An Endonuclease from *Neurospora crassa* Specific for Polynucleotides Lacking an Ordered Structure

I. PURIFICATION AND PROPERTIES OF THE ENZYME*

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To identify enzymes with properties and enzymatic specificities that would permit their use as reagents for the determination of nucleic acid structure and nucleotide sequence, we have undertaken the purification and characterization of nucleases from microorganisms which, in an earlier survey,[1] were found to possess particularly high levels of nucleolytic activity. One of these organisms, *Neurospora crassa*, is of special interest, for, although it has been the subject of much genetic investigation, its nucleic acid metabolism has remained relatively unexplored (1–5).

A preliminary examination of extracts of *N. crassa* showed that there were at least two physically separable nucleases at roughly comparable levels of activity in mycelia and conidia. We have purified one of these nucleases approximately 10,000-fold from extracts of conidia and found it to be an endonuclease with a high degree of specificity for polyribo- or polydeoxyribonucleotides lacking an ordered conformation. Moreover, it appears to possess a distinct, although not absolute, preference for diester bonds involving guanosine or deoxyguanosine residues.

This paper describes the purification and some of the properties of the enzyme, while the following paper (6) deals with its specificity.

EXPERIMENTAL PROCEDURE

Materials and Methods

Escherichia coli DNA labeled with ^{32}P was prepared as described previously (7); it was denatured by heating at 100° for 10 minutes in 0.02 M NaCl at a concentration of 1 μmole of nucleotide per ml, then cooled quickly by immersion in an ice bath. Salmon sperm DNA (Grade A) was purchased from the California Corporation for Biochemical Research, and dissolved in distilled water. Ribosomal RNA was prepared according to Littauer and Eisenberg (8).

Crystalline pancreatic ribonuclease was purchased from the Worthington Biochemical Corporation.

Phosphocellulose (cation exchange powder, P70) was a Whatman product. In order to process it for use, 200 g of the dry powder were first washed thoroughly with distilled water by decantation and then in a column with 4 liters of 0.02 M K$_2$HPO$_4$ containing 0.001 M EDTA and 0.001 M 2-mercaptoethanol, followed by 2 liters of 0.02 M potassium phosphate buffer, pH

7.4, containing 0.01 M 2-mercaptoethanol. It was stored in the phosphate-mercaptoethanol solution at 4°.

Hydroxylapatite (Hypatite-C) was purchased from Clarkson Chemical Company, Williamsport, Pennsylvania, and was used without further treatment.

Polyethylene glycol (Carbowax 6000) was a product of Union Carbide Chemicals Company. Streptomycin was generously donated by Merck Sharp and Dohme.

Concentrations of DNA and RNA are expressed in terms of nucleotide residues.

Assay of N. Crassa Nuclease

Assay A: Denatured DNA as Substrate—This assay measures the conversion of ^{32}P-labeled denatured DNA to fragments which are soluble in perchloric acid. Unless otherwise noted, the reaction mixture (0.3 ml) contained 30 μmoles of Tris-chloride buffer, pH 7.5, 3 μmoles of MgCl$_2$, 25 mμmoles of ^{32}P-labeled *E. coli* DNA, and 0.0025 to 0.015 unit of enzyme diluted in 0.05 M Tris, pH 7.5. After incubation for 30 minutes at 37°, the reaction mixture was chilled in ice, and 0.2 ml of "carrier" (salmon sperm DNA, 2.5 mg per ml) and 0.5 ml of 0.35 N perchloric acid were added. The mixture was mixed and kept at 0° for 5 minutes; then the precipitate was removed by centrifugation for 5 minutes at 17,000 × *g*. An aliquot (usually 0.2 ml) of the supernatant fluid was pipetted onto a planchet and 1 drop of 1 N KOH and 1 drop of a 1% of Duponol were added to the planchet. The sample was then taken to dryness and its radioactivity was determined.

A unit of enzyme is defined as that amount which catalyzes the formation of 1 μmole of acid-soluble ^{32}P in 30 minutes. Throughout the purification the ^{32}P made acid-soluble was proportional to the amount of enzyme added in the range from 10 to 65% of the substrate utilized. This assay served as the basis for enzyme purification, and, unless otherwise indicated, was used in all the experiments describing the properties of the enzyme.

Assay B: Native DNA as Substrate—Conidial extracts contain a second nuclease which is capable of hydrolyzing native as well as denatured DNA. The reaction mixture for assay of this enzyme (0.3 ml) contained 30 μmoles of sodium acetate buffer, pH 5.6, 3 μmoles of MnCl$_2$, 25 mμmoles of ^{32}P-labeled *E. coli* DNA, and enzyme diluted in 0.05 M sodium acetate, pH 5.6. The remainder of the procedure was as described for Assay A. This assay was linear in the range of 25 to 70% of the substrate made acid-soluble.

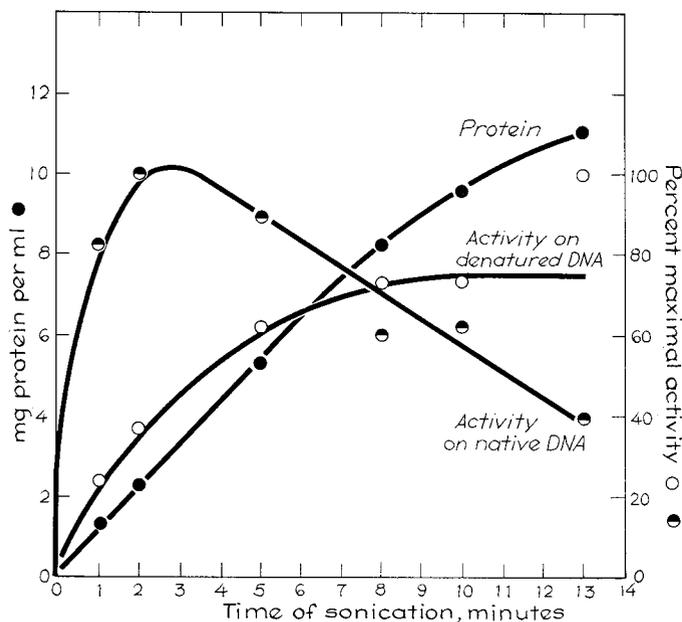[1] E. A. Pratt and I. R. Lehman, unpublished observations.

FIG. 1. Liberation of nucleases from conidia during sonic disruption. Conidia harvested from seven Fernbach flasks were suspended in 0.05 M glycylglycine buffer, pH 7.0, to a final volume of 150 ml. The suspension was exposed to sonic irradiation as described in the text; at the times indicated, 1-ml samples were removed and centrifuged for 15 minutes at 17,000 × g, and the supernatant fluids were assayed as described in "Experimental Procedure."

Assay C: RNA as Substrate—The incubation mixture contained (in 0.3 ml) 110 to 120 mμmoles of ribosomal RNA, 30 μmoles of Tris buffer, pH 7.5, and sufficient enzyme to produce 25 to 35 mμmoles of perchloric acid-soluble nucleotide. After 30 minutes at 37°, the reaction mixture was chilled and 0.2 ml of "carrier" DNA and 0.5 ml of 0.35 N perchloric acid were added. The mixture was kept at 0° for 5 minutes; then the precipitate was removed by centrifugation at 17,000 × g for 5 minutes. The absorption at 260 and 280 mμ was determined against a blank sample from which enzyme had been omitted. The A_{260} for the acid-soluble ribonucleotide produced in the course of digestion was assumed to be 10.0. In each case the $A_{280}:A_{260}$ was determined to check for extraneous ultraviolet-absorbing material. This assay was linear only in the range of 25 to 35 mμmoles of RNA made acid-soluble.

Other Assays

The protein concentrations of fractions through the first dialysis step were estimated according to the method of Lowry *et al.* (9). An aliquot of the fraction to be assayed was treated with 2 volumes of 7% trichloroacetic acid for 10 minutes at room temperature, then centrifuged for 5 minutes at 17,000 × g. The pellet was dissolved in the copper tartrate solution used in the assay.

Because of the extremely low concentrations of protein obtained in fractions subsequent to the phosphocellulose chromatography (20 μg or less per ml), protein concentrations of these fractions were determined by reading the difference in absorption between 215 mμ and 225 mμ (10, 11). By this method crystalline bovine plasma albumin and crystalline pancreatic RNase gave identical standard curves which were linear for protein concentrations ranging from 2 to 200 μg per ml. The absorbances were

corrected for a slight interference due to the presence of phosphate ion; these corrections never exceeded 10%.

The nucleic acid concentration of crude extracts was estimated by measuring the optical density of the extracts at 280 mμ and 260 mμ according to the method of Warburg and Christian (12).

[32]P was measured in a Nuclear-Chicago model D-47 gas flow counter equipped with a Micromil window.

Spectrophotometric measurements were made with a Zeiss PMQ II spectrophotometer.

RESULTS

Purification of Enzyme

Unless otherwise noted, all operations were carried out at 0–4° and all centrifugations were done at 11,000 × g.

Growth of N. crassa—A wild-type strain, ATC9279, obtained from the American Type Culture Collection, was used in all experiments. The organism was grown in 2800-ml Fernbach flasks in Vogel's synthetic medium (13) supplemented with 1% glycerol and 1% sucrose. Stock slants and cultures of conidia were further supplemented with 2% agar. Maximum nuclease activities were found when the organism was maintained between 23° and 26° throughout its growth period.

Large Scale Production of Conidia—Fernbach flasks, plugged with cotton and containing 500 ml of solid medium, were inoculated with 2 ml of a conidial suspension in distilled water. The inoculum was spread evenly over the surface of the agar, and the cultures were allowed to grow for 3 days with normal lighting. At this time the cotton plugs were replaced by a double layer of sterile tissue paper taped over the neck of the flask, and the cultures were grown for 3 more days illuminated by fluorescent lights positioned just above the flasks.

The conidia were harvested by pouring 250 ml of distilled water over the culture, scraping the mycelial mat off of the agar, and swirling the flask until a fine conidial suspension resulted. The suspension was filtered through cheesecloth to remove the mycelial fragments, and the filtrate was centrifuged for 15 minutes. The pellets of conidia could be processed immediately or stored at −15° for at least 6 weeks without loss of activity.

Sonic Disruption of Conidial Suspension—The rates at which the two nuclease activities thus far identified in *N. crassa* were liberated during sonic irradiation of a conidial suspension differed markedly (Fig. 1). Thus, the level of activity with denatured DNA as substrate reached a maximum in the supernatant fraction after about 6 minutes of treatment, then remained relatively constant; the activity determined with native DNA as substrate reached a maximum after about 3 minutes of treatment, then dropped off. Protein, on the other hand, was released continuously for at least 13 minutes, while nucleic acid leveled off after about 11 minutes of treatment. Thus, by sonic irradiation for a limited time, an extract could be prepared which contained essentially all of the nuclease activity measured with denatured DNA, but which contained only about 50% of the conidial protein.

In a typical purification (Table I), the conidia harvested from 301 Fernbach flasks were suspended in 0.05 M glycylglycine buffer, pH 7.0, to a final volume of 3670 ml. The suspension was treated for 6 minutes in 160-ml aliquots with a Branson model 75 Sonifier with the standard "step-horn" (1½ × ½ inches) at full power, then centrifuged for 25 minutes. The supernatant

fluid (2870 ml) served as the starting material for the purification.

To 2870 ml of the crude extract (nucleic acid concentration of 2.55 mg per ml) were added 182.8 ml of a solution of 5% streptomycin sulfate.[2] The resulting suspension was stirred for 10 minutes, then centrifuged for 10 minutes, and the precipitate was discarded.

Ammonium Sulfate Fraction—The light yellow streptomycin supernatant fraction (3000 ml) was mixed with 300 ml of 1 M potassium phosphate buffer, pH 7.5, and the resulting solution was divided into three 1100-ml aliquots. To each portion were added 399 g of finely powdered ammonium sulfate. The solutions were stirred until the salt dissolved, stirred for an additional 5 minutes, and then centrifuged for 12 minutes, and the precipitates were discarded.

To each of the three supernatant solutions were added an additional 273 g of ammonium sulfate, and the same procedure as above was followed. The supernatant fluids were discarded, and the precipitates were combined and dissolved in 0.05 M Tris-chloride, pH 7.5, yielding 610 ml of a dark yellow solution.

Acetone Fractionation—To 610 ml of the ammonium sulfate fraction (at 0°) were added, with stirring, 372 ml of acetone (at −20°) over a 5-minute period. The suspension was stirred for 1 additional minute, then centrifuged for 10 minutes. The precipitate was discarded, and 288 ml of acetone were added to the supernatant fluid over a 5-minute period. After stirring for 1 minute more, the suspension was centrifuged as above, the supernatant fluid was discarded, and the precipitate was extracted by stirring with 250 ml of 0.05 M potassium phosphate, pH 7.5, for 2½ hours at 0°. The undissolved material was then removed by centrifugation for 10 minutes and discarded.

Dialysis of Acetone Fractions—The acetone fraction was dialyzed overnight against two 6-liter portions of 0.01 M potassium phosphate, pH 6.5. This dialysis resulted in a loss of protein and an increase in activity (Table I). The specific activity after dialysis was quite reproducible, although the actual change depended upon the yields and purifications in the ammonium sulfate and acetone steps. This change of specific activity was also dependent upon both the pH and composition of the buffer used for the dialysis. In every case examined there appeared to be some correlation between loss of protein and increase of activity; in general the greatest increase in specific activity was observed after dialysis at pH 6.5. No increase in activity was observed upon dialysis of crude extracts under any conditions which were examined.

Phosphocellulose Chromatography—A column (3.6 × 20 cm) containing 200 ml of packed phosphocellulose was equilibrated with 0.01 M potassium phosphate, pH 6.5. The dialyzed acetone fraction (250 ml) was applied to the column at a rate of 4.0 ml per minute. The column was washed first with 650 ml of 0.01 M potassium phosphate, pH 6.5, then with 400 ml of 0.05 M potassium phosphate, pH 6.5. A linear salt gradient was applied with 0.05 and 0.35 M potassium phosphate, pH 6.5, as limiting concentrations, the total volume of the gradient being 1300 ml. Fractions (16 ml) were collected at 12-minute intervals.

Eighty per cent of the protein was recovered in the pass-through and wash fractions, although no enzyme activity was

[2] This step was quite reproducible if the amount of streptomycin added was based on nucleic acid content rather than protein concentration of the crude extract and if the streptomycin solution was prepared immediately before use.

TABLE I

Purification of N. crassa nuclease

Fraction	Volume	Units	Total protein	Specific activity
	ml		*mg*	*units/mg*
Crude extract	2,870	1.37×10^4	12,900	1.1
Streptomycin	3,000	1.39×10^4	6,700	2.1
Ammonium sulfate	610	1.10×10^4	4,600	2.4
Acetone*	242		581	
Dialyzed acetone	254	1.64×10^4	360	45.2
Phosphocellulose	128	2.78×10^3	4.5	614.0
Concentrated phosphocellulose	42	3.02×10^3	3.5	855
Hydroxylapatite	55	1.37×10^3	0.26	5,350
Concentrated hydroxylapatite†	1.7	9.38×10^2	0.08	11,500

* Assays of the acetone fraction were found to be poorly reproducible.

† Pooling and concentration of fractions purified 3.4- to 5-fold over the concentrated phosphocellulose fraction yield an additional 250 units with a specific activity of 5130.

detectable. A major protein peak, which was eluted between 0.07 and 0.17 M potassium phosphate, also contained no enzymatic activity. The enzyme was eluted between 0.18 and 0.25 M potassium phosphate, and the fractions purified from 8- to 21-fold in this step were pooled. The pooled fractions contained 58% of the activity recovered; the total recovery was 30% of that applied to the column.

Dialysis against Polyethylene Glycol—Two 64-ml portions of the pooled phosphocellulose fraction were each dialyzed against 1.1 liters of a 30% (w/v) solution of polyethylene glycol in 0.007 M potassium phosphate, pH 6.5. After about 12 hours, the dialysis bags, which contained little, if any, visible liquid, were thoroughly washed with distilled water, then opened, and the contents of each bag were collected by washing with six 3.5-ml aliquots of 0.02 M potassium phosphate, pH 6.5. The phosphate buffer washes were combined, yielding 42 ml of concentrated enzyme solution.

An increase in activity and loss of protein also accompanied this dialysis step (Table I). As noted for the dialysis of the acetone fraction, the extent of increase in activity and loss of protein depended upon the purification and recovery achieved in the previous step.

Hydroxylapatite Chromatography—A column of hydroxylapatite (1 × 15 cm) was equilibrated with 0.02 M potassium phosphate, pH 6.5. The concentrated phosphocellulose fraction (42 ml) was applied to the column at the rate of 0.4 ml per minute. The column was washed first with 75 ml of 0.02 M potassium phosphate, pH 6.5, and then with 35 ml of 0.05 M potassium phosphate, pH 6.5. A linear gradient was applied with 0.05 and 0.30 M potassium phosphate as limiting concentrations, the total volume of the gradient being 330 ml. Fractions (7.0 ml) were collected at 20-minute intervals. All elutions were carried out with an applied pressure of 1.3 p.s.i.

The pass-through and wash fractions contained about 80% of the protein and none of the enzymatic activity added to the column. Most of the remainder of the protein was eluted between 0.12 and 0.27 M phosphate, while the peak of enzymatic activity was eluted between 0.14 and 0.23 M phosphate (Fig. 2).
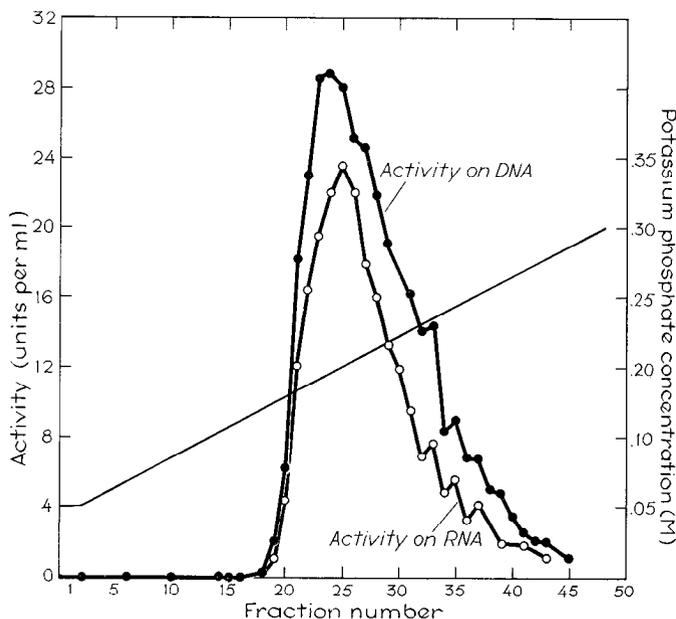
FIG. 2. Hydroxylapatite chromatogram. Assays were performed as described in "Experimental Procedure."
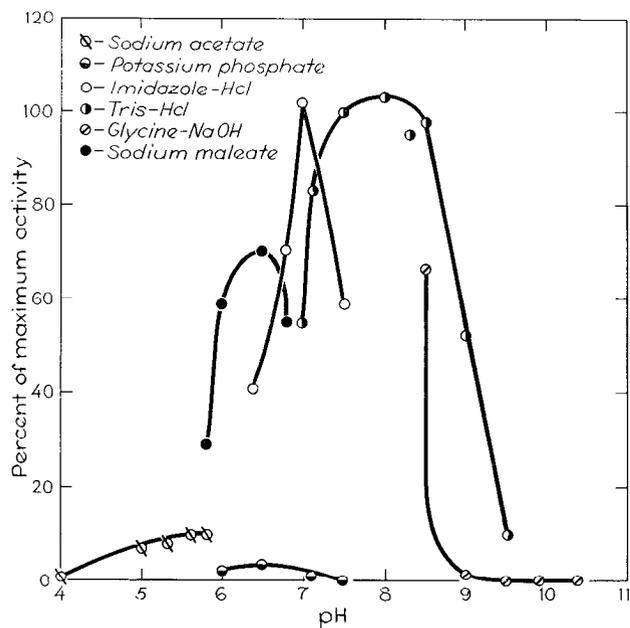

FIG. 3. pH-activity curve. Assays were performed as described in "Experimental Procedure." The indicated buffers were added at a final concentration of 0.1 M.

The fractions which were purified 5- to 8-fold over that put onto the column were pooled. These fractions represented 56% of the activity recovered; the total activity recovered was 81% of that applied.

A background of ultraviolet-absorbing material, giving an apparent protein concentration of 3 to 5 μg per ml, was present in the column effluent. This background was due to an impurity in the polyethylene glycol used in the previous step and was removed in the subsequent dialysis (below), causing an apparent increase in specific activity.

Concentration of Hydroxylapatite Fraction by Pervaporation—

The pooled hydroxylapatite fractions were concentrated by pressure dialysis against 2 liters of 0.05 M potassium phosphate, pH 6.5, over a 21-hour period as described by Richardson and Kornberg (14). The concentrated fraction was removed from the dialysis tubing, which then was washed with several 0.1-ml aliquots of 0.05 M potassium phosphate, pH 6.5. The resulting 1.7 ml of solution were stored either in liquid nitrogen ($-185°$) or at $0°$.

Properties of Enzyme

Unless otherwise indicated, the concentrated hydroxylapatite fraction was used in all studies to be described.

*Absence of 5'-Nucleotidase Activity—*The concentrated hydroxylapatite fraction had no detectable 5'-nucleotidase activity (less than 0.1% of its nuclease activity) at pH 5.5, 7.5, or 9.5 as measured by the liberation from 5'-dAM ^{32}P of ^{32}P that did not adsorb to Norit.

*Stability—*The fractions up to and including the acetone step were unstable, and consequently it was necessary to carry the purification through this step and the subsequent dialysis in 1 day. After the first dialysis, only the highly diluted column fractions (less than 10 μg of protein per ml) were unstable, showing daily losses of activity of 5 to 10% when stored at $0°$; more concentrated fractions were stable for several days. Phosphate buffer helped to stabilize the enzyme even though it strongly inhibited enzymatic activity (see below).

The concentrated hydroxylapatite fraction could be stored for at least 8 months at $-185°$ without loss of activity. Storage at $0°$ resulted in a loss of about 30% of the initial activity in the first month with no additional loss thereafter. Storage at $0°$ of fractions previously kept at $-185°$ showed the same initial loss of activity followed by apparent stabilization. Some of the activity lost on storage at $0°$ could be recovered by addition of oxidized glutathione (0.004 M) to the reaction mixture or, as shown below, by incubation of the enzyme at $55°$ for short periods.

*pH Optimum—*The rate of hydrolysis of denatured DNA in various buffers is shown in Fig. 3. Maximal activity of the most purified fraction was between pH 7.5 and 8.5, but the enzyme had significant activity at pH values ranging from about 5.8 to 9.3. The low level of activity observed with acetate buffer may be due to contamination of the enzyme by a second nuclease which is optimally active at pH 5.6.

*Effect of Ionic Strength—*There was a marked effect of ionic strength on enzymatic rate with denatured DNA as substrate. In the presence of 0.01 M MgCl$_2$ the enzyme showed optimal activity between 0.1 and 0.2 M Tris and was significantly less active at lower salt concentrations. For example, at 0.017 and 0.067 M Tris, the activity observed was 31 and 81% of maximum, respectively. Addition of NaCl to reaction mixtures containing 0.033 M Tris (and 0.01 M MgCl$_2$) increased the rate to the same extent as found with increasing concentrations of Tris. Sodium or potassium sulfate (adjusted to pH 7.5) was as effective as NaCl in this respect.

In the absence of added magnesium ion, a similar but smaller dependence upon ionic strength was observed. In this case the rate observed was 30% higher at 0.13 M Tris-chloride than at 0.03 M; with lower buffer concentrations, sodium chloride, sodium sulfate, or potassium sulfate were again stimulatory.

*Inhibition by Potassium Phosphate—*Enzymatic activity was strongly inhibited in the presence of potassium phosphate. If potassium phosphate was the buffer used in the reaction mixture,

1 to 3 % of the optimal activity was found from pH 6.0 to 7.0; no activity was detectable at pH 7.5 (Fig. 3). When potassium phosphate was added to reaction mixtures containing 0.1 M Tris buffer, inhibition increased with increasing levels of phosphate (Fig. 4). Only 0.5% of the activity remained in the presence of 0.1 M phosphate, and no activity was detectable at higher concentrations.

Requirement for Divalent Cations—As shown in Table II, an appreciable rate was observed with denatured DNA as substrate even in the absence of added metal ion; however, the activity was enhanced by the addition of Ca^{++}, Mg^{++}, or Fe^{++} at 0.01 M. The stimulation by $FeCl_2$ was variable, presumably because of uncontrolled oxidation of ferrous to ferric ion. Addition of monovalent or trivalent cations produced no significant stimulation.

The enzymatic rate was strongly inhibited by EDTA; in the presence of 10^{-2} M $MgCl_2$, while there was no inhibition by 1.5×10^{-6} M EDTA, there was about 80% inhibition by 7×10^{-6} M EDTA. In the range of 7×10^{-6} to 10^{-4} M EDTA, inhibition varied from 80 to 95%, but never reached 100%.

Since it was apparent that $MgCl_2$ could not reverse this inhibition, a variety of other cations were tested. Co^{++} and Fe^{++} were found to be the most effective (Table III). Cobaltous ion not only overcame the inhibition by EDTA, but stimulated the activity beyond that observed in the absence of EDTA. In the presence or absence of 10^{-2} M $MgCl_2$, activity was maximal in 5×10^{-5} to 5×10^{-4} M $CoCl_2$, in both cases about 150% that found without added $CoCl_2$. The presence of 10^{-5} M EDTA under these conditions was without effect. Concentrations of $CoCl_2$ above 5×10^{-4} M, however, were distinctly inhibitory. In the presence of 10^{-2} M $CoCl_2$ the enzyme was 99% inhibited in the absence of $MgCl_2$ and 94% inhibited in the presence of 10^{-2} M $MgCl_2$.

When the enzyme was first exposed to levels of EDTA capable of producing about 99% inhibition, then dialyzed overnight
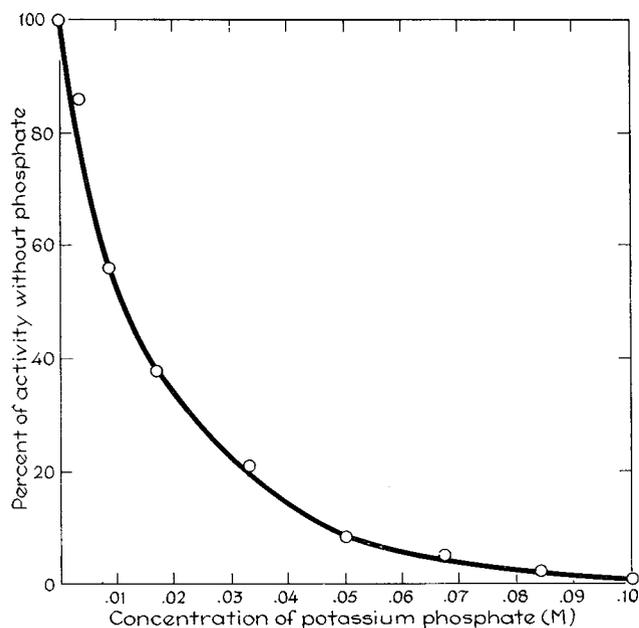

FIG. 4. Effect of potassium phosphate on enzymatic rate. Potassium phosphate buffer at pH 7.5 was added to the reaction mixtures as indicated. The remainder of the assay was as described in "Experimental Procedure."

TABLE II

Effect of cations on activity

Reactions were carried out at pH 7.5, as described in "Experimental Procedure," with cation added as indicated.

Cation	Concentration	Activity in absence of cation
	M	%
None		100
$MgCl_2$	0.0001	119
	0.001	172
	0.01	248
	0.05	28
	0.10	0.7
$CaCl_2$	0.001	148
	0.01	255
$FeCl_2$	0.01	288
$CuSO_4$	0.01	51
$MnCl_2$	0.01	50
$CoCl_2$, $ZnCl_2$, $NiCl_2$, or $Cd(NO_3)_2$	0.01	0
$FeCl_3$	0.01	118
NaCl	0.01	110
KCl	0.01	121
$KCl + MgCl_2$	0.01 (each)	273

TABLE III

Reversal of EDTA inhibition by cations

Cations were added at a concentration of 3×10^{-4} M. The assays were carried out as described under "Experimental Procedure," except that $MgCl_2$ was replaced by the metals as indicated and 10^{-4} M EDTA was present.

Cation added	Activity in absence of EDTA
	%
None	20
$FeCl_2$	0 (92)*
$ZnCl_2$	56 (26)*
$CoCl_2$	150
$CuSO_4$	26
$Cd(NO_3)_2$	40
$NiCl_2$	22
$MgCl_2$	27
$MnCl_2$	71
$FeCl_3$	0

* With cation concentration of 1×10^{-4} M.

against 0.05 M potassium phosphate, pH 6.5, a considerable amount of activity was recovered (greater than 50% of a control dialysis without added EDTA), implying that the effect of EDTA might not be due simply to the removal of metal from the enzyme. However, the presence of trace contaminants of divalent cation in the buffer used in dialysis has not been ruled out.

Effect of Temperature on Enzymatic Rate—The dependence of initial rate upon temperature of incubation is shown in Fig. 5. In the range from 15° to 47°, the reaction exhibited an unusually high Q_{10}, ranging from 5 to 6. From 47° to 62°, the rate was virtually independent of temperature and approximately 5 times that found at 37°, but above 62° the rate quickly fell off. The residual activity observed above about 70° may simply have
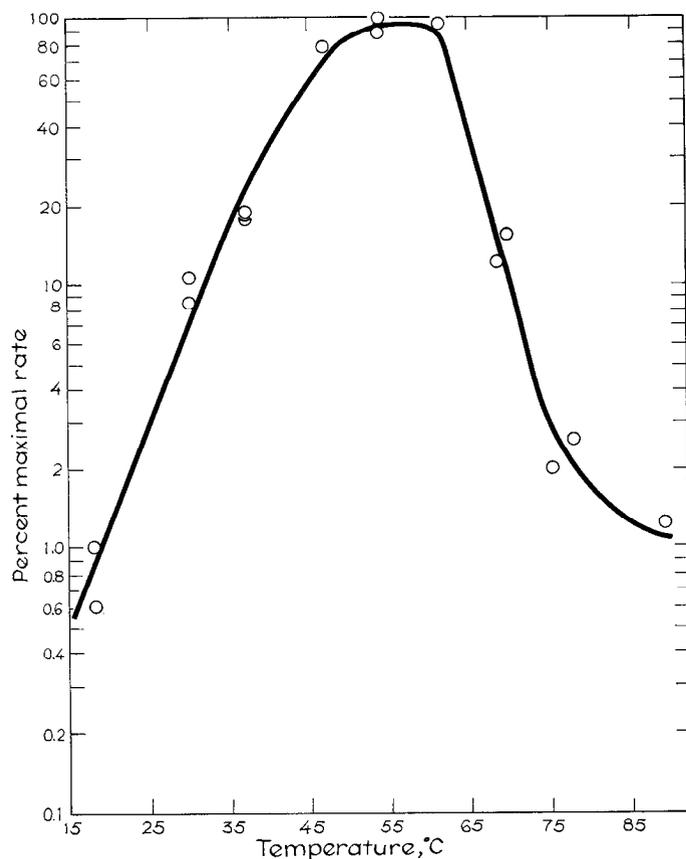
FIG. 5. Effect of temperature on enzymatic rate. The reaction mixtures were prepared as described in "Experimental Procedure" and were incubated at the temperatures indicated. The reaction mixtures were at 0° at the beginning of the incubations.

reflected limited enzymatic activity before the reaction mixture achieved thermal equilibrium.

In the absence of magnesium ion the Q_{10} between 27° and 37° was 6.5, a value slightly higher than was found in the presence of magnesium ion.

Evidence That Single Enzyme Catalyzes Hydrolysis of DNA and RNA—The concentrated hydroxylapatite fraction hydrolyzes polyribonucleotides at nearly the same rate as polydeoxyribonucleotides. Several lines of evidence suggest that the two activities are associated with the same protein.

1. Cochromatography: The ratio of the two activities throughout the peak obtained upon hydroxylapatite chromatography is essentially constant, varying from 1.2 to 1.5 (Fig. 2).

2. Similar pH optima: Both activities exhibit maximal activity between pH 7.5 and 8.5, are strongly inhibited by potassium phosphate, and show similar levels of activity at acid and alkaline pH values.

3. Similar temperature optima: The two activities both show an unusually strong dependence upon temperature. Thus the RNase activity at 45° is 3.6 times that at 37°, compared with 3.3 times for DNase activity; at 37° the RNase activity is 10 times as active as at 27° compared with 5 times for DNase activity.

4. Similar rates of heat inactivation: The two activities were inactivated at a similar rate upon incubation of the preparation at 55° during 90 minutes (Fig. 6). It is of interest to note that although no loss in RNase or DNase activity was observed upon storage of the concentrated hydroxylapatite fraction at −185°,

the RNase activity, unlike the DNase activity, was unstable at 0°, showing continual losses of about 15% per week. However, incubation of such an aged preparation for 5 minutes at 55° resulted in full restoration of its RNase activity (Fig. 6). Furthermore, in cases when DNase activity showed losses during storage at 0°, the RNase activity found after heat treatment decreased correspondingly; as in the case of the DNase activity, addition of 0.004 M oxidized glutathione to reaction mixtures caused partial recovery of the lost activity.

5. Similar effects of EDTA and divalent cations: RNase activity was inhibited by the same concentrations of EDTA as the DNase activity. Thus 1.3×10^{-6} M EDTA gave no inhibition, but 10^{-5} M EDTA produced 84% inhibition, and concentrations of EDTA ranging from 10^{-5} to 10^{-4} M produced
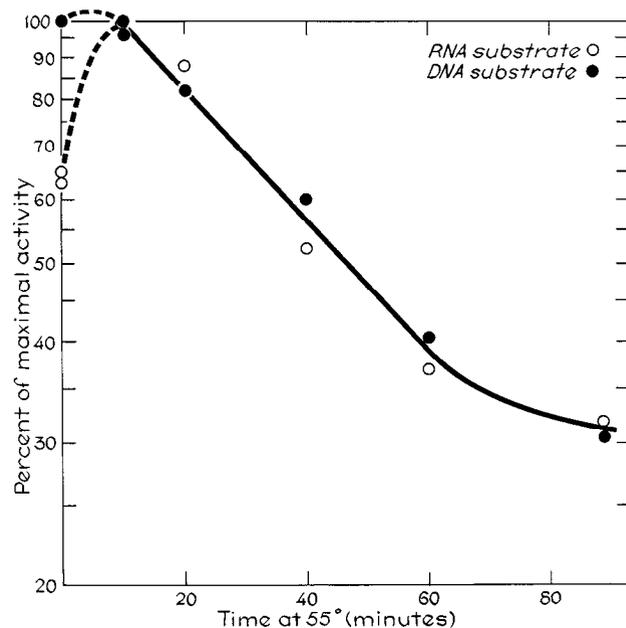

FIG. 6. Parallel heat inactivation of RNase and DNase activation. An enzyme preparation which had been stored for several weeks at 0° was incubated at 55° in 0.05 M Tris, pH 7.5, at a concentration of 3 units per ml. At the times indicated, samples were removed, added to previously prepared reaction mixtures, and immediately assayed as described in "Experimental Procedure."

TABLE IV

Inhibition of DNase activity by RNA

The composition of the incubation mixtures was as indicated, and the reaction was carried out as described under "Experimental Procedure."

RNA added	Inhibition at various concentrations of reactants			
	Enzyme, 0.017 unit; Mg++, 0.01 M; DNA, 25 mμmoles	Enzyme, 0.027 unit; No Mg++; DNA, 25 mμmoles	Enzyme, 0.011 unit; Mg++, 0.01 M; DNA, 25 mμmoles	Enzyme, 0.011 unit; Mg++, 0.01 M; DNA, 16 mμmoles
mμmoles	%	%	%	%
0.00	0	0	0	0
3.85			5	14
7.70	6		10	28
15.4	17	16	29	42
23.1			45	52
30.8	29	23	48	57
61.6	63	55		

about 95% inhibition (but, as with the DNase activity, never 100%). In this case, however, Co^{++} restored activity only partially; for example, 2×10^{-5} M Co^{++} restored only 18% of the activity inhibited by 10^{-5} M EDTA, and 2×10^{-4} M Co^{++} reduced the inhibition of 10^{-4} M EDTA from 97% to only 84%.

6. RNA inhibition of DNase activity: Addition of unlabeled RNA to reaction mixtures containing ^{32}P-labeled, denatured DNA resulted in the inhibition of hydrolysis of the DNA, the amount of inhibition depending upon the concentrations of enzyme, DNA, and RNA (but not Mg^{++}) (Table IV).

Studies on the mechanism of inhibition were complicated, however, by the degradation of the inhibitor itself, particularly at low RNA concentrations, where an appreciable fraction of the RNA would be degraded to oligonucleotides which might be less inhibitory.

7. Similar requirements for substrates in nonordered conformation and formation of similar products: As shown in the succeeding paper (6), the *N. crassa* nuclease attacks polyribonucleotide and polydeoxyribonucleotides with ordered structures at a much lower rate than those lacking an ordered structure. Moreover, the distribution of products formed after hydrolysis of RNA and DNA by the *N. crassa* nuclease is strikingly similar.

DISCUSSION

Although the *N. crassa* nuclease has been purified some 10,000-fold, it is not certain that a physically homogeneous preparation was achieved. The yield of enzyme (0.08 mg from approximately 13 g of conidial protein) was far too low to permit physical characterization. It is worth noting, however, that the specific activity of the *N. crassa* nuclease is approximately 3- to 4-fold higher than crystalline pancreatic DNase when the two enzymes are assayed under conditions optimal for each. As shown in the following paper (6), the purified enzyme, which is highly specific for denatured or single stranded DNA, is in fact contaminated with traces of another activity which is able to attack native DNA and which can under certain conditions be largely inhibited. Although the contaminant would appear to be the nuclease acting optimally at acid pH which is described in Fig. 1, this possibility has by no means been established, and purification of the latter enzyme would be required to verify this point.

The purified nuclease is able to attack RNA and DNA lacking an ordered structure at nearly equivalent rates, and it appears that the same enzyme catalyzes both types of hydrolytic reactions. Thus the two activities cochromatograph on hydroxylapatite; have similar pH temperature, and ionic optima; have a similar requirement for nonordered polynucleotide substrates; produce a similar distributon of products; and show similar rates of heat inactivation. Moreover, RNA is an inhibitor of DNase activity.

The stimulation of enzymatic activity by Mg^{++} and Co^{++} seems to operate through independent mechanisms. In particular, the stimulation of activity by the two cations is additive, maximal activity being found only in the presence of both. Another indication of a difference in the mode of action of the two cations is the facility with which stoichiometric amounts of Co^{++}, but not 100-fold excessess of Mg^{++}, can overcome inhibition of the enzyme by EDTA. It is also significant that optimal stimulation by Mg^{++} (or Ca^{++}) requires concentrations of cation higher than the substrate nucleotide concentration while Co^{++} is maximally effective at concentrations significantly lower than that of the substrate, becoming inhibitory at higher concentra-

tions. These observations suggest that the Mg^{++} (or Ca^{++}) might act by inducing a more favorable substrate conformation, or by facilitating substrate-enzyme interaction, while Co^{++} or a similar cation might act upon the enzyme itself. The ability of the enzyme to act in the absence of added cation and the extreme sensitivity of the enzyme to EDTA further suggest that the enzyme as isolated contains a significant amount of Co^{++}, or similar cation, which is necessary for activity.

The effect of temperature on enzymatic rate would also appear to be related to substrate conformation and may reflect the requirement of the enzyme for polynucleotides without an ordered conformation. Thus, the unusually high Q_{10} values observed are very possibly related to the suppression of spurious hydrogen bond formation in denatured or single stranded polynucleotides at the elevated temperatures.

The wide range of conditions under which the *N. crassa* nuclease is active, including temperatures ranging from 25° to 65°, the presence or absence of added divalent cation, pH values ranging from 6 to 9, salt concentrations from 0.03 to greater than 0.20 M, and the possibility of strong inhibition of the enzyme by 0.1 M potassium phosphate, pH 7.5, or by EDTA even in the presence of a large excess of Mg^{++}, coupled with its high degree of selectivity for substrates without an ordered structure, should make the enzyme a valuable reagent for polynucleotide study.

SUMMARY

A nuclease has been purified 10,000-fold from conidia of *Neurospora crassa*. This preparation hydrolyzes heat-denatured deoxyribonucleic acid and ribosomal ribonucleic acid at approximately the same rate. Both activities appear to be catalyzed by the same enzyme. The enzyme is active under a variety of conditions, including temperatures ranging from 25° to 65°, pH values from 6 to 9, the presence or absence of added divalent cations, and salt concentrations ranging from 0.03 to greater than 0.20 M. It is inhibited by potassium phosphate and by ethylenediaminetetraacetate; the latter inhibition can be overcome by stoichiometric amounts of Co^{++}, but not by a large excess of magnesium ion. These properties, along with the specificity described in the subsequent paper, should make this enzyme useful for the study of polynucleotide structure and conformation.

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