# Characterization of a Guanylic Acid Specific Ribonuclease from *Aspergillus fumigatus*†

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ABSTRACT: *Aspergillus fumigatus* produces an extracellular ribonuclease when the organism, cultured in a glucose–peptide broth, is transferred to RNA solution. Lesser amounts of nuclease are detected if other polynucleotide inducers are used. The enzyme has been purified about 3000-fold in six steps. It has the same specificity as RNase $T_1$ of *Aspergillus oryzae:* RNA is hydrolyzed at guanylic acid residues *via* the cyclic phosphate. Kinetic comparisons show the nuclease differs from RNase $T_1$ most obviously in cyclic phosphate hydrolysis and action on macromolecular substrates; the $K_m$ for RNA is low, and partial degradation products of bacteriophage RNA are not all identical with those in RNase $T_1$ digests. Amino acid and peptide analyses show some apparently identical sequences in the two enzymes. Antibodies to RNase $T_1$ also precipitate the *A. fumigatus* RNase, but less effectively. The enzyme and RNase $T_1$ are inactivated at equivalent rates by iodoacetate, succinic anhydride, or trinitrobenzenesulfonate. Phenylglyoxal treatment showed a single rate of *A. fumigatus* RNase inactivation if assayed with cyclic phosphate, and a two-step inactivation if assayed with RNA; RNase $T_1$ shows a single-step inactivation using either substrate. This result is interpreted to indicate a function in binding macromolecular substrates for the second arginine residue in the *A. fumigatus* enzyme.

Common characteristics of many ribonucleases make these enzymes especially attractive for the comparative study of structure–function relationships. The enzymes are often small, stable, and easily purified, with a broad distribution and sometimes great specificity. The cyclizing ribonucleases catalyze two reactions: transphosphorylation and hydrolysis of the resulting cyclic phosphate. These reactions can be studied independently using both natural and synthetic substrates. Thus a considerable literature has accumulated in the description of the pancreatic ribonucleases, RNase[1] $T_1$ and related enzymes (guanylonucleases), and the Staphylococcal nuclease. (See Barnard (1969), Egami and Nakamura (1969), and Uchida and Egami (1971) for reviews and references.)

We have undertaken a study of a guanylic acid specific ribonuclease from *Aspergillus fumigatus* which appears to be very closely related to RNase $T_1$ of *Aspergillus oryzae*. In this paper we wish to report on the isolation of the enzyme and its direct comparison with RNase $T_1$.

## Materials and Methods

*Materials.* All unspecified chemicals were of reagent grade or the equivalent. A stock culture of *Aspergillus fumigatus* was obtained from the collection of the Bacteriology Department, UCLA.

Ribonuclease $T_1$ (Sankyo Ltd.) and pancreatic ribonuclease (5X crystallized) were purchased from Calbiochem, and *Rhizopus oligosporus* ribonucleases were prepared as described by Woodroof and Glitz (1971). RNase $N_1$ was purchased from Miles Laboratories, while RNase $U_1$ was the generous gift of Dr. C. A. Dekker. Bovine serum albumin (Pentex, fraction V or crystalline) and crystalline chymotrypsin were purchased from Calbiochem, and triphenyl chloromethyl ketone trypsin from Worthington Biochemicals.

DEAE-cellulose was the standard grade product from Schleicher and Schuell, and aminoethylcellulose was purchased from Bio-Rad Laboratories. Sephadex was purchased from Pharmacia, and Bio-Gel and Dowex resins from Bio-Rad Laboratories.

Guanosine 2′,3′-cyclic phosphate was purchased as the barium salt from the Sigma Chemical Co., and was converted to the sodium salt by passage through a column of Dowex 50 ($Na^+$). Dinucleoside monophosphates were purchased from Calbiochem or Miles Laboratories, and were chromatographed on a 1 × 30 cm column of Sephadex G-15 or Bio-Gel P-2 prior to use as substrates. RNA was the high molecular weight fraction from wheat germ described by Glitz and Dekker (1963). Bacteriophage F-2 RNA was prepared as described by Glitz (1968) for MS2 RNA.

*Methods.* The assay for ribonuclease measures production of perchloric acid soluble material from RNA and was described by Woodroof and Glitz (1971). Protein was measured by a modification of the procedure of Lowry *et al.* (1951). Amino acid composition was determined by the method of Moore *et al.* (1958) with a Spinco Model 120B automatic amino acid analyzer. Samples (about 0.2 mg) were hydrolyzed *in vacuo* in 6 N HCl at 110° for 24 or 72 hr. Performate-oxidized samples (Moore, 1963) were hydrolyzed and analyzed for cysteic acid. Tryptophan was estimated spectrophotometrically essentially as described by Bencze and Schmid (1957). Protein was reduced and carboxymethylated as described by Hirs (1967) for RNase A, and digested with 1% by weight of chymotrypsin (or 2% triphenyl chloromethyl ketone trypsin) in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 2 hr at 37°. Peptides were fractionated by paper electrophoresis for 1 hr at 3000 V (40 V/cm) with an acetic acid–pyridine–water (10:100:890, v/v) buffer, pH 3.5. Visualiza-

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[1] Abbreviations used are: RNase, ribonuclease; PEI-cellulose, polyethyleneimine cellulose; AE-cellulose, aminoethylcellulose.

tion was attained by dipping the paper in 0.2% ninhydrin plus 5% collidine in 95% ethanol.

Sedimentation equilibrium was performed with a Spinco Model E centrifuge at protein concentrations of 0.2–1.0 mg/ml, and the molecular weight determined from the plot of log $C$ *vs.* $X^2$ according to the equation of Van Holde and Baldwin (1958). Partial specific volume was calculated from the amino acid composition as described by Smith *et al.* (1954).

Polyacrylamide gel electrophoresis of proteins was conducted in 7.5% gels of cyanogum 41 (E.-C. Apparatus Co.). Gels were stained in 1% Amido Blue-Black (Matheson Chemicals) dissolved in methanol–acetic acid–$H_2O$ (5:1:4, v/v) and destained by diffusion in the same solvent. Gel electrophoresis of partial RNA hydrolysates was done in 5% or 10% gels of Cyanogum 41 with a slab apparatus (E.-C. Co.), as described by Peacock and Dingman (1968). Gels were stained with "Stains All" (1-ethyl-2-[3-(1-ethylnaphtho[1,2-*d*]-thiazolin-2-ylidine)-2-methylpropenyl]naphtho[1,2-*d*]thiazolium bromide, Eastman 2718) according to Dahlberg *et al.* (1969).

Immunization of young adult male New Zealand rabbits was by injection of 1.6 mg of RNase $T_1$ as a 0.5% aqueous solution emulsified with four volumes of Freund's adjuvant (Difco Laboratories) into the footpads. Fourteen days later an intradermal booster injection of 1 ml of 0.5 mg of RNase/ml in saline was given. Blood was collected 14 and 24 days later, and active sera pooled and frozen. Immunodiffusion was conducted in 1% Ionagar No. 2 (Oxoid Ltd., purchased from Colab) in 0.1 M NaCl plus 0.01 M Tris-HCl, pH 7.0. The gels were run at room temperature overnight, washed for 16–24 hr in 0.85% NaCl, and stained with 0.6% Amido Black (Matheson Chemicals) dissolved in methanol–acetic acid–water (5:1:5, v/v). Quantitative precipitation reactions were conducted using 50 μl of serum plus variable amounts of antigen in a total volume of 0.5 ml of 0.9% NaCl. The samples were incubated 1 hr at 37° and then overnight at 5°, centrifuged 10 min at 7000*g*, and the precipitates washed three times with cold 0.9% NaCl. Protein in the precipitate was measured by the Lowry *et al.* (1951) technique.

*Chromatography of Nucleotides.* Column chromatography on DEAE-cellulose with 0.01 M Tris-HCl, pH 7.5, containing 7 M urea (Tomlinson and Tener, 1962) and a linear 0–0.4 M gradient of NaCl was used to fractionate oligonucleotides. Paper chromatography was done on Whatman 3MM paper with 1-propanol–$NH_4OH$–$H_2O$ (6:3:1, v/v). Thin-layer chromatography on plates of PEI-cellulose (Brinkman) was conducted according to Randerath and Randerath (1964).

*Enzyme Kinetics.* RNA breakdown was estimated by production of acid-soluble material from wheat germ RNA, measured at 5-min intervals over a 15-min period. Dinucleoside monophosphate cleavage was determined by nucleoside release. Both techniques have been detailed by Woodroof and Glitz (1971). Hydrolysis of guanosine 2′,3′-cyclic phosphate was determined by paper chromatographic fractionation of reaction mixtures, and elution and spectrophotometric measurement of cyclic phosphate and 3′-nucleotide. In the case of RNase $T_1$, cyclic phosphate hydrolysis was also estimated from the rate of uptake of 3 mM KOH in a Radiometer automatic titrator. Hydrolysis of cyclic phosphate termini of oligonucleotides was estimated from the rate of KOH uptake with an RNA substrate. Data were plotted according to Lineweaver and Burk (1934) ($1/V_i$ *vs.* 1/[S] and [S]/$V_i$ *vs.* [S]) and values of $V_{max}$ calculated as moles of product per mole protein per minute.

*Protein Modification Reactions.* The reaction of ribonucleases with iodoacetic acid was based on the studies of Takahashi *et al.* (1968). Enzyme (*ca.* 1 mg/ml) was mixed with an equal volume of 1% iodoacetic acid (Mann), pH 5.5, and incubated in foil-wrapped tubes at 37°. Samples (10 μl) were withdrawn at various times and diluted to 1 ml with 1 mg/ml of cold bovine serum albumin solution. The dilutions were assayed as soon as was convenient, although activity was not changed by up to 2 days of storage at 4°. Nucleases were incubated under similar conditions but for 24 hr with [$^{14}$C]-iodoacetic acid (New England Nuclear) of specific activity 0.8 mCi/mmole.

Succinylation followed the general procedures of Frist *et al.* (1965). Solid crystalline succinic anhydride was added to 5 ml of 0.2 mg/ml of RNase at 5° in the vessel of a Radiometer automatic titrator, and pH 7.0 maintained by addition of 1 M NaOH; 50-μl samples were withdrawn at various times and diluted to 0.5 ml with 0.2 M glycine, 1 mg/ml of bovine serium albumin, pH 7. The dilutions were assayed for RNase as soon as was practical, and the results corrected for dilution by the NaOH added by the titrator.

Trinitrophenylation was based on the work of Kasai *et al.* (1969). Nucleases (0.5 mg/ml) were incubated with 5 mM sodium trinitrobenzene sulfonate (Eastman) in 0.2 M sodium phosphate buffer, pH 7.7, for up to 70 hr at 0°. At various times 50-μl samples were withdrawn and diluted with 150 μl of 0.2 M glycine, 2 mg/ml of the albumin, pH 7. Samples were assayed as soon as was practical, although no changes were apparent after up to 3-days storage.

The inactivation of nucleases with phenylglyoxal followed the work of Takahashi (1970). RNase (0.3–1.2 mg/ml) in water was mixed with an equal volume of 4% solution (w/v) of phenylglyoxal (K and K Laboratories) in 0.4 M morpholine ethanesulfonate buffer, pH 8.0. At various times 10-μl samples were withdrawn and diluted to 0.5 ml with 0.1 M sodium acetate, 1 mg/ml of the albumin, pH 4.0, and assayed as quickly as was practical. If hydrolysis of cyclic phosphate was to be assayed, 25-μl portions of RNase–phenylglyoxal reaction mixture were added directly to 175 μl of assay solution to give a final concentration of sodium guanosine 2′,3′-cylic phosphate of 7.25 mM in 0.05 M imidazole, 1 mg/ml of the albumin, pH 7.0. At various times 25-μl portions of this mixture were spotted on Whatman 3MM paper and chromatographed. Spots corresponding to the cyclic phosphate substrate and guanosine 3′-phosphate were located by ultraviolet light, eluted with 0.1 M $NH_4OH$, and measured spectrophotometrically, and enzyme activity evaluated by the rates of guanosine 2′,3′-cyclic phosphate consumption and of guanosine 3′-phosphate formation. Results were corrected for spontaneous hydrolysis of the cyclic phosphate under the conditions used.

*Ribonuclease Production.* Formation of ribonuclease in trial experiments was measured as follows: 5-ml cultures of *Aspergillus fumigatus* in Antibiotic Medium No. 3 (Difco) were incubated at 28° on a rotary shaker for 40 hr, the mycelium was collected by filtration, washed with *ca.* 5 ml of distilled water, and then incubated in the test medium (0.01 M Tris-HCl plus desired additions, pH 7.0) for up to 4 days. Samples were withdrawn periodically for assay of extracellular ribonuclease.

*Ribonuclease Purification.* Approximately 5-ml samples of a 24-hr culture of *A. fumigatus* in Antibiotic Medium No. 3 were used as inocula for 1500-ml portions of the same medium in 6-l. erlenmeyer flasks. The cultures were incubated at 25 or 28° for 40–44 hr on reciprocal shakers operated at

TABLE I: Formation of Extracellular RNase by *Aspergillus fumigatus*.

| Substance Added[a] | Amount (mg/ml) | Maximum Enzyme Level (units/ml) |
|---|---|---|
| None | | 0 |
| Sodium phosphate | 0.5[b] | 0–0.3 |
| | 0.1[b] | 1–3 |
| | 0.01[b] | 3–6 |
| Xanthine | 0.01–1 | 0 |
| Guanine | 0.01–1 | 0 |
| Guanosine | 0.01–1 | 0 |
| Guanosine 2′,3′-cyclic phosphate | 0.2–1 | 0–2 |
| 3′(2′)-Guanylic acid | 0.2–1 | 2–6 |
| Poly(G) | 1 | 3 |
| Poly(A), poly(U), poly(C) | 1 | 3–10 |
| DNA | 0.2 | 2 |
| | 1 | 4 |
| | 5.0 | 8 |
| RNA, yeast (commercial) | 2.5 | 20–25 |
| Wheat germ, ribosomal | 2.5 | 20–25 |

[a] Five ml of 0.01 M Tris-HCl, pH 7.0, containing the appropriate substance, was inoculated with mycelium from 5 ml of a 40-hr culture, incubated at 28° on a rotary shaker, and assayed periodically for RNase. [b] M.

90 cpm, and filtered through Whatman No. 1 paper. The mycelium was washed with a few 100-ml portions of water, drained, and transferred to 1500-ml portions of 2.5 mg/ml of RNA solution, pH 7 (Schwarz/Mann, Nutritional Biochemicals, or Sigma Chemical Co.). This suspension was shaken at 25 or 28° for 40–44 hr and filtered as above, and the filtrate was chilled and stored for up to 3 months at 4°. All further manipulations were carried out at 4°.

The filtrate above was run onto a 5 × 120 cm column of DEAE-cellulose which had been equilibrated with 0.002 M Tris-HCl, pH 9.0. Enzyme addition was stopped when the effluent activity was greater than 0.5 unit/ml (usually after addition of about 100 l. of filtrate). The column was washed with 4 l. of 0.002 M Tris-HCl, pH 9, and then eluted with 10 l. of the same buffer containing a linear gradient of 0–2 M NaCl, followed by 3 l. of 3 M NaCl in Tris-HCl, pH 9.0. Enzyme was usually eluted as a broad, trailing peak. Fractions (500–1000 ml) containing more than 75 units/ml were pooled (step 2). This step was repeated with fresh filtrate until $1.5 \times 10^6$ to $2 \times 10^6$ units of enzyme had been collected.

The material from step 2 was brought to 80% saturation by the addition of 516 g of solid ammonium sulfate per liter and centrifuged for 15 min at 11,000*g* (Sorvall GS-3 rotor), and the pellet discarded. The supernatant was brought to saturation by addition of 198 g of ammonium sulfate per liter, adjusted to pH 2.5 with *ca.* 1 M $H_2SO_4$, and stored cold for 3 days. The precipitate was collected by centrifugation as above, dissolved in about 800 ml of water, and dialyzed overnight against three 12-l. portions of water (step 3). An equal volume of acetone was slowly added to the contents of the dialysis bag, and the resulting suspension was centrifuged as above. The supernatant was decanted and an equal volume of acetone added dropwise (bringing the solution to 75% acetone, v/v). After centrifugation as above, the precipitate was dissolved in about 100 ml of distilled water (step 4).

The preparation was diluted fourfold with 0.01 M Tris-HCl, pH 9, applied to a DEAE-cellulose column (2.0 × 100 cm) equilibrated with the same buffer, and eluted with 2 l. of buffer containing a linear gradient of 0–0.4 M NaCl. Nuclease activity was eluted at about 0.2 M; fractions of specific activity greater than $10^4$ units/ml of protein were pooled (step 5), dialyzed against four 6-l. portions of distilled water, and lyophilized. The solid was dissolved in about 5 ml of water and chromatographed on a 1.8 × 100 cm column of Sephadex G-200. Elution was with water at a flow rate of 0.1 ml/min; fractions of specific activity greater than $2.5 \times 10^4$ units/mg of protein were pooled (step 6). This material was applied to a 1.8 × 20 cm column of AE-cellulose (equilibrated with 0.01 M Tris-HCl, pH 7.5) and eluted with 1800 ml of this buffer containing a parabolic gradient of 0–0.4 M NaCl. Nuclease, eluted as a broad peak with a specific activity of 41,000 units/mg of protein, was dialyzed, lyophilized, and dissolved in a small volume of water (step 7).

Chromatography on AE-cellulose was sometimes replaced by chromatography on a similar column of DEAE-cellulose with 0.02 M Tris-HCl, pH 7.5, and a linear gradient of 0–0.4 M NaCl; the enzyme eluted as a sharp peak at about 0.27 M NaCl. The final purity was not always as great as when AE-cellulose was employed, but the yield was very high.

All specific activities are expressed on the basis of protein measurement in the assay of Lowry *et al.* (1951) using bovine serum albumin as a standard.

## Results

*Formation of the Enzyme.* Ribonuclease activity of less than 0.1 unit/ml was found in supernatants from sand- or alumina-ground mycelia of *A. fumigatus*, either after growth on Antibiotic Medium No. 3 (or other media) or after transfer to RNA. After growth on Antibiotic Medium No. 3 little activity was detected in the medium, but extracellular ribonuclease levels of 8–25 units/ml were found after shaking the washed mycelium in 2.5 mg/ml of RNA. No further growth was seen after transfer to the RNA solution. The amount of enzyme formed and the time course of production varied with pH, RNA concentration, temperature, and aeration. Conditions were chosen for maximal enzyme yield with reasonable reproducibility in preparative scale cultures.

Substances other than RNA were tested for their ability to induce extracellular RNase activity. These results, presented in Table I, indicated that RNase was formed in the presence of almost any polynucleotide, as well as nucleotide or inorganic phosphate, although none were equivalent to RNA. Nucleosides or free bases were ineffective, and the size of the RNA appeared to be unimportant. No attempt was made to demonstrate that the same protein was assayed in all cases.

*Enzyme Purification.* Table II summarizes the purification of the ribonuclease. In early stages of purification quantities of degraded RNA are present and interfere in the assay; thus the yield in early steps may not be as great as Table II suggests. The RNase was stable at all stages, except that protease activity is present through step 4 and may inactivate the nuclease if temperatures rise above 37°.

*Criteria of Purity.* Polyacrylamide gel electrophoresis of 60 μg of the enzyme preparation at pH 9.5 showed a single component. Gels are shown in Figure 1 which indicate that

TABLE II: Purification of *Aspergillus fumigatus* Ribonuclease.

| Step | Volume (ml) | Activity (units/ml) | (Total) | Protein (mg/ml)[a] | Sp Act. (units/mg of Protein)[a] | % Yield |
|---|---|---|---|---|---|---|
| 1 Crude | $165 \times 10^3$ | 13 | $2.14 \times 10^6$ | 0.95 | 13.7 | 100 |
| 2 DEAE I | $11 \times 10^3$ | 193 | $2.12 \times 10^6$ | 0.84 | 230 | 99 |
| 3 $(NH_4)_2SO_4$ | $1.45 \times 10^3$ | 1,176 | $1.70 \times 10^6$ | 0.96 | 1,225 | 80 |
| 4 Acetone | 125 | 15,000 | $1.88 \times 10^6$ | 4.79 | 3,130 | 88 |
| 5 DEAE II | 240 | 4,856 | $1.16 \times 10^6$ | 0.31 | 15,645 | 54 |
| 6 G-200 | 90 | 10,960 | $0.99 \times 10^6$ | 0.31 | 35,350 | 46 |
| 7 AE-cellulose | 9.85 | 77,000 | $0.76 \times 10^6$ | 1.86 | 41,000 | 35.5 |

[a] Based on a bovine serum albumin standard in the assay of Lowry *et al.* (1951).

under the conditions used the new enzyme is barely separable from RNase $T_1$, although more clearly distinguishable from RNase $U_1$ of *Ustilago sphaerogena* and RNase $N_1$ of *Neurospora crassa*. The *N. crassa* commercial preparation was stabilized with albumin, which appears to be the major band in these gels; the nuclease is apparently the fainter, slower moving band. Sedimentation equilibrium of the *A. fumigatus* nuclease in 0.1 M sodium acetate, pH 4.5, gave a linear plot of log $C$ *vs.* $X^2$ with no indications of heterogeneity.

In addition to experiments intended to investigate the homogeneity of the protein, other observations failed to indicate measurable impurities. Ion-exchange chromatography in the final step of the purification gave one peak with constant specific activity. This specific activity ($4.1 \times 10^4$ units per mg of Lowry protein, equivalent to 78,000 units per mg of dry weight since the tyrosine content is high) was equivalent to or greater than other nucleases in the same assay: RNase $T_1$, pancreatic ribonuclease (commercial products, not necessarily of maximal activity), and *Rhizopus oligosporus* RNases had specific activities of $9 \times 10^3$, $1.6 \times 10^4$, and $3.1 \times 10^4$ units per mg of Lowry protein, respectively. Amino acid analysis and peptide patterns (Table III and Figure 2) did not reveal unexpected or trace components indicative of contamination.

*Molecular Weight.* Sedimentation equilibrium indicated a molecular weight of 11,200 in normal or dissociating solvents, in good agreement with the data from the amino acid analysis (Table III), which gives a calculated molecular weight of 10,934 (not including any carbohydrate which might be present).

*Chemical Composition.* The amino acid composition of the *A. fumigatus* RNase is presented in Table III. As a control and comparison, samples of RNase $T_1$ were analyzed by identical methods and found to agree well with published values for its composition (see Uchida and Egami, 1971). Reduced and carboxymethylated *A. fumigatus* RNase and RNase $T_1$ were treated with chymotrypsin and the result-

TABLE III: Amino Acid Composition of Guanyloribonucleases.

| Component | *Aspergillus fumigatus* RNase μmole | *Aspergillus fumigatus* RNase Residues | RNase $T_1$[d] Residues | RNase $N_1$[d] Residues | RNase $U_1$[d,e] Residues |
|---|---|---|---|---|---|
| Lysine | 0.016 | 1 | 1 | 3 | 3 |
| Histidine | 0.028 | 2 | 3 | 3 | 2 |
| Arginine | 0.027 | 2 | 1 | 3 | 2 |
| Aspartic acid | 0.180 | 14 | 15 | 14 | 16 (15) |
| Threonine | 0.062[a] | 5 | 6 | 4 | 8 (9) |
| Serine | 0.154[a] | 12 | 15 | 14 | 13 |
| Glutamic acid | 0.094 | 8 | 9 | 4 | 6 |
| Proline | 0.057 | 5 | 4 | 5 | 4 |
| Glycine | 0.139 | 11 | 12 | 13 | 15 |
| Alanine | 0.147 | 11 | 7 | 10 | 5 |
| Half-cysteine | 0.045[b] | 4 | 4 | 4 | 4 |
| Valine | 0.075 | 6 | 8 | 4 | 6 |
| Methionine | Nd[b] | 0 | 0 | 2 | 0 |
| Isoleucine | 0.037 | 3 | 2 | 5 | 2 |
| Leucine | 0.042 | 3 | 3 | 4 | 1 |
| Tyrosine | 0.129 | 10 | 9 | 9 | 12 |
| Phenylalanine | 0.046 | 4 | 4 | 5 | 4 |
| Tryptophan | *c* | 1 | 1 | 1 | 0 |
| Total residues | | 102 | 104 | 107 | 103 |

[a] Values obtained by linear extrapolation to zero time. [b] Values from performate-oxidized samples. [c] Determined spectrophotometrically. [d] From Uchida and Egami (1971). [e] From Kenney and Dekker (1971); values in parentheses are those which disagree with Uchida and Egami (1971).

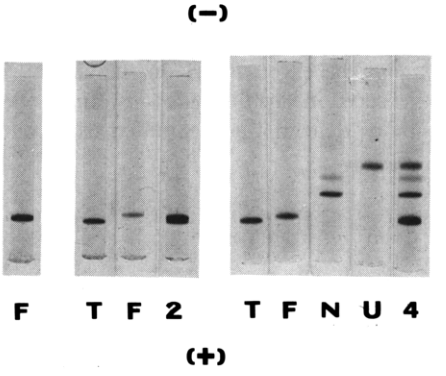


FIGURE 1: Polyacrylamide gel electrophoresis of guanyloribonucleases. Electrophoresis was at pH 9.5 in 7.5% gels for 45 min at 2 mA/gel. Gels labeled F, T, N, and U are of *A. fumigatus* RNase, RNase $T_1$, RNase $N_1$, and RNase $U_1$; gel 2 is a mixture of F and T, and gel 4 is a mixture of *ca.* 20 μg each the four nucleases. The preparation of RNase $N_1$ was stabilized by serum albumin which is the major band visible; the RNase migrates less rapidly.

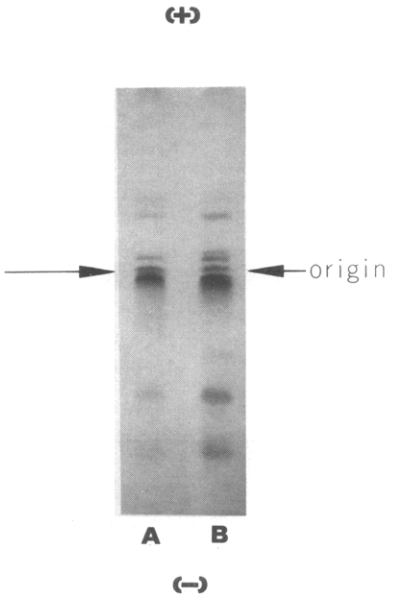


FIGURE 2: Chymotryptic peptides of guanyloribonucleases. About 0.5 mg of reduced carboxymethylated protein was hydrolyzed with 5 μg of chymotrypsin and the digest subjected to electrophoresis for 1 hr at 3000 V (40/cm) in pyridine–acetic acid–water (100:10:890, v/v), pH 3.5. Column A, *A. fumigatus* RNase; column B, RNase $T_1$.

ing peptides subjected to paper electrophoresis. The peptide patterns of Figure 2 are consistent with the amino acid composition of each enzyme, and emphasize their considerable similarity. Hydrolysis of either carboxymethylated nuclease with triphenyl chloromethyl ketone trypsin produced only a few peptides of low mobility, as might be expected from the small number of basic residues present in each enzyme.

*Immunology*. Antibodies to ribonuclease $T_1$ were induced in rabbits, and the resulting sera tested for cross reactivity to the *A. fumigatus*, *N. crassa*, and *U. sphaerogena* ribonucleases. Ouchterlony double diffusion gels, as in Figure 3, showed a strong precipitation line with homologous antigen, a weaker concentration-dependent double band with *A. fumigatus* ribonuclease, and no detectable reaction between the *N. crassa* or *U. sphaerogena* enzymes and antibodies to RNase $T_1$. Reduced and carboxymethylated RNase $T_1$ gave a weak reaction, while no precipitin lines could be detected if carboxymethyl *A. fumigatus* ribonuclease was examined under identical conditions. Quantitative precipitation tests, shown in Figure 4, confirm the weak interaction between *A. fumigatus* RNase and antibodies to RNase $T_1$.

*pH Optimum*. The effect of pH on the activity of the nuclease was measured in various buffers. Both pH optimum and maximal activity depend on the buffer used, as shown in Table IV. The pH response in imidazole buffer is plotted in Figure 5; curves of similar shape were obtained in other buffers. Parallel measurements of RNase $T_1$ showed similar curves, except in imidazole buffer when the optimum was displaced about 0.3 pH lower.

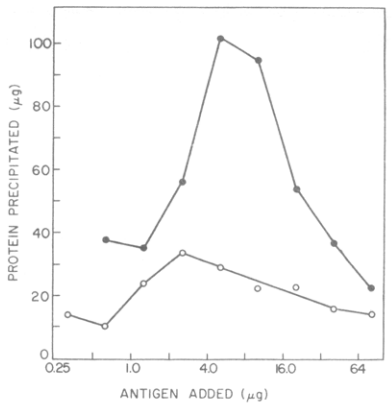


FIGURE 4: Precipitation of RNase $T_1$ and *A. fumigatus* RNase by anti-RNase $T_1$. Variable amounts of nuclease were incubated with 50 μl of serum and the precipitate analyzed by the method of Lowry *et al.* (1951). Closed circles, RNase $T_1$; open circles *A. fumigatus* RNase

*Substrate Specificity*. RNA (5 mg) was incubated for 18 hr at 30° with 800 units of *A. fumigatus* ribonuclease, and the products separated by DEAE-cellulose chromatography with buffers containing urea. The elution pattern, shown in Figure 6, was similar to that obtained in parallel digests using an equivalent amount of ribonuclease $T_1$, but with one exception: the *A. fumigatus* RNase digest contained a large peak of guanosine 2′,3′-cyclic phosphate (the first large peak of Figure 6), equivalent to 40–50% of the mononucleotide released. A much smaller amount of cyclic phosphate was produced in similar RNase $T_1$ digests. No mononucleotide other than guanylic acid was detected by thin-layer or paper chromatography of *A. fumigatus* RNase digests of RNA.

Synthetic homopolynucleotides (1 mg/ml) were treated with 5 units/ml of *A. fumigatus* ribonuclease and hydrolysis products separated by chromatography on thin layers of PEI-cellulose after up to 45-hr incubation at 30°. No degradation of poly(A), poly(C), or poly(U) was detected. Poly(X) was slowly degraded; the first small products were visible after 60–90 min of hydrolysis. Poly(G) was degraded more rapidly as cyclic nucleotide was seen after 30 min. Poly(I) appeared to be the most susceptible homopolymer tested, with cyclic nucleotide present after 5–10 min of digestion.

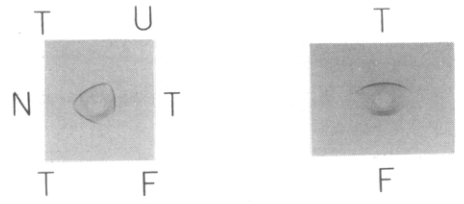


FIGURE 3: Immunodiffusion of guanyloribonucleases. The center well contained antiserum to RNase $T_1$, and peripheral wells contained 0.5-mg/ml solutions of RNase $T_1$ (T), RNase $U_1$ (U), RNase $N_1$ (N), or *Aspergillus fumigatus* RNase (F). Lower concentrations of *A. fumigatus* RNase gave only a single precipitin line.

TABLE IV: pH Optimum of *A. fumigatus* Ribonuclease.

| Buffer | pH Optimum | Relative Activity at Optimum |
|---|---|---|
| Imidazole | 6.5–7.2 | 100 |
| Tris-HCl | 6.3–7.0 | 60 |
| NaEDTA | 6.0–6.4 | 55 |
| $KHPO_4$ | 5.3–6.3 | 12 |

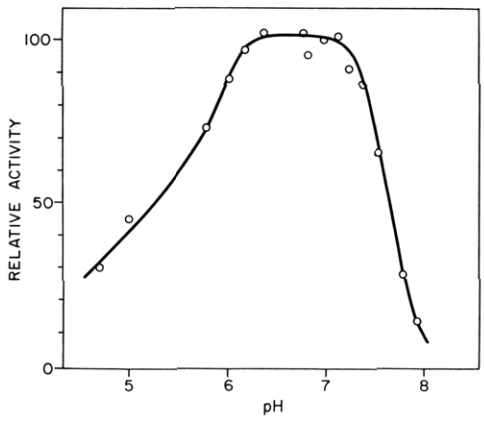


FIGURE 5: pH optimum of *A. fumigatus* RNase. Activity was measured in 0.05 M imidizole, 1 mg/ml of bovine serum albumin using the standard assay.

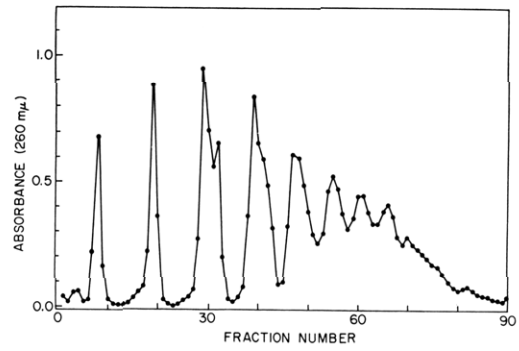


FIGURE 6: DEAE-cellulose chromatography of an *A. fumigatus* RNase digest of RNA. RNA (5 mg) was digested at 30° for 18 hr with 800 units of enzyme, and applied to a 0.8 × 12 cm column of DEAE-cellulose, and eluted with 500 ml of 0.01 M Tris-HCl plus 7 M urea, pH 7.5, with a linear gradient of 0–0.4 M NaCl. Fractions (5 ml) were collected at a flow rate of 0.4 ml/min.

The effect of substrate concentration on reaction velocity was determined using a number of substrates. Identical experiments were performed with RNase $T_1$. The results are shown in Table V; values of $V_{max}$ are expressed as moles of product per mole of enzyme per min.

Polyacrylamide gel electrophoresis was used to compare the products of partial RNA digestion, using equivalent quantities of each ribonuclease (on a basis both of weight and activity in the standard assay procedure) and homogenous macromolecular RNA from bacteriophage F2. Gel patterns are shown in Figure 7. Although many bands appeared common to all digests at a given time, characteristic bands or very different intensities of a given common band (noted by arrows in Figure 7) indicated that the oligonucleotide products of RNA digestion by the *A. fumigatus* RNase were in part peculiar to the action of this ribonuclease, and were either lacking or quantitatively different in digests with RNase $T_1$.

*Iodoacetate Inactivation.* Samples of RNase $T_1$ and *A. fumigatus* RNase were treated with iodoacetic acid at pH 5.5, and the rates of inactivation measured. The enzymes, indistinguishable in this experiment, were 50% inactivated in 75 min.

TABLE V: Ribonuclease Activity Using Different Substrates.

| | Enzyme | | | |
|---|---|---|---|---|
| | RNase $T_1$ | | *A. fumigatus* RNase | |
| Substrate | $K_m$ (mM) | $V_{max}$ (moles/mole of E/min) | $K_m$ (mM) | $V_{max}$ (moles/mole of E/min) |
| Guo-cyclic phosphate | 0.85 | 10 | 9.5 | 15 |
| $(Np)_xG$-cyclic phosphate[a] | 1.05[b] | 560 | 1.0[b] | 580 |
| GpC | 0.33 | 65,000 | 0.50 | 312,000 |
| GpA | 0.41 | 21,000 | 0.63 | 38,000 |
| GpG | 0.39 | 11,750 | 0.23 | 11,000 |
| GpU | 0.48 | 5,650 | 0.60 | 29,500 |
| IpU | 0.63 | 1,800 | 1.22 | 2,040 |
| RNA | 1.32[b] | 11,000[c]<br>3,350[d] | 0.19[b] | 24,000[c]<br>7,150[d] |

[a] Measured with an RNA substrate in a pH-Stat. [b] Expressed as the concentration of Gp in the RNA substrate. [c] As trinucleotide solubilized in the usual assay. [d] As decanucleotide solubilized in the usual assay.

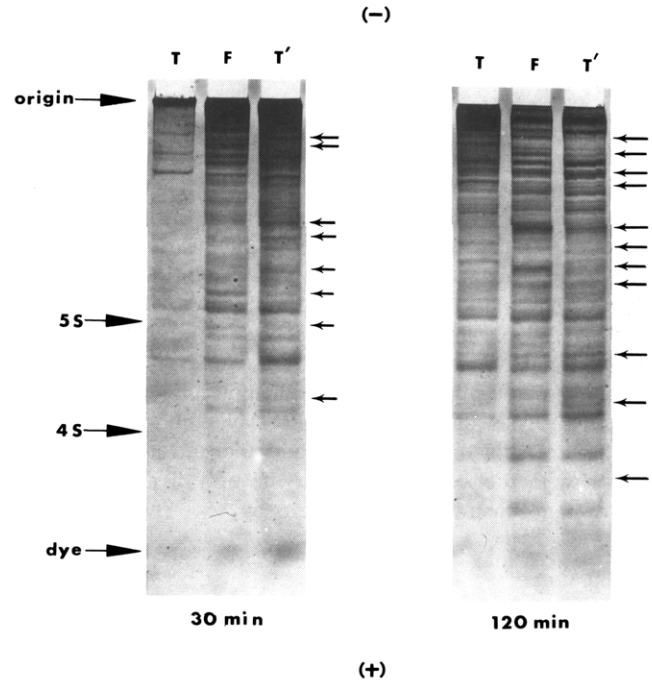


FIGURE 7: Polyacrylamide gel electrophoresis of nuclease digests of bacteriophage F2 RNA. The digestion was carried out at 30° with 900 μg of RNA in a total volume of 225 μl of 0.05 M imidazole, 1.0 mg/ml of bovine serum albumin, pH 7.0. At various times 50-μl samples were withdrawn, extracted for 1 min at room temperature with an equal volume of 90% phenol, and centrifuged. The aqueous phase was separated and *ca.* 60 μg of RNA per slot applied to 10% slabs of polyacrylamide for electrophoresis at 4° and 200 V for 5 hr. *Escherichia coli* RNA and Bromophenol Blue were used as markers. Column F: *A. fumigatus* RNase digest using 0.03 unit ($7.3 \times 10^{-4}$ μg of Lowry protein) of enzyme in the initial reaction mixture. Column T: RNase $T_1$ digest using an equivalent weight of enzyme ($7.3 \times 10^{-4}$ μg of Lowry protein or 0.0066 unit). Column T′: RNase $T_1$ digest using an equivalent activity of enzyme (0.03 unit or $3.3 \times 10^{-3}$ μg of Lowry protein). Arrows to the right of each pattern are only to point out some clear differences between columns F and T′.

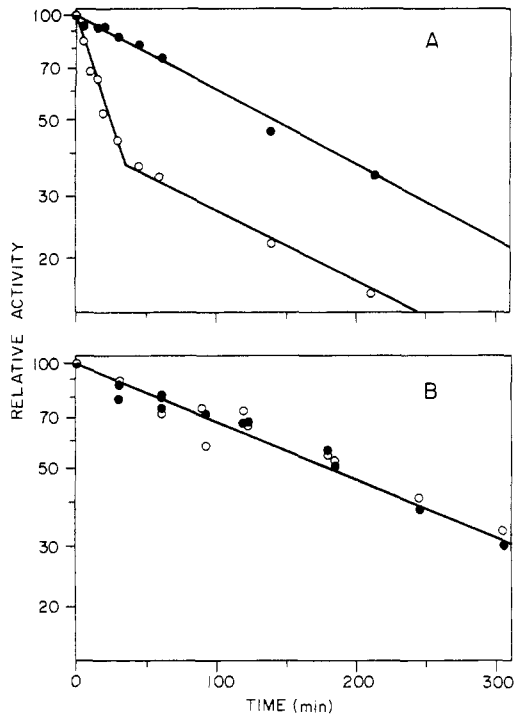


FIGURE 8: Inactivation of nucleases with phenylglyoxal. RNases were incubated with 2% (w/v) phenylglyoxal at pH 8.0 and assayed for enzymic activity at various times. Closed circles, RNase $T_1$; open circles, *A. fumigatus* RNase. Section A shows activity in hydrolysis of wheat germ high molecular weight RNA. Section B shows activity in the hydrolysis of guanosine 2′,3′-cyclic phosphate.

Iodoacetate labeled with $^{14}C$ was incorporated at an equivalent level of 1 mole per mole of protein in each case.

*Trinitrophenylation or succinylation* of RNase $T_1$ and the *A. fumigatus* RNase inactivated both enzymes, although plots of log activity *vs.* time were not linear. The *A. fumigatus* and $T_1$ enzymes were 50% inactivated in 35 and 60 hr (trinitrophenylation) or in about 1 min (succinylation).

*Phenylglyoxal* inactivation of RNase $T_1$ and the *A. fumigatus* RNase was monitored using two assays: formation of acid-soluble materials from high molecular weight RNA, and hydrolysis of guanosine 2′,3′-cyclic phosphate. These results are shown in Figure 8, and indicate a two-step reaction with the *A. fumigatus* enzyme only if a high molecular weight substrate is used in the assay.

## Discussion

*Enzyme Formation and Purification.* This *Aspergillus fumigatus* ribonuclease is probably an inducible extracellular enzyme. There appears to be little relationship between ribonuclease levels measured and the potential of the inducer as a substrate, since DNA and nuclease-resistant homopolymers also induce some RNase activity (Table I). However, it is possible that simpler degradation products of these polymers are the actual inducers, since a variety of phosphate esters and inorganic phosphate itself induce to some extent. Induction of an extracellular guanylic acid specific ribonuclease was also found in *Ustilago sphaerogena* cultured in an RNA medium (Glitz and Dekker, 1964a,b), but other ribonucleases were also found (Arima *et al.*, 1968a,b; Blank *et al.*, 1971). It is thus quite possible that the RNase activity measured in our experiments results from more than the single guanylic acid specific enzyme studied in detail.

Purification of the *A. fumigatus* RNase is simple once the solution volume is reduced and the large quantities of degraded RNA are removed. The final preparation appears to be pure by many of the usual criteria. The specific activity of the enzyme is 41,000 units/ml of protein based on the Lowry determination with a bovine serum albumin standard. Because of the high tyrosine content of the enzyme (see Table III) this assay overestimates the quantity of protein, and a specific activity of 78,000 units/mg dry weight has been calculated. This is comparable with the specific activity in a similar assay reported by Kenney and Dekker (1971) for purified RNase $U_1$, since their unit is about one-third of the unit used here. (Direct comparison is difficult since temperature, substrate, and buffer concentrations are not identical in the two procedures.) The *A. fumigatus* RNase shows a considerably higher specific activity than the commercial preparations of RNase $T_1$ or pancreatic RNase used as comparisons, but these preparations probably do not have the maximal activity possible for the enzymes.

*Enzyme Specificity and Activity.* The base specificity of the *Aspergillus fumigatus* ribonuclease is clearly like RNase $T_1$ (see Uchida and Egami, 1971). RNA is split at guanylic acid residues, producing 2′,3′-cyclic phosphate intermediates which then are opened, presumably to form the 3′ isomers. Depolymerization of poly(G), poly(I), and poly(X) but not poly(A), poly(C), or poly(U) is also like RNase $T_1$ and indicates a probable recognition of 6-ketopurines.

Comparison of kinetic constants for the nucleases using defined substrates indicates similarity but not identity between RNase $T_1$ and the new enzyme. The dinucleoside phosphates (Table V) show the effect on $K_m$ and especially $V_{max}$ of the nucleoside residue adjacent to the guanosine 3′-phosphate. The data for RNase $T_1$ are comparable to $K_m$ and relative rate results reported by Irie (1968), considering that different conditions of pH and buffer were used. Since the RNase $T_1$ used was a commercial crystalline preparation and no attempt was made at further purification, we do not believe our values of $V_{max}$ for RNase $T_1$ to be the highest values possible, but their interrelationships should be significant.

Although considerably more cumbersome than the method of Irie (1968), the column chromatographic fractionation used here permits study of a wide range of substrate concentrations and gives data which can be used to determine kinetic constants with some precision (Woodroof and Glitz, 1971). All of the dinucleoside phosphates except GpG show slightly higher values of $K_m$ with the *A. fumigatus* RNase than with RNase $T_1$. Hydrolysis of guanosine 2′,3′-cyclic phosphate was measured by a paper chromatographic technique which is not very accurate, but the low value of $V_{max}$ and tenfold difference in $K_m$ help to explain the tendency of this compound to accumulate in RNA digests produced with the *A. fumigatus* RNase (Figure 6).

The $K_m$ of GpG with the *A. fumigatus* ribonuclease does not fit the pattern for dinucleoside phosphates seen in the comparison of this enzyme and RNase $T_1$. Guanylic acid oligonucleotides have a tendency toward self-aggregation in solution (see *e.g.*, Young and Fraenkel-Conrat, 1971); it is thus possible that GpG behaves almost as a polymer under our conditions (see below). Aggregation of polyguanylic acid could also explain why this polymer is a relatively poor substrate for the nuclease.

The RNA hydrolysate of Figure 6 and the data on guanosine cyclic phosphate hydrolysis (Table V) both indicate that the cyclic mononucleotide is a poor RNase substrate. In contrast, the hydrolysis of terminal guanosine cyclic phosphate residues

on oligonucleotides, as estimated from rates of RNA hydrolysis in the pH-Stat, proceeds at an equivalent and (relative to guanosine cyclic phosphate) fairly rapid rate for both nucleases. These rates may be only semiquantitative, but certainly do not approach those of the transphosphorylation step; they do indicate that the *A. fumigatus* RNase is capable of catalyzing the hydrolysis step in a manner roughly equivalent to RNase $T_1$.

The *A. fumigatus* nuclease can be clearly differentiated from RNase $T_1$ using high molecular weight substrates such as RNA. The data of Table V are based on the formation of perchloric acid soluble material from high molecular weight RNA, and are difficult to quantitate on an absolute basis, but comparison of the two enzymes on any arbitrary base shows a clear dissimilarity. If $K_m$ is accepted as a measure of substrate binding, the *A. fumigatus* RNase shows a much stronger interaction with a polymeric substrate than was found with dinucleoside phosphates; RNase $T_1$ shows no such differences.

Since the interaction of the two nucleases with RNA is not identical, it is reasonable to expect that the initial products of RNA breakdown might be qualitatively or quantitatively dissimilar. Such a result was predicted by Pinder and Gratzer (1970), who reported dissimilar RNA degradation patterns for RNases $T_1$ and $N_1$, although both enzymes are guanylic acid specific. The gel patterns of Figure 7 illustrate the differences in bacteriophage F2 RNA cleavage with either molar (weight) or enzyme unit equivalence of RNase $T_1$ and the *A. fumigatus* enzyme. Although considerable similarity is obvious, particularly when the nucleases are compared on the basis of activity in our assay, the pattern of digestion products remains characteristic for each nuclease. A possible application in RNA sequence analysis is suggested, since the *A. fumigatus* enzyme and RNase $T_1$ would give some different oligonucleotides of possible use in overlapping and sequence reconstruction (see Gilham, 1970).

The *Aspergillus fumigatus* ribonuclease and RNase $T_1$ clearly share characteristics in addition to base specificity in the hydrolysis of RNA. The amino acid composition (Table III) suggests a relatedness which is amplified by the similarity of the chymotryptic peptide patterns (Figure 2). These data suggest that the *A. fumigatus* RNase is structurally related to RNase $T_1$, and support the prediction that the two enzymes should behave quite similarly. Comparison of the amino acid composition of the *A. fumigatus* RNase with reported values for the *Ustilago sphaerogena* or *Neurospora crassa* guanyloribonucleases (Table III) indicates that the new enzyme most closely resembles RNase $T_1$.

Similar interrelationships are suggested by the interaction of antibodies to RNase $T_1$ with the *A. fumigatus* enzyme as opposed to RNases $U_1$ and $N_1$; only the *A. fumigatus* enzyme is capable of forming a visible precipitate with antibodies to RNase $T_1$ (Figure 3). This precipitate is quantitatively small compared to the precipitate in the homologous reaction (Figure 4). Uchida (1970) has reported that antibodies to RNase $T_1$ showed no interaction with RNase $N_1$ and a very weak reaction with RNase $U_1$. As in our hands, no $U_1$ precipitation line was seen in Ouchterlony gels, but Uchida (1970) did find precipitation in quantitative tests, and inhibition of RNase $U_1$ by anti-RNase $T_1$ serum. In our experiments reduced and carboxymethylated RNase $T_1$ retained a low level of precipitation activity while similarly modified *A. fumigatus* RNase was ineffective; we interpret this result as indicating that the antibodies recognize primarily determinants due to secondary or tertiary structure which would not probably survive reduction and carboxymethylation, as opposed to recognition of amino acid sequences which would be unaffected by the modification. Thus it would appear that in addition to sequence similarities, there may be considerable similarity in the three dimensional structures of RNase $T_1$ and this enzyme.

Carboxymethylation of RNase $T_1$ has been shown by Takahashi *et al.* (1968) to occur at a single glutamic acid in the active site of the enzyme. While we have not yet attempted full characterization of the product of carboxymethylation of the *A. fumigatus* RNase, the identical kinetics and equivalent uptake of radioactive iodoacetate suggest that an essentially identical reaction is taking place. Thus it appears that considerable similarity to RNase $T_1$ exists in the active site of the new enzyme.

The terminal $\alpha$-amino group and single lysine $\epsilon$-amino group of RNase $T_1$ have been modified by deamination (Shiobara *et al.*, 1962) and trinitrophenylation (Kasai *et al.*, 1969) with only slight loss in nuclease activity; the *A. fumigatus* RNase is similarly only slowly and partially inactivated by trinitrophenylation. Succinylation results in a rapid inactivation of both enzymes. This radical modification, in which positively charged amino groups are converted to the negatively charged succinate-substituted derivatives, has been used, *e.g.*, to dissociate virus particles (Frist *et al.*, 1965). Thus it should not be surprising that succinylation has a much greater effect on the nucleases than modifications which simply neutralize a charged group.

Modification of the single arginine residue in RNase $T_1$ with phenylglyoxal led Takahashi (1970) to postulate that this amino acid was present at or near the active site of the enzyme. We have confirmed the report that RNase $T_1$ inactivation by phenylglyoxal occurs at a similar rate whether RNA or guanosine 2′,3′-cyclic phosphate is used as substrate, but find that a parallel loss of activities does not occur in the inactivation of the *A. fumigatus* RNase (Figure 8). Activity with cyclic phosphate is lost at a rate approximately equal to that seen for RNase $T_1$, but if *A. fumigatus* RNase activity is measured with RNA a two-step inactivation seems to occur. Since the *A. fumigatus* enzyme contains two arginine residues, we interpret these results as follows: the most rapid reaction is with an arginine residue not immediately at the active site and involved in hydrolysis, since activity against cyclic phosphate is largely retained. Instead, this first arginine residue is involved in binding larger substrates such as RNA; the *A. fumigatus* RNase shows rather great dissimilarities to RNase $T_1$ in values of $K_m$ determined with an RNA substrate. A second slower reaction of phenylglyoxal with the second arginine residue results in loss of both activities at a rate approximately equal to RNase $T_1$ inactivation. This second reaction would appear to directly involve the catalytic center of the enzyme.

Our interpretation of the phenylglyoxal inactivation experiment clearly requires characterization of the products of the modification reaction at points of partial and extensive inactivation. It might also suggest that partial inactivation would result in RNA degradation patterns different from those seen in Figure 7, and perhaps more similar to the patterns seen for an equivalent weight or activity of RNase $T_1$.

Sequence analysis of the *A. fumigatus* RNase is desirable as a next step in the comparison of this enzyme and RNase $T_1$. It may then be possible to more clearly identify residues involved in catalysis and substrate binding by the nucleases.

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References

Arima, T., Uchida, T., and Egami, F. (1968a), *Biochem. J. 106*, 601.
Arima, T., Uchida, T., and Egami, F. (1968b), *Biochem. J. 106*, 609.
Barnard, E. A. (1969), *Annu. Rev. Biochem. 38*, 677.
Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.
Blank, A., Holloman, W. K., and Dekker, C. A. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1093.
Dhalberg, A. E., Dingman, C. W., and Peacock, A. C. (1969), *J. Mol. Biol. 41*, 139.
Egami, F., and Nakamura, K. (1969), Microbiol Ribonucleases, New York, N. Y., Springer-Verlag.
Frist, R. H., Bendet, I. J., Smith, K. M., and Lauffer, M. A. (1965), *Virology 26*, 558.
Gilham, P. T. (1970), *Annu. Rev. Biochem. 39*, 227.
Glitz, D. G. (1968), *Biochemistry 7*, 927.
Glitz, D. G., Angel, L., and Eichler, D. C. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1094.
Glitz, D. G., and Dekker, C. A. (1963), *Biochemistry 2*, 1185.
Glitz, D. G., and Dekker, C. A. (1964a), *Biochemistry 3*, 1391.
Glitz, D. G., and Dekker, C. A. (1964b), *Biochemistry 3*, 1399.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 199.
Irie, M. (1968), *J. Biochem. (Tokyo) 63*, 649.
Kasai, H., Takahashi, K., and Ando, T. (1969), *J. Biochem. (Tokyo) 66*, 591.
Kenney, W. C., and Dekker, C. A. (1971), *Biochemistry 10*, 4962.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Peacock, A. C., and Dingman, C. W. (1968), *Biochemistry 7*, 668.
Pinder, J. C., and Gratzer, W. B. (1970), *Biochemistry 9*, 4519.
Randerath, K., and Randerath, E. (1964), *J. Chromatogr. 16*, 111.
Shiobara, Y., Takahashi, K., and Egami, F. (1962), *J. Biochem. (Tokyo) 52*, 267.
Smith, E. L., Stockell, A., and Kimmel, J. R. (1954), *J. Biol. Chem. 207*, 551.
Takahashi, K. (1970), *J. Biochem. (Tokyo) 68*, 659.
Takahashi, K., Stein, W. H., and Moore, S. (1968), *J. Biol. Chem. 242*, 4682.
Tomlinson, R. V., and Tener, G. M. (1962), *J. Amer. Chem. Soc. 84*, 2644.
Uchida, T. (1970), *J. Biochem. (Tokyo) 68*, 255.
Uchida, T., and Egami, F. (1971), *in* The Enzymes, Vol. 4, Boyer, P. D., Ed., New York, N. Y., Academic Press, 3rd ed, p 205.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Woodroof, E. A., and Glitz, D. G. (1971), *Biochemistry 10*, 1532.
Young, R. J., and Fraenkel-Conrat, H. (1971), *Biochim. Biophys. Acta 228*, 446.

# A Phospholipase in *Bacillus megaterium* Unique to Spores and Sporangia†

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ABSTRACT: A phospholipase activity which first appears in sporulating *Bacillus megaterium* is then found in mature, free spores. The enzyme is released from spores during germination or by mechanical disruption. The phospholipase is not essential for germination because it is destroyed by heating that does not affect the viability of the spore. The enzyme released from germinated spores behaves as a water-soluble enzyme and has been purified 170-fold to near homogeneity. It is characterized as specific in cleaving the 1-acyl linkage. The purified enzyme requires either a nonionic or anionic detergent for a negatively charged substrate, phosphatidylglycerol, but an anionic detergent (sodium taurocholate) for hydrolysis of neutral phospholipids. Thus the enzyme seems to prefer negatively charged substrate–detergent complexes. The phospholipase activity in sporangial extracts has properties similar to those of the purified spore enzyme, including $A_1$ specificity, pH and detergent responses, and the lack of any requirement for calcium or magnesium ions.

Phospholipid metabolism must play an important part in the membrane alterations involved in the development of the spore and in its germination. A fall of about 50% in the total phospholipid level during sporulation in *Bacillus megaterium* was found in an earlier study (Bertsch *et al.*, 1969; for a review of sporulation, see Kornberg *et al.*, 1968). We detected at about the same time in the sporulation process the appearance

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