed with at least 2 liters of distilled water before reuse.

Fraction 3 was brought to 65% saturation with ammonium sulfate by the slow addition of 39.8 g of solid per 100 ml of solution. After stirring for about an hour the suspension was centrifuged for 15 minutes at 10,000 × *g* and the precipitate was discarded. The supernatant was then brought to 100% saturation with ammonium sulfate by the addition of 30 g of solid per 100 ml of fraction 3, the *p*H was adjusted to 3–4 with 0.5 M sulfuric acid, and the suspension was stirred slowly overnight. The precipitate was collected by centrifugation for 15 minutes at 25,000 × *g* and the supernatant was discarded. The precipitate was dissolved in a minimal volume of water (fraction 4) and dialyzed against two 3-liter portions of distilled water for 8 hours.

The contents of the dialysis tubing was applied to a column of DEAE-cellulose (Cellex D, Bio-Rad Laboratories, capacity 0.65 meq/g), of dimensions 4.5 cm diameter by 25 cm length, which had been equilibrated with 0.05 M Tris hydrochloride buffer (Sigma 121), *p*H 9.0. The column was washed with 1 liter of the same buffer and the effluent was discarded. The enzyme was eluted using 3 liters of Tris hydrochloride buffer including a linear gradient of 0.0–1.0 M sodium chloride. Fractions from the column were examined using the qualitative assay for ribonuclease activity (see Assay Procedures) and those fractions giving a positive RNAase test were pooled (fraction 5).

The column effluent was brought to 75% saturation with respect to ammonium sulfate by the slow addition of 47.6 g of solid per 100 ml of fraction 5. The final portions of ammonium sulfate were added over a period of a few hours. A fine crystalline precipitate formed and was removed by centrifugation at 15,000 × *g* for 15 minutes. The crystals were dissolved in a minimal volume of distilled water, recrystallized from 75% saturated ammonium sulfate solution, and centrifuged as above. The crystals were discarded and the 75% saturated ammonium sulfate supernatant solutions then were combined and brought to 100% saturation with respect to ammonium sulfate by the addition of 17.4 g of solid per 100 ml of solution. The *p*H was adjusted to 3–4 with 0.5 M sulfuric acid, and the enzyme suspension was stirred slowly for about 10 hours to complete precipitation. The precipitate was collected by centrifugation and extracted successively with 100-ml portions of 100%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, and 50% saturated ammonium sulfate solution, centrifuging for 10 minutes at 10,000 × *g* between each extraction. The fractions containing nuclease were combined, and the enzyme was precipitated by bringing the solution to saturation with ammonium sulfate and stirring the suspension slowly overnight. The precipitate was dissolved in a minimal volume of distilled water and dialyzed for 24 hours against five 3-liter portions of distilled water. The contents of the dialysis tubing was dried by lyophilization and dissolved in a quantity of distilled water sufficient to give a concentration of about 1 mg protein per ml (fraction 6).

Fraction 6 was extracted with 0.5 volume of water-saturated phenol, the layers were separated by centrifugation for 10 minutes at 10,000 × *g*, and the aqueous layer was re-extracted twice with 0.3-volume portions of water-saturated phenol solution. The combined phenol layers were lyophilized until no phenol odor could be detected, and the solid was dissolved in a minimal volume of distilled water, applied to a column of DEAE-cellulose, and chromatographed as was done earlier, with the exceptions that all operations were carried out at room temperature and only 1 liter of elution buffer containing a sodium chloride gradient was used. Active fractions were located using the qualitative RNAase assay, pooled, and dialyzed for 24 hours against five 3-liter portions of distilled water. The contents of the dialysis bag was lyophilized and the solid was dissolved in a volume of distilled water sufficient to give a concentration of about 4 mg/ml (fraction 7). Data summarizing the purification of enzyme from 50 liters of growth medium are presented in Table I.

Assay Procedures.—The quantitative assay for ribonuclease activity which was used was based on the measurement of perchloric acid–soluble nucleotides formed from wheat germ RNA (Glitz and Dekker,

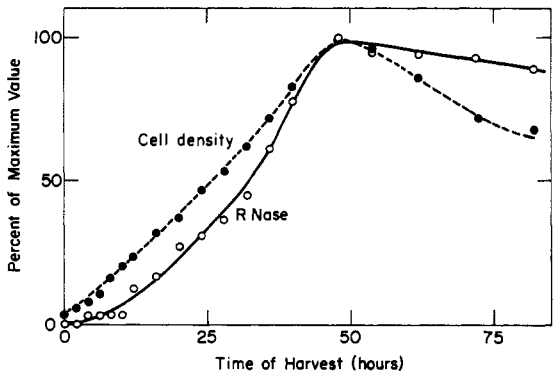

FIG. 1.—Rate of nuclease formation and cell growth. Portions (12 ml) of culture medium were inoculated with 0.1 ml of a suspension of *U. sphaerogena* cells grown in the same medium (0.5% yeast RNA, 0.05% ammonium chloride, 0.01% yeast extract). The cultures were incubated for the periods indicated using a reciprocal shaker operated at a rate of 90 cycles/min and a temperature of 30°. Ribonuclease activity was measured in the usual quantitative assay, while growth was estimated by measurement of turbidity at 650 mμ.

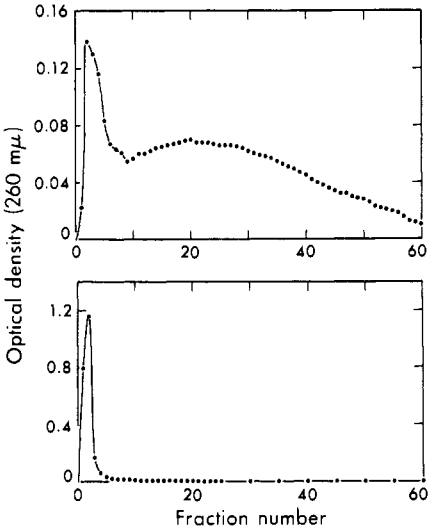

FIG. 2.—Methylated albumin-kieselguhr chromatography of *Ustilago* medium. About 1.5 mg of RNA contained in the *U. sphaerogena* growth medium was adsorbed on a column of methylated albumin-kieselguhr of dimensions 2.3 cm diameter by 2.8 cm length and chromatographed using 600 ml of 0.05 M NaH_2PO_4 buffer employing a linear gradient of 0.05–0.95 M NaCl. The upper pattern was obtained using sterilized medium, while the lower pattern was given by the same volume of medium after growth of the organism for 72 hours (i.e., the crude *Ustilago* nuclease preparation). Fractions of 10 ml were collected using a flow rate of 2.5 ml/min. (Note the difference in scale of the ordinates of the two graphs.)

1963) upon incubation with the enzyme. The assay mixture contained the following: 0.1 M imidazole, *p*H 7.0, 0.6 ml; 1% bovine plasma albumin, *p*H 7.0, 0.2 ml; 1.5% wheat germ RNA, 0.2 ml; enzyme (*ca.* 1 unit) plus water, 0.5 ml. The reaction materials were brought to a temperature of 28° and the reaction was started by the addition of substrate RNA. The solution was mixed thoroughly. Fifteen seconds after the addition of RNA, 0.5 ml of the assay mixture was withdrawn and immediately added to 1.0 ml of cold 10% perchloric acid solution contained in a 10-ml polyethylene centrifuge tube. Immediate and vigorous mixing was essential to obtain a fine, particulate precipitate. Fifteen minutes after the removal of the initial aliquot a second 0.5-ml sample was withdrawn from the assay mixture and added to a second 1.0-ml portion of cold 10% perchloric acid. About 10 minutes after the addition of the aliquot of reaction mixture, the perchloric acid suspensions were diluted with 2.0-ml portions of cold distilled water and centrifuged for 10 minutes at 10,000 × *g*, and the optical densities of the supernatant solutions were measured at 260 mμ. The optical density of the initial (15-second) aliquot was subtracted from that of the final aliquot to give the increase in acid-soluble ultraviolet-absorbing materials resulting from enzymic action. An increase in optical density at 260 mμ of 0.250 was defined as one unit of enzyme activity.

The qualitative assay for ribonuclease activity was adapted from the spectrophotometric assay of Shapira (1962). The assay mixture consisted of the following: 0.01% thionine (Calbiochem), 1.0 ml; 0.1 M imidazole, *p*H 7.0, 3.0 ml; 1% bovine plasma albumin, *p*H 7.0, 1.0 ml; 1.5% wheat germ RNA, 0.1 ml. One-tenth ml of the above assay mixture was placed in a depression of a white spot-plate and 5 μl of enzyme solution (containing at least 0.05 μg of pancreatic ribonuclease or the equivalent) was added. After about 5 minutes the color was compared to the color of an aliquot of assay mixture to which no enzyme had been added. In the presence of ribonuclease the blue color of the assay solution changed to purple.

Phosphomonoesterase activity was estimated spectrophotometrically using the *p*-nitrophenyl phosphate method of Garen and Levinthal (1960), except that the total increase in optical density after 10 minutes of incubation was measured. Protein was measured using the method of Lowry *et al.* (1951) with a bovine plasma albumin (Sigma) standard. The specific activities of all enzyme preparations reported here are based on this measurement of protein concentration. The approximate concentration of protein was estimated by measurement of the optical density at 280 mμ using an extinction coefficient ($\epsilon_{1\%}$) of 10. Nucleic acid concentration was estimated in a similar manner using the OD at 260 mμ and an $\epsilon_{1\%}$ of 250.

Criteria of Purity of the Enzyme.—Sedimentation velocity of the *Ustilago* enzyme was measured in sucrose density gradients using a Spinco Model L preparative ultracentrifuge with an SW 39 swinging-bucket rotor. Gradients were prepared and fractions were collected using the techniques and apparatus described by Martin and Ames (1961). The phosphomonoesterase of *E. coli* (Worthington) was used as a marker enzyme. Sedimentation velocity in the analytical ultracentrifuge was measured by Miss F. Putney using a Spinco Model E unit equipped with a schlieren optical system.

Starch-gel electrophoresis was performed according to the method of Barrett *et al.* (1962), using a discontinuous buffer system at *p*H 8.0. The spectrum of the enzyme at a concentration of 0.19 mg/ml was measured using a Beckman DU spectrophotometer. A correction for light scattering was made using the technique described by Bonhoeffer and Schachman (1960).

Properties of the Enzyme.—Inhibition studies of the enzyme were performed using either the usual quantitative assay for ribonuclease activity or an automatic constant-*p*H-recording titrator (*p*H-stat) manufactured by the International Instrument Co., Canyon, Calif. In the latter case a *p*H of 7.0 was maintained by the addition of *ca.* 0.025 M potassium hydroxide, using a 10-ml reaction mixture containing 10 mg of substrate and 1 mg of protective bovine plasma albumin. The temperature was maintained at 28° and enzyme activity was estimated by the rate of alkali

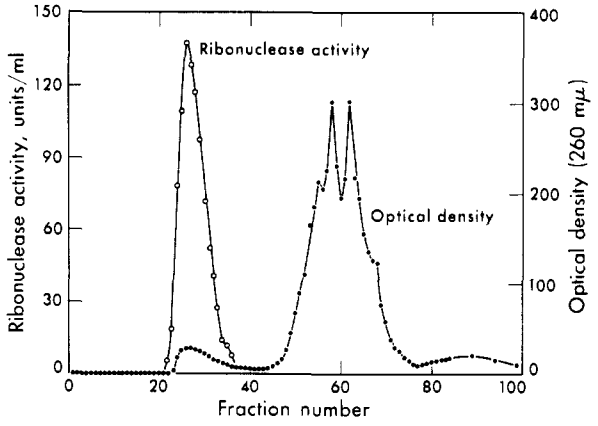

FIG. 3.—Sephadex G-25 gel filtration of *Ustilago* nuclease. Forty ml of enzyme concentrate was chromatographed using a column of Sephadex G-25 of dimensions 4.8 cm diameter by 35 cm length. Elution was carried out with distilled water using a flow rate of 2 ml/min and collecting fractions of 10 ml. Nuclease activity was measured in the usual quantitative assay, while nucleotide concentration was approximated by measurement of the optical density at 260 mμ.

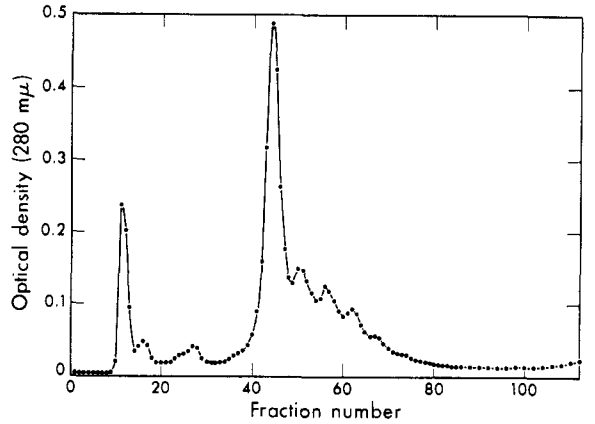

FIG. 4.—DEAE-cellulose chromatography of phenol-extracted *Ustilago* nuclease. About 10^7 units of enzyme were freed of phenol by lyophilization and applied to a column of DEAE-cellulose of dimensions 4.5 cm diameter by 25 cm length. The enzyme was chromatographed using 1 liter of 0.05 M Tris hydrochloride buffer, *p*H 9.0, employing a linear gradient of NaCl ranging from 0.0 to 1.0 M. The protein concentration was estimated by measurement of the optical density at 280 mμ, while RNAase activity was located in fractions 40–48 using the qualitative thionine assay.

uptake. Polyvinyl sulfate was prepared from polyvinyl alcohol using the method of Bernfeld *et al.* (1960). Poly-L-lysine of an average chain length of 50 residues was purchased from Mann Research Laboratories, while spermine tetrahydrochloride and spermidine phosphate were products of Hoffmann LaRoche. Guanosine-2′,3′-cyclicphosphate (G-cyclic-p) was prepared according to the method of Michelson (1959) and purified by paper chromatography using a 2-propanol-NH_3–H_2O solvent.

Incubation of nucleases with iodoacetate was carried out at 37° and *p*H 6.0. The concentration of nuclease was 1–2 mg/ml, while iodoacetate was present in a concentration of 3 mg/ml. Samples were withdrawn as desired and immediately diluted by a factor of 1000, using cold 0.1% bovine plasma albumin, before measurement of ribonuclease activity in the usual quantitative-assay procedure. The reaction with *p*-mercuribenzoate (Sigma) was carried out according to the method of Boyer (1954) except that the solutions were 7.5 M with respect to urea. Acetate buffer was used at *p*H 4.6 and a concentration of 0.33 M, while 0.05 μmole of *p*-mercuribenzoate was present. The total volume of the reaction mixture was 4.0 ml. Optical densities at 260 mμ were measured using a Beckman DU spectrophotometer, and ultraviolet spectra were measured with a Cary Model 14 unit. For purposes of calculation of equivalents of sulfhydryl groups the molecular weight of β-lactoglobulin was taken as 35,500 (Boyer, 1954), and that of pancreatic RNAase A as 13,700 (Spackman *et al.*, 1960). The molecular weight of the *Ustilago* enzyme was assumed to be about 11,000, as has been reported for the T_1 ribonuclease (Ui and Tarutani, 1961).

RESULTS

Formation of the Enzyme.—A comparison has been made of the rates of cell growth and ribonuclease appearance employing the usual (0.5% RNA) medium. The results are indicated in Figure 1. Ribonuclease activity appears to closely parallel cell growth: the apparent drop in cell density after about 50 hours of incubation represents aggregation of the organism. The effect of RNA concentration in the medium on both cell density and ribonuclease activity has been examined. Growth and nuclease activity in the medium are both negligible in the absence of substrate RNA, but show a parallel increase as the RNA level is raised to about 0.5%. The presence of greater quantities of RNA substrate does not appreciably increase the level of nuclease, while only a slightly greater cell density is observed. Replacement of the substrate RNA with glucose resulted in heavy growth of *Ustilago* cells, but no ribonuclease activity could then be detected in the medium.

The method of culture employed appears to have a strong effect on the level of nuclease in the medium in which cells have been grown. Ten to twenty times as much nuclease activity was found in the medium if liter portions of culture medium in 4-liter Erlenmeyer flasks were inoculated and incubated under the same conditions used in the examination of 2.5-cm culture tubes, but with the period of growth extended to 72 hours to obtain maximal cell densities. An attempt was made to grow the organism in an 18-liter carboy using 15 liters of growth medium and forced-air aeration and agitation. In this case growth was very heavy, but no ribonuclease activity could be detected in the medium.

U. sphaerogena cells grown in the RNA med um were ground with alumina and the nuclease activity of the resulting extract was determined. Only 0.05% of the total nuclease activity was found in the extract The remainder was located in the growth medium after removal of the cells.

Chromatography using methylated albumin-kieselguhr was performed on the RNA-containing growth medium both before and after culture of *U. sphaerogena* cells, as shown in Figure 2. Considerable degradation of the substrate RNA is apparent, al hough only about 30% of the initial ultraviolet-absorbing materials have been removed from the medium by growth of the organism. Under a variety of conditions of culture and levels of substrate RNA concentration, it was found that no more than 30–35% of the substrate supplied was removed from the medium during the growth period.

Purification of the Enzyme.—The results of the purification of *Ustilago* nuclease from 50 liters of growth medium are presented in Table I. Concentration of the crude medium could be performed in the rotary evaporator with only slight loss in activity. In prepa-

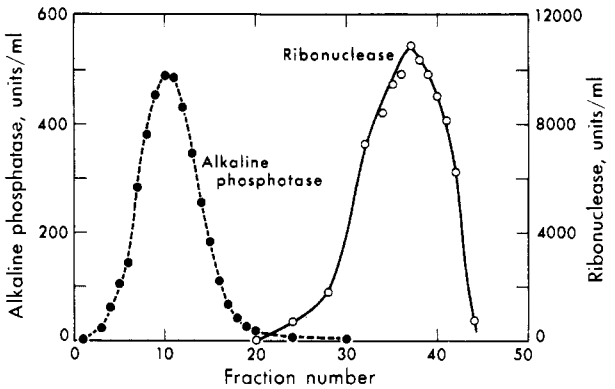

FIG. 5.—Sucrose-density-gradient centrifugation of *Ustilago* nuclease. Nuclease solution (0.2 ml; containing *E. coli* alkaline phosphatase as a marker enzyme) was layered on top of 4.6 ml of 5–20% sucrose density gradient and centrifuged for 24 hours at 38,000 rpm using a Spinco Model L centrifuge and an SW-39 swinging-bucket rotor. Fractions of 6 drops per tube were collected and assayed in the usual manner.

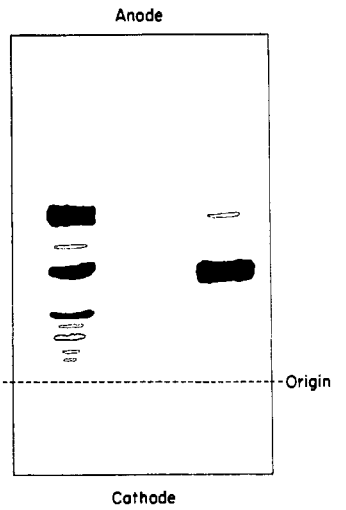

FIG. 6.—Starch-gel electrophoresis of *Ustilago* nuclease. Electrophoresis was carried out at *p*H 8.0 according to the method of Barrett *et al.* (1962). The pattern on the right was obtained with the *Ustilago* enzyme preparation, while that on the left was obtained using crystalline bovine plasma albumin (Sigma).

ration of smaller quantities of enzyme, concentration was accomplished by lyophilization, resulting in yields of about 100% of the initial enzyme activity. After gel filtration (step 3), 95% or more of the contaminating nucleotides were removed (Fig. 3) and ordinary fractionation procedures could be applied. Attempts to use common protein-fractionation methods, such as precipitation with acetone or ammonium sulfate, were unsuccessful or difficult to reproduce before concentration of the enzyme and removal of most of the degraded RNA.

In step 4, nuclease activity was precipitated most completely and reproducibly at *p*H values of 3–4. The second ammonium sulfate fractionation (step 6), following DEAE-cellulose chromatography, removed a crystalline impurity of unknown function from the nuclease. When the precipitate of step 6 had a specific activity of 1.6×10^5 units/mg protein, ammonium sulfate extraction resulted in no further increase in specific activity. Moreover, after this stage of purification had been attained, no improvement could be achieved by fractionation with acetone, further treatment with ammonium sulfate, or rechromatography using DEAE-cellulose. In the last case a single symmetrical peak accounting for all the nuclease activity was found to be eluted from the column at a salt concentration of about 0.3 M.

Extraction of the *Ustilago* enzyme into water-saturated phenol, as accomplished with other nucleases by Rushizky *et al.* (1963), resulted in a decrease in the specific activity of the preparation to *ca.* 10^5 units/mg protein. However, rechromatography of this material using DEAE-cellulose resulted in considerable fractionation, as shown in Figure 4. The main protein peak possessed a specific activity of 175,000 units/mg protein; no greater specific activity has yet been attained.

Criteria of Purity of the Enzyme.—Enzyme of specific activity 1.6×10^5 units/mg protein has been sedimented in sucrose density gradients with the alkaline phosphomonoesterase of *E. coli* added as a marker enzyme. In a separate but identical gradient in the same rotor the *Ustilago* enzyme was sedimented independently to determine protein distribution. Because of the small amount of enzyme available for the experiment, protein could be measured only in tubes 34–40 with a maximum at tube 37. This is coincident with the peak of maximum nuclease activity as shown in Figure 5. Using a value of 6.3 S for the sedimentation coefficient of the *E. coli* phosphomonoesterase (Garen and Levinthal, 1960), an approximate sedimentation coefficient of 2 S was calculated for the nuclease using the data of Figure 5. Sedimentation of the same nuclease preparation in the analytical ultracentrifuge resulted in the formation of a very sharp boundary which accounted for an estimated 90% of the material present. A sedimentation coefficient of 1.6 S was calculated for this boundary.

Starch-gel electrophoresis of the best preparation showed one very heavy band and a second, barely visible component. In the same run crystalline bovine plasma albumin was split into four major bands and a number of minor components. A drawing of the gel is shown in Figure 6. The ultraviolet spectrum of the same preparation was measured and is shown in Figure 7. At any stage of purification earlier than step 7 the spectrum showed an absorption maximum at 260 mμ, indicating probable contamination by nucleic acid or degradation products thereof.

As noted earlier, the specific activity of the best *Ustilago* nuclease preparation was 175,000 units/mg protein. In the same assay procedure the specific activity of various preparations of pancreatic ribonuclease (Schwarz or Armour) was about 40,000, while rechromatographed pancreatic ribonuclease A (Worthington) showed a specific activity of 55,000 units/mg protein. A commercial sample of ribonuclease T_1 had a specific activity of 24,000.

Properties of the Enzyme.—When measured in the usual assay procedure using imidazole buffer, the *Ustilago* enzyme shows a *p*H optimum of 7.0–7.5, dropping sharply on each side of this range. The buffer in which enzyme activity was measured was found to have a great effect on the absolute activity and the apparent *p*H optimum. The relative activity of the enzyme at an early stage of purification (specific activity about 5000 units/mg protein) at *p*H 6.0 and 7.0 in a number of different buffers is presented in Table II. Similar results were obtained in less extensive experiments using highly purified enzyme. The greatest absolute activity thus far measured was found using imidazole buffer at *p*H 7.0. Protective bovine plasma albumin was found necessary for the measurement of enzyme activity even at very early stages of purification;

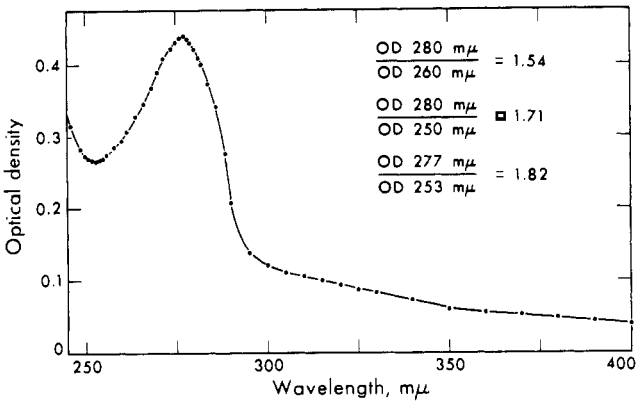

FIG. 7.—Spectrum of *Ustilago* nuclease. Enzyme of specific activity 175,000 units/mg protein at a concentration of 0.19 mg/ml was used. Measurements were made using a Beckman DU spectrophotometer and corrected for light scattering according to the method used by Bonhoeffer and Schachman (1960).

TABLE II

EFFECT OF BUFFER ON ACTIVITY OF *Ustilago* NUCLEASE[a]

Buffer	*p*H 6.0 Units/ml	*p*H 6.0 Relative Activity	*p*H 7.0 Units/ml	*p*H 7.0 Relative Activity
Imidazole	1362	1.00	2375	1.00
Bicarbonate	1532	1.12	274	0.10
Cacodylate	934	0.68	760	0.28
β-Glycerol phosphate	416	0.30	271	0.10
Sodium phosphate	330	0.24	65	0.02
EDTA	890	0.65	1230	0.45
Sodium citrate	388	0.28		
Ethylenediamine			440	0.16
2-Aminopyridine			1440	0.53
Tris hydrochloride			1335	0.49
Pyrophosphate			<10	<0.01

[a] Enzyme activity was measured in the usual quantitative assay procedure. All buffers were added to the assay mixture at a concentration of 0.1 M, resulting in a final concentration of buffer of 0.04 M in the reaction medium. The specific activity of the enzyme preparation used was about 5000 units/mg protein.

in the absence of added protective protein the activities measured were generally low and quite difficult to reproduce. The effect of substrate concentration on the apparent activity of the *Ustilago* enzyme has been examined employing the usual quantitative assay procedure, but varying the quantity of RNA present in the incubation mixture. Considerable substrate inhibition was found as the RNA concentration was increased beyond 0.75 mg/assay, using about 1.5 units of nuclease activity in each case. Under the conditions employed in the assay procedure about 60% of the maximal quantity of acid-soluble material was formed by incubation with the enzyme, but minor variations in the quantity of RNA present did not alter the amount of acid-soluble nucleotides formed by a given quantity of enzyme.

No organic or metallic cofactor has been found necessary for the action of the *Ustilago* nuclease, and, in fact, added salts appear to be inhibitory even at low levels. The degree of inhibition of hydrolysis of wheat germ RNA (either s-RNA or high-molecular-weight RNA) and G-cyclic-p by added sodium or magnesium chloride is shown in Figure 8.

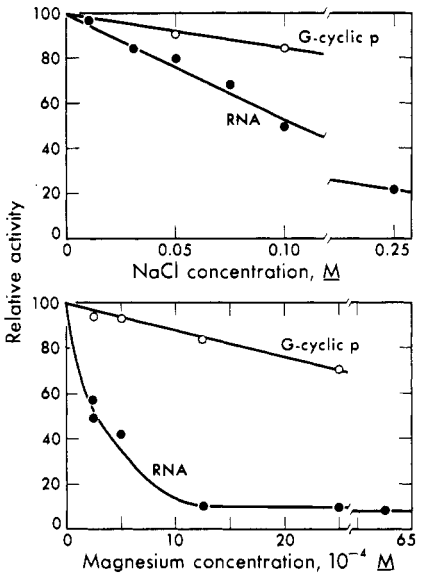

FIG. 8.—Effect of salts on the activity of *Ustilago* nuclease. Ribonuclease activity was measured at *p*H 7.0 using an automatic recording titrator. Comparison was made of the relative rates of consumption of 0.025 M KOH using 10 ml of reaction mixture containing 10 mg of substrate, 1 mg of protective bovine plasma albumin, and added salt at the level indicated. About 10^3 units of nuclease activity were present in each measurement of RNA hydrolysis, using either wheat germ s-RNA or high-molecular-weight RNA. Measurement of G-cyclic-p activity required 3×10^4 units of enzyme, as measured in the usual assay procedure.

Polyvinyl sulfate has been found to be an effective inhibitor of pancreatic ribonuclease (Fellig and Wiley, 1959). In contrast, the *Ustilago* nuclease appears to be unaffected by the presence of at least ten times the quantity of polyvinyl sulfate needed to reduce the activity of pancreatic ribonuclease by 50%. Added polyamines such as spermine, spermidine, and poly-L-lysine, on the other hand, are quite inhibitory to the action of *Ustilago* nuclease on RNA. Spermine and spermidine, at a relative concentration of 0.2 mole of nitrogen per mole of the phosphate in the substrate RNA, result in essentially complete loss of enzyme activity as measured in the usual assay procedure. Poly-L-lysine is somewhat less effective, showing no inhibition at a lysine-to-phosphate ratio of 0.2, but almost complete inhibition as this ratio is increased to 0.4.

The enzyme appears to be very stable to a number of possible denaturants. Heating to a temperature of 100° for at least 5 minutes resulted in no loss of nuclease activity. Overnight incubation of the enzyme at *p*H values of 2–10 and temperatures of 0–25° similarly resulted in no inactivation. At any stage of purification after removal of cells, storage of the enzyme at 0–4° or frozen for periods of at least 3 months has been found possible with no loss in activity.

Incubation of the *Ustilago* enzyme with iodoacetate at *p*H 6 caused no loss of nuclease activity, while similar treatment of pancreatic ribonuclease resulted in over 60% inactivation in the same time period (4 hours). An attempt was made to titrate possible sulfhydryl groups of the enzyme using *p*-mercuribenzoate, as illustrated in Figure 9. The expected increase in optical density at 250 mμ was found using glutathione and β-lactoglobulin, while essentially no reaction occurred with pancreatic ribonuclease. Addition of the *Ustilago* enzyme to *p*-mercuribenzoate solution resulted in a decrease in optical density at 250 mμ. This abnormal reaction is apparent from the spectral shift

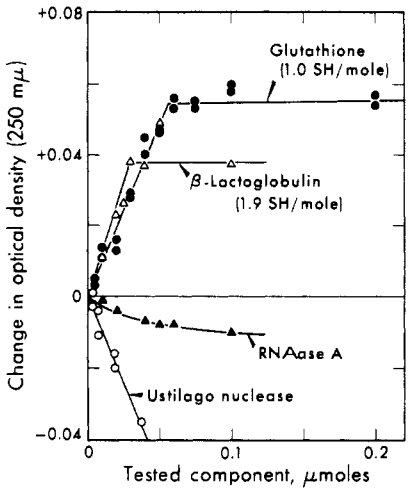

FIG. 9—Titration of sulfhydryl groups with *p*-mercuribenzoate. The reaction mixture contained 0.05 μmole of *p*-mercuribenzoate, 1.3 mmoles of sodium acetate buffer, *p*H 4.6, 0.022 mole of urea, and the quantity of test component indicated in a total volume of 4.0 ml. The optical density at 250 mμ was measured after 12 hours of incubation at room temperature.

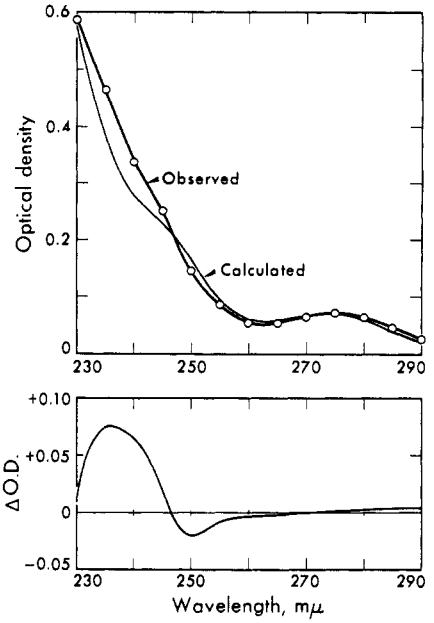

FIG. 10.—Spectra of *Ustilago* nuclease plus *p*-mercuribenzoate. The individual spectra of *p*-mercuribenzoate (0.05 μmole) and *Ustilago* nuclease (76 μg) were measured separately and summed to give the calculated curve. The same quantity of each component was then mixed and the observed curve was measured. The value of the observed curve was subtracted from that of the calculated curve to give the difference spectrum shown in the lower figure. All solutions were 7.5 M with respect to urea, and spectra were measured using a Cary 14 spectrophotometer.

illustrated in Figure 10. Incubation of *Ustilago* nuclease with *p*-mercuribenzoate in 7.5 M urea did not inactivate the enzyme to any greater extent than similar incubation in 7.5 M urea alone. About 70% of the enzymic activity remained after treatment of the *Ustilago* enzyme with excess *p*-mercuribenzoate in the presence of urea for 12 hours at room temperature.

DISCUSSION

Formation of the Enzyme.—It is likely that the *Ustilago* nuclease is an induced extracellular enzyme. The enzyme is not found if cells are grown on a glucose medium. When the organism is grown on RNA, enzyme formation is nearly parallel to growth, but nuclease activity is only apparent after some hours of exposure of the cells to the RNA of the medium (see Fig. 1). This lag period may represent the utilization of low-molecular-weight materials present in the commercial RNA used in the medium. Essentially all of the nuclease is found in the medium, and there is no indication that enzyme is released by cell lysis.

Purification of the Enzyme.—Under the best conditions of culture thus far attained, only about 2 mg of enzyme per liter of medium can be found. The same volume of medium contains 3–3.5 g of highly degraded RNA (see Fig. 2), an excess of *ca.* 1500-fold over the enzyme. This RNA interferes greatly in fractionation procedures, and makes measurement of ribonuclease activity difficult. Much of the highly degraded RNA is insoluble in the concentrated medium of step 2, and a major portion of that remaining in solution is removed by gel filtration (see Fig. 5). Nevertheless, some nucleotide material remains associated with the nuclease activity throughout the purification procedure, and is removed only in the final phenol extraction step.

The crystalline impurity removed from the nuclease preparation at step 6 remains uncharacterized. This fraction does not inhibit the enzyme and possesses no hydrolytic activity toward RNA or *p*-nitrophenyl phosphate. Before removal of the impurity the *Ustilago* enzyme is eluted from DEAE-cellulose columns at a sodium chloride concentration of about 0.5 M, but after the crystallization step rechromatography of nuclease using the same type of column results in enzyme elution at a lower salt concentration, *ca.* 0.3 M (see Fig. 4). Thus there appears to be some interaction between the impurity and the *Ustilago* nuclease. It is interesting to note that a similar crystalline impurity was isolated by Nishimura (1960) during the purification of another extracellular ribonuclease, in this case from *Bacillus subtilis*. Again, the crystalline impurity was precipitated at a lower concentration of ammonium sulfate than that required to precipitate the nuclease, and no function for the crystalline impurity was apparent.

Criteria of Purity of the Enzyme.—Evidence from a number of sources indicates that the *Ustilago* nuclease is relatively pure. Sucrose-density-gradient centrifugation (see Fig. 5) and sedimentation in the analytical ultracentrifuge indicate that the large majority of protein and of ribonuclease activity possess the same low sedimentation coefficient. The more accurate of the two values calculated is 1.6 S, and may be compared to the value of 1.62 S reported for RNAase T_1 by Ui and Tarutani (1961), a value of 1.4 S obtained by Nishimura (1960) using the *B. subtilis* ribonuclease, and the sedimentation coefficient of 1.85 S reported for pancreatic ribonuclease (from Edsall, 1953). Some purification of the *Ustilago* nuclease beyond that of the preparation which was sedimented has been obtained, and it is possible that the contaminants thus removed could account for the impurities observed in the earlier analytical ultracentrifuge run. Starch-gel electrophoresis of the more highly purified enzyme preparation (see Fig. 6) provides further evidence for the purity of the enzyme, since contaminating material was barely visible. The ultraviolet spectrum of Figure 7 indicates that little nucleotide contamination is likely, since no peak is seen at 260 mμ. On the other hand, the absorption of the enzyme preparation at 400 mμ indicates that some colored material is still present, probably as a

contaminant since there appears to be no correlation between yellow color and nuclease activity.

The specific activity of the most highly purified preparation of *Ustilago* nuclease is three to four times that of crystalline pancreatic ribonuclease, when RNAase activity is measured in the assay procedure used throughout this work. Nishimura (1960) has reported that the specific activity of the crystalline ribonuclease of *B. subtilis* is equal to that of pancreatic ribonuclease, and Takahashi (1961) has found the highly purified ribonuclease T_1 to be four times as active as pancreatic RNAase. Takahashi used an assay similar to that used in these studies, except that the *p*H was 7.5 and EDTA was present in the assay buffer. Thus the specific activity of the *Ustilago* enzyme is of the same order of magnitude as has been found using other highly purified ribonucleases. The fact that the *Ustilago* enzyme is about seven times as active as the sample of ribonuclease T_1 with which it was compared is probably not significant, since the previous history of the T_1 sample was not known. In addition, the relative activities of this sample of T_1 ribonuclease and the pancreatic enzyme are quite different from those values reported by Takahashi (1961) using well-characterized enzyme.

Properties of the Enzyme.—Examination of the data of Table II indicates that the enzyme is about six times as active at *p*H 6 as at *p*H 7 when measured using a bicarbonate buffer. The relative activity at *p*H 6 is also higher in cacodylate, phosphate, and β-glycerol phosphate buffers, while at *p*H 7 the activity is greater in EDTA and imidazole buffers. Some but not all of the effect of the different buffers of Table II may be due to ionic-strength variations, since all assays were performed at the same molarity of buffer. Since similar results were obtained using crude and purified *Ustilago* nuclease, it is quite doubtful that the variations in activity are due to other enzymes in the preparation. The absolute activity of the enzyme was found to be considerably greater using imidazole buffer at *p*H 7 than was found under any other condition of *p*H or buffer medium.

The stabilizing effect of bovine plasma albumin on the *Ustilago* nuclease is appreciable: the apparent activity of the crude enzyme preparation was about five times as great when protective protein was present in the assay mixture. More important for purposes of assay, the reproducibility of activity measurements made in the presence of added protein is greatly increased. Study of the saturation of the nuclease with substrate indicates that RNA at high concentrations is somewhat inhibitory to the enzyme, and, in fact, the optimal concentration of RNA is not that used in the assay procedure for ribonuclease estimation. On the other hand, the assay as it was used was found to give a linear relation between the volume of enzyme solution added and the increase in acid-soluble ultraviolet-absorbing materials formed from RNA.

The very strong inhibition of RNA hydrolysis by added salts, as observed with the *Ustilago* enzyme (see Fig. 8), is not observed with either pancreatic ribonuclease or the T_1 enzyme. For instance, 0.1 M sodium chloride reduces the rate of RNA hydrolysis by the *Ustilago* nuclease to about 55% of the maximal value, while Sato and Egami (1957) found ribonuclease T_1 unaffected by the same salt level. This result is of particular interest in view of the similar specificity of the T_1 enzyme and the *Ustilago* nuclease (see Sato and Egami, 1957; Glitz and Dekker, 1964). In similar measurements a 0.1 M concentration of sodium chloride resulted in a considerable stimulation of the activity of pancreatic RNAase using an RNA substrate. A comparable result has been reported by Kalnitsky *et al.* (1959). Even more striking than the sodium chloride effect is the inhibition of RNA hydrolysis by magnesium salts added to *Ustilago* nuclease reaction mixtures. At a magnesium chloride concentration of 10^{-3} M about 90% of the activity of the *Ustilago* enzyme is lost, while ribonuclease T_1 is only about 25% inhibited under the same conditions (see Fig. 8, and Sato and Egami, 1957).

It is apparent from the data of Figure 8 that the effect of added salts upon *Ustilago* nuclease activity is substrate dependent. Inhibition of the hydrolysis of G-cyclic-p is much less marked than the inhibitory effect on RNA hydrolysis at the same concentration of added salt. It is likely that some of the inhibitory effect of added salt is due to alteration of the secondary structure of the RNA substrate. The effect of added salts on the activity of pancreatic ribonuclease has also been found to be substrate dependent. Again, an effect on secondary structure is suggested (see, e.g., Kalnitsky *et al.*, 1959; Doty *et al.*, 1959; Nishimura and Novelli, 1963).

Polyamines such as spermine and spermidine have been found to strongly inhibit the hydrolysis of RNA by the *Ustilago* nuclease. These multivalent cations are known to react with and perhaps stabilize polyanions such as RNA (see, e.g., Tabor *et al.*, 1961). In contrast, polyvinyl sulfate does not appear to inhibit the *Ustilago* enzyme, although pancreatic ribonuclease is greatly affected. Polyvinyl sulfate would not be expected to react with a polyanion such as RNA, but will bind to a basic protein such as RNAase A (pI = 9.45) which bears a net positive charge under the conditions of the assay used here, *p*H 7.0 (see Tanford and Hauenstein, 1956). The lack of an inhibitory effect by polyvinyl sulfate, the electrophoretic mobility at *p*H 8, the fact that ammonium sulfate precipitation occurs most completely and reproducibly at acid *p*H values, and the chromatographic properties using DEAE-cellulose all tend to indicate that the *Ustilago* enzyme is not a basic protein, such as pancreatic ribonuclease, but more likely an acidic protein such as ribonuclease T_1.

The reaction of pancreatic ribonuclease with iodoacetate at *p*H 6 results in enzyme inactivation by carboxymethylation of histidine residues (see Gundlach *et al.*, 1959). The failure of the *Ustilago* enzyme and of T_1 ribonuclease (Sato and Egami, 1957) to be inactivated under these conditions must be interpreted with caution until the reaction can be investigated over a wide range of *p*H. Figures 9 and 10 indicate that some interaction occurs between the *Ustilago* enzyme and *p*-mercuribenzoate, but this is not the expected reaction of *p*-mercuribenzoate with free sulfhydryl groups, which would result in an increase in the optical density at 250 mμ. The retention of enzymic activity after incubation with *p*-mercuribenzoate provides further evidence that the reaction is not the titration of SH groups which was intended. Measurement of the extent of reaction at 246 mμ would have indicated no *p*-mercuribenzoate reaction with the *Ustilago* and pancreatic enzymes, but would have shown the expected titration of glutathione and β-lactoglobulin.

Many of the properties of the *Ustilago* enzyme are quite similar to those of other nucleases, and especially ribonuclease T_1. Both enzymes possess a similar high specific activity and a low sedimentation coefficient, about 1.6 S. The *p*H optimum of the two nucleases is similar, and each is inhibited by salts added to a reaction system, although apparently not to the same degree. Neither enzyme is inactivated by iodoacetate at *p*H 6, and it is likely that neither enzyme possesses any free sulfhydryl groups (see Yamagata *et al.*, 1962).

Finally, as will be detailed in the following paper, the specificity of the two enzymes appears to be the same.

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We would like to thank Dr. H. K. Schachman and Miss F. Putney, who performed and analyzed the analytical ultracentrifuge run, and Drs. F. Egami and H. Fraenkel-Conrat, who provided the sample of ribonuclease T_1 used here. We are also indebted to Dr. R. D. Cole for valuable discussion regarding the reaction of *p*-mercuribenzoate with proteins.

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Studies on a Ribonuclease from *Ustilago sphaerogena*. II. Specificity of the Enzyme*

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Hydrolysis of RNA by the ribonuclease from *Ustilago sphaerogena* results in the formation of 3′-guanylic acid and oligonucleotides ending in guanosine-3′-phosphate. The enzyme acts first through the formation of guanosine-2′,3′-cyclicphosphate, and then more slowly hydrolyzes the cyclic ester to form the 3′-nucleotide. A copolymer of adenylic acid and guanylic acid is attacked at all guanylic acid residues, resulting in the formation of guanosine-2′,3′-cyclicphosphate, guanosine-3′-phosphate, and a series of oligonucleotides composed of adenylic acid but ending in a single guanylic acid residue. The enzyme is equally active on a number of RNA and polynucleotide substrates containing guanylic acid, and slightly less active on polymers containing inosinic acid. Polyadenylic acid is hydrolyzed at 0.002% the rate of RNA, while polyuridylic and polycytidylic acids, DNA, and bis-(*p*-nitrophenyl)phosphate are inert to the enzyme. Methylated albumin-kieselguhr chromatography has been used to examine partial degradation products of s-RNA during the digestion by a number of nucleases. The *Ustilago* enzyme initially produces large fragments from the RNA substrate, and then gradually reduces these fragments to small oligonucleotides.

In the preceding paper (Glitz and Dekker, 1964) the purification of an extracellular ribonuclease produced by the smut fungus *Ustilago sphaerogena* was described. The enzyme was obtained in a form which was judged to be highly purified, as indicated by electrophoretic homogeneity, the formation of a single sharp boundary in the ultracentrifuge, and a specific activity equal to or greater than those of other nucleases. In this paper the specificity and the mode of action of the nuclease will be considered.

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MATERIALS AND METHODS

Enzymes.—An extracellular ribonuclease from *U. sphaerogena* was prepared as described in the preceding paper (Glitz and Dekker, 1964). Unless otherwise stated, enzyme of specific activity 175,000 units/mg protein was used. Pancreatic ribonuclease A (Worthington Biochemical Co.) was rechromatographed by Dr. M. Irie of this laboratory and was a homogeneous preparation of specific activity 55,000 units/mg pro-