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

II. STUDIES OF ENZYME SPECIFICITY

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The preceding paper described the purification and some of the properties of a nuclease from Neurospora crassa (1). The purpose of this communication is to describe its mode of attack as an endonuclease with a high degree of specificity for polynucleotides (both ribo- and deoxyribo-) lacking an ordered structure. Moreover, it has a distinct preference for diester bonds within a polynucleotide in which guanine residues are involved.

EXPERIMENTAL PROCEDURE

Materials

Nucleic Acids—32P-Labeled Escherichia coli DNA was isolated as described previously (1). Unlabeled E. coli DNAs were 

32P-labeled preparations which had fully decayed (less than 0.01 µc per µmole). 32P-Labeled T7 phage DNA was prepared by the method of Davison and Freifelder (2); phage λ DNA was prepared as described by Kaiser and Hogness (3); T4 phage DNA was isolated as described previously (4); calf thymus DNA was isolated by the method of Kay, Simonne, and Donnies (5); salmon sperm DNA (Grade A) was purchased from California Corporation for Biochemical Research. Ribosomal RNA from E. coli was prepared as described by Littauer and Eisenberg (6), and soluble RNA was isolated from E. coli by a modification of the method of Zalusky (7). Unless otherwise indicated, all DNA samples were denatured by heating at 100° for 10 minutes in 0.02 M NaCl at a concentration of 1 µmole of nucleotide per ml; they were then cooled rapidly by immersion in an ice bath. Denaturation with alkali was performed by mixing 0.2 ml of DNA (1 µmole per ml in 0.02 M NaCl) with 0.1 ml of 1 M KOH (final pH, 12.0 to 12.5) and incubating this mixture for 30 minutes at room temperature; 0.02 ml of 1 M Tris, pH 7.2, was then added (final pH, 8 to 8.5). DNA was denatured with formamide by dialyzing 0.2 ml of DNA first against 20 ml of formamide by dialyzing 0.2 ml of DNA first against 20 ml of 

0.02 M NaCl at a concentration of 1 µmole of nucleotide per ml; they were then cooled rapidly by immersion in an ice bath. Denaturation with alkali was performed by mixing 0.2 ml of DNA (1 µmole per ml in 0.02 M NaCl) with 0.1 ml of 1 M KOH (final pH, 12.0 to 12.5) and incubating this mixture for 30 minutes at room temperature; 0.02 ml of 1 M Tris, pH 7.2, was then added (final pH, 8 to 8.5). DNA was denatured with formamide by dialyzing 0.2 ml of DNA first against 20 ml of 99% formamide for 45 hours at room temperature, then exhaustively against 0.02 M NaCl at 4°. This treatment resulted in a about a 3-fold concentration of the DNA.

Synthetic Polynucleotides—The copolymer dAT is prepared

* This work was supported in part by grants from the National Institutes of Health, United States Public Health Service.

† The abbreviations used are: dAT, an alternating copolymer of deoxyadenylate and deoxycytidylate; dGdC, a polymer consisting of homopolymers of deoxyguanylate and deoxythymylolate, hydrogen-bonded together; dIdC, a polymer consisting of homopolymers of deoxynosinate and deoxyctydylate, hydrogen-bonded together; dG, dC, and dT, the homopolymers of deoxyguanylate, deoxycytidylate, and deoxyinosinate, respectively, bonding not specified; rAU, an alternating copolymer of adenylate and uridylylate; d(pT)₆, refers to an oligonucleotide composed of 6 numbers of residues of deoxythymidine 5'-phosphate; d(pT)₆-acetate is the hexamer, (pT)₆-acetate, in which the 3'-hydroxyl group has been acetylated; dT(pT)₆, dT(pT)₆, and dT(pT)₆ refer to thymidine oligonucleotides containing 6, 7, and 8 residues, and lacking a terminal phosphomonoester group; dT(pT)₆ is the thymidine pentamer with both 3'- and 5'-phosphomonoester groups. sRNA is used to designate soluble ribonucleic acid.

‡ We are grateful to Dr. H. G. Khorana for his guidance in the preparation of these oligonucleotides.
**Methods**

**Enzyme Assays**—Assays of the *N. crassa* nuclease with *32^P*-labeled DNA or unlabeled RNA were performed as described in the preceding paper (1). Assay of the enzyme by measurement of the liberation of acid-soluble, ultraviolet-absorbing products from DNA or synthetic polynucleotides was performed in the following way. The reaction mixtures contained (in 0.3 ml) 110 to 120 μmoles of substrate, 30 μmoles of Tris, pH 7.5, 3 μmoles of MgCl₂ (for deoxyribonucleotide substrates only), and enzyme as indicated. After 30 minutes at 37° the reaction mixture was chilled, 0.2 ml of carrier (salmon sperm DNA, 2.5 mg per ml) and 0.5 ml of 0.35 n perchoric acid were added, and the mixture was kept at 0° for 5 minutes. The precipitate was removed by centrifugation, at 17,000 × g for 5 minutes, and the absorption at 260 μ and 280 μ was determined by reading against a blank containing no enzyme with a Zeiss PMQ II spectrophotometer. The blank was also read against distilled water in order to determine that the untreated substrate was totally acid-precipitable. The A₂₆₀ for the acid-soluble fragments produced from DNA was assumed to be 10.0, and the A₂₆₀ for the products formed from synthetic polynucleotides was assumed to be equal to that of its constituent mononucleotides at acid pH. The A₂₆₀∶A₂₈₀ was determined in order to check against extraneous ultraviolet absorption and, in the case of the synthetic polymers, to verify the composition of the products. The assays were linear from 15 to 65% of the substrate made acid-soluble and, in the case of *E. coli* DNA, gave excellent agreement with the *32^P* assays.

**Sedimentation Analyses**—Sedimentation analyses were performed with a Spinco model E ultracentrifuge equipped with an ultraviolet optical system. Zone sedimentation of DNA was performed in 0.1 M NaOH-0.9 M NaCl or in 1 M NaCl-0.01 M Tris, pH 7.5, as described by Studier (23). Estimates of the degree of degradation were made by measuring the proportion of trailing material in tracings made with a Joyce-Loebel densitometer.

**Analysis of Products of Digestion**—The susceptibility of digestion products to bacterial alkaline phosphatase was determined by measuring the liberation of *32^P* which did not adsorb to Norit as described earlier (10). Blank without enzyme gave less than 2% of the assay values. The incubation mixture for treatment of products with 5'-nucleotidase contained (in 0.3 ml) 5 to 10 μmoles of degraded *32^P*-DNA, 30 μmoles of glycine buffer, pH 8.5, 3 μmoles of MgCl₂, and 1.7 units of 5'-nucleotidase. After incubation for 30 minutes at 37°, the *32^P* which did not adsorb to Norit was determined as for the alkaline phosphatase.

Descending paper chromatography was carried out with the 1-propanol-ammonia-water solvent of Hanes and Isherwood (24) for 50 to 60 hours at room temperature. In the case of RNA digests, all four mononucleotides were well separated by this solvent. These were quantitatively estimated by elution from paper chromatograms with distilled water and measurement of ultraviolet absorption against appropriate blanks. *A₂₆₀∶A₂₈₀ and *A₂₆₀∶A₂₈₀ were checked in order to assure purity of the spots. In the case of DNA digests, dCMP was well resolved, but dAMP and dTMP were nearly coincident and dGMP was contaminated with about 15% dinucleotides. The dGMP was purified further by paper electrophoresis, and, when indicated, dTMP and dCMP were also resolved electrophoretically. Paper electrophoresis was carried out at room temperature by the method of Markham and Smith (25) in 0.05 M sodium citrate, pH 3.3, at a potential of 1100 volts for 2 hours. The amounts of mono- and oligonucleotides were then measured either with a Vanguard Autoscaner 800 chromatogram scanner or by elution of the spots with water and determination of their radioactivity with a Nuclear-Chicago model D-17 gas flow counter equipped with a Mieromil window; results by the two methods were in good agreement.

**RESULTS**

**Activity of *N. crassa* Nuclease toward Native and Denatured DNA**

A comparison of activities with native and denatured DNA as substrates is shown in Table I. Under a variety of conditions, the activity with native DNA was about 2% of that found with denatured DNA. Furthermore, native DNA was degraded optimally at acid pH or in the presence of Mn++, while denatured DNA was degraded optimally in the presence of Mg++ at alkaline pH.

With crude extracts, optimal hydrolysis of native DNA was also observed in the presence of MnCl₂ and at pH 5.5. Partial purification of this activity showed it to be physically separable from the enzyme described here. It is therefore possible that the residual activity observed with native DNA in the most purified preparation is, in fact, due to contamination by another nuclease, and indeed the following lines of evidence indicate this to be the case.

### Table I

<table>
<thead>
<tr>
<th>pH</th>
<th>Cation added</th>
<th>Acid-soluble nucleotide formed</th>
<th>Denatured DNA</th>
<th>Native DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mmoles/30 min/unit enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>Mg++</td>
<td>1000</td>
<td>15 (16)</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>Mg++</td>
<td>128</td>
<td>18 (23)</td>
<td></td>
</tr>
<tr>
<td>8.2</td>
<td>Mg++</td>
<td>1180</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>Mn++</td>
<td>200</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>Mn++</td>
<td>111</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

*a* Values in parentheses were obtained with 0.44 unit of enzyme.
First, it was found that hydrolysis of native DNA was considerably more sensitive to the action of thiols. Thus, while addition of 0.003 M 2-mercaptoethanol to an assay mixture at pH 7.5 produced a 53% inhibition of the hydrolysis of denatured DNA by 0.014 unit of enzyme, it resulted in a 96% inhibition of the hydrolysis of native DNA by 0.55 unit of enzyme (Fig. 1). The residual activity observed with native DNA as substrate might have been due to contamination of the native DNA with denatured fragments, so that the actual extent of inhibition by the 2-mercaptoethanol could conceivably have been greater than the 96% observed. At pH 8.2 the effect of 2-mercaptoethanol was qualitatively the same, although both activities showed considerably greater sensitivity to this reagent.

Reduced glutathione at 0.003 M, pH 7.5, produced a similar differential inactivation, causing a 32% inhibition of the hydrolysis of denatured DNA by 0.014 unit of enzyme while inhibiting 92% of the activity of 0.55 unit of enzyme when native DNA was used as substrate. Similarly, the hydrolysis of native DNA was found to be more sensitive to the presence of p-chloromercuribenzoate, Cu++, or EDTA than the hydrolysis of denatured DNA. Inhibition by EDTA of the hydrolysis of native as well as denatured DNA could be overcome by addition of stoichiometric amounts of Co++.

Second, the two activities showed different rates of heat inactivation. When the enzyme preparation was incubated at 55° in 0.05 M Tris, pH 7.5, a more rapid loss of the activity with native DNA as substrate was observed than with denatured DNA. A stimulation of activity (10 to 20%) on denatured but not native DNA after 5 minutes of incubation at 55° was noted consistently (Fig. 2a). Incubation of the enzyme at 55° in 0.05 M Tris, pH 7.5, in the presence of 0.001 M 2-mercaptoethanol produced a similar differential inactivation, although both activities diminished at an increased rate (Fig. 2b). Heating under a variety of other conditions, including potassium phosphate at pH 6.5 in the presence or absence of 2-mercaptoethanol and Tris at pH 8.2, all produced a more rapid rate of inactivation of the activity observed with native DNA as substrate than with denatured DNA.

Third, the products formed from native DNA were predominantly mononucleotides in the early phases of hydrolysis, in contrast to the high proportion of oligonucleotides formed from denatured DNA at a comparable phase of hydrolysis (see below).

These observations, in addition to suggesting that the activity observed with native DNA as substrate is largely associated with an enzyme distinct from the one described here, provided a means of conferring on the N. crassa nuclease a high degree of specificity for denatured DNA. In order to minimize the complications due to denatured regions within native DNA molecules or to denatured fragments in preparations of native DNA, DNA from phage T7, which can be isolated as a relatively pure preparation, was used as substrate for the assay of activity.

3 Units of enzyme refer to the activity measured with denatured E. coli DNA as substrate (Assay A of the preceding paper (1)).

4 Unpublished observations.

5 The rate at which denatured T7 DNA is attacked by the N.
DNA had occurred yielding fragments that were not acid-soluble error (less than 0.1% of the rate found with denatured DNA). DNA had been reduced essentially to the limit of experimental criterion, the activity of the N. crassa nuclease on native T7 DNA was observed during this time. Moreover, at pH 8.2, even in the absence of 2-mercaptoethanol or with 0.4 unit in its presence, degradation of the native DNA was markedly reduced ethanol for 11 hours, essentially no formation of acid-soluble products, more than 20 units of enzyme per pmole of substrate was required. When native 32P-labeled T7 DNA was incubated under usual assay conditions with 0.02 unit of enzyme in the presence of 0.004 M 2-mercaptoethanol for 11 hours, essentially no formation of acid-soluble products was observed. With 0.02 unit of enzyme in the absence of 2-mercaptoethanol or with 0.4 unit in its presence, considerable degradation of the DNA was observed during this time. Moreover, at pH 8.2, even in the absence of 2-mercaptoethanol, degradation of the native DNA was markedly reduced (Table II). With the formation of acid-soluble products as a criterion, the activity of the N. crassa nuclease on native T7 DNA had been reduced essentially to the limit of experimental error (less than 0.1% of the rate found with denatured DNA). The possibility that limited endonucleolytic action upon native DNA had occurred yielding fragments that were not acid-soluble

<table>
<thead>
<tr>
<th>Enzyme added</th>
<th>2-Mercaptoethanol</th>
<th>pH</th>
<th>Acid-soluble nucleotide formed in 30 min</th>
<th>60 min</th>
<th>180 min</th>
<th>360 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>unit</td>
<td>-</td>
<td>7.5</td>
<td>0.69</td>
<td>0.66</td>
<td>1.34</td>
<td>10.33</td>
</tr>
<tr>
<td>0.02</td>
<td>-</td>
<td>8.2</td>
<td>0.85</td>
<td>0.78</td>
<td>1.33</td>
<td>1.33</td>
</tr>
<tr>
<td>0.02</td>
<td>+</td>
<td>7.5</td>
<td>0.70</td>
<td>0.50</td>
<td>0.67</td>
<td>0.70</td>
</tr>
<tr>
<td>0.40</td>
<td>+</td>
<td>7.5</td>
<td>0.45</td>
<td>0.61</td>
<td>2.06</td>
<td>31.5</td>
</tr>
</tbody>
</table>

**Table II**

**Time course of hydrolysis of native DNA**

Conditions were as described for Table I except that 32P-labeled phage T7 DNA was used and 120 mmoles of 2-mercaptoethanol were added where indicated. Denatured DNA under all conditions shown would have been made at least 75% acid-soluble.

**Table III**

*Hydrolysis of phage λ DNA measured by zone sedimentation in alkali*

Reaction mixtures (0.2 ml) contained 20 μoles of Tris, pH 7.5 (Experiments 1, 2, and 3) or 8.2 (Experiment 4); 2 μoles of MgCl2; 16 μoles of native λ DNA; 0.8 μole of 2-mercaptoethanol; and enzyme as indicated. Incubation was conducted for 30 minutes at 37°C, after which 20 μl of 0.2 M EDTA were added to stop the reaction. The digests were then dialyzed for 18 hours against 0.05 M NaCl-0.01 M Tris, pH 8.0, containing 10 μ M EDTA. The samples were analyzed by zone sedimentation as described under "Methods." The control sample was treated similarly, except that EDTA was added immediately after addition of the enzyme and there was no incubation. Parallel incubation mixtures containing denatured 32P-labeled E. coli DNA yielded products which were 60 to 70% acid-soluble in all cases.

**Table IV**

*Hydrolysis of denatured DNA*

The reaction mixture contained (in 1.2 ml) 120 μoles of Tris, pH 7.5, 12 μoles of MgCl2, 93 mmoles of denatured, 32P-labeled E. coli DNA, and 0.03 unit of enzyme. At the times indicated, 0.1 ml samples were removed and acid-soluble 32P was determined.

**Fig. 3. Time course of hydrolysis of denatured DNA.** The reaction mixture contained (in 1.2 ml) 120 μoles of Tris, pH 7.5, 12 μoles of MgCl2, 93 mmoles of denatured, 32P-labeled E. coli DNA, and 0.03 unit of enzyme. At the times indicated, 0.1 ml samples were removed and acid-soluble 32P was determined.

**Rate and Extent of Hydrolysis of Denatured DNA**

Hydrolysis of denatured DNA proceeded at a linear rate until 60 to 70% of the substrate was made acid-soluble. Then a second phase began, in which degradation continued at a much diminished rate (Fig. 3). During the initial phase the rate of reaction was proportional to the amount of enzyme added; however, in the second phase both the rate and the final extent of acid solubility were dependent upon, but not directly proportional to, the amount of enzyme present. To convert the denatured DNA quantitatively (more than 98%) to acid-soluble DNA to fragments of which 60% are acid-soluble. Furthermore, under these conditions, as shown by sedimentation of the treated DNA in 1 M NaCl at pH 7.5, there was little if any degradation of the double stranded structure of the λ DNA molecules. At pH 8.2 in the absence of 2-mercaptoethanol, a significant number of molecules also remained intact.

Rate and Extent of Hydrolysis of Denatured DNA

Hydrolysis of denatured DNA proceeded at a linear rate until 60 to 70% of the substrate was made acid-soluble. Then a second phase began, in which degradation continued at a much diminished rate (Fig. 3). During the initial phase the rate of reaction was proportional to the amount of enzyme added; however, in the second phase both the rate and the final extent of acid solubility were dependent upon, but not directly proportional to, the amount of enzyme present. To convert the denatured DNA quantitatively (more than 98%) to acid-soluble products, more than 20 units of enzyme per μ mole of substrate were required.

E. coli DNA denatured by treatment with either alkali or formamide was attacked at the same rate as heat-denatured DNA; moreover, the linear phase of hydrolysis proceeded to the same point (60 to 70% acid-soluble products) (Table IV).
The rates at which heat-denatured DNAs from a variety of sources were degraded were very nearly equivalent (Table IV).

TABLE IV
Effect of source of DNA and method of denaturation on rate of hydrolysis

DNAs were denatured as described under "Methods." E. coli DNA was labeled with $^{32}$P; the other DNAs were unlabeled. Several levels of enzyme were used in each case to obtain an estimate of the point at which the initial rate of hydrolysis terminated. Assays measured $^{32}$P or ultraviolet-absorbing material made acid-soluble as described under "Methods."

<table>
<thead>
<tr>
<th>DNA source</th>
<th>Denaturation method</th>
<th>Acid-soluble nucleotide formed</th>
<th>% of enzyme at initial rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Heat</td>
<td>1.00</td>
<td>60-70</td>
</tr>
<tr>
<td>E. coli</td>
<td>Formamide</td>
<td>0.97</td>
<td>60-70</td>
</tr>
<tr>
<td>E. coli</td>
<td>pH 12</td>
<td>1.00</td>
<td>60-70</td>
</tr>
<tr>
<td>Phage T7</td>
<td>Heat</td>
<td>1.00</td>
<td>60-70</td>
</tr>
<tr>
<td>Phage λ</td>
<td>Heat</td>
<td>0.63</td>
<td>30-40</td>
</tr>
<tr>
<td>Phage T4</td>
<td>Heat</td>
<td>1.08</td>
<td>40-50</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>Heat</td>
<td>1.20</td>
<td>60-70</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>Heat</td>
<td>1.06</td>
<td>60-70</td>
</tr>
</tbody>
</table>

Thus DNAs from E. coli, salmon sperm, calf thymus, and phages T7 and T4 were attacked at the same initial rate, although the extent of hydrolysis of the T4 DNA at the end of the first phase was somewhat lower. Phage λ DNA was hydrolyzed at a lower initial rate, and to a lesser extent at this rate. It is possible that the greater resistance to hydrolysis of T4 and λ phage DNAs was due to renaturation after heating.

Rate of Hydrolysis of Synthetic Polydeoxyribonucleotides

As might be anticipated from the resistance of native DNA to the nuclease, the rate at which enzymatically synthesized poly- deoxynucleotides were attacked was related to the extent to which these polymers possessed an ordered structure (Table V). Thus dC in the presence or absence of magnesium ion and dI in the absence of magnesium ion are in the form of random coils and were hydrolyzed at rates comparable to that observed for denatured DNA. On the other hand, dAT and dI under conditions in which the helical forms predominate were degraded at the low rates characteristic of native DNA, while dG and dGdC, which possess highly ordered structures, appeared to be totally resistant to the nuclease.

At 37° in the absence of magnesium ion, dIdC is presumably in the range of the helix to coil transition, and indeed under these conditions it was attacked at a lower rate than would be expected for a mixture of the individual homopolymers. However, the greater susceptibility of dI as compared with dC was reflected in a predominance of deoxyinosine nucleotides in the acid-soluble products. At 27°, where the structure of dIdC is presumably helical, the same disproportion of products was noted; furthermore, the $Q_{10}$ between 27° and 37° was no different from that found with denatured DNA. On the other hand, in the presence of magnesium ion, dIdC was attacked at an extremely low rate. In this case the hydrolysis products were composed predominantly of deoxythymidine nucleotides, in agreement with the greater susceptibility of the dC as compared with dI under these conditions.

Hydrolysis of Deoxythymidine Oligonucleotides

In order to determine the effect of chain length on enzymatic rate, a series of deoxythymidine oligonucleotides terminated by a 5'-phosphomonoester group was exposed for 30 minutes to 1 unit of enzyme per $\mu$ mole of deoxythymidine and then examined for degradation by paper chromatography (Fig. 4). The trimer remained undegraded under these conditions, while the pentamer exhibited perhaps a trace of degradation. On the other hand, the hexamer was degraded, yielding smaller oligonucleotides.

Acetylation of the terminal 3'-hydroxyl group of the hexamer did not significantly alter the extent of its hydrolysis, but phosphorylation of the terminal 3'-hydroxyl group of the pentamer yielded a substrate which was hydrolyzed to the extent characteristic of the hexamer (Fig. 4). Removal of the terminal 5'-phosphate from either the hexamer, heptamer, or octamer yielded products which showed no degradation under the conditions noted above. The relative insusceptibility of these large oligonucleotides lacking phosphomonoester groups is curious, since neither the pretreatment of DNA with phosphatase nor the inclusion of large excesses of phosphatase in reaction mixtures in which DNA served as substrate had any effect on the rate of hydrolysis of the DNA as measured by the production of acid-soluble $^{32}$P.

These studies have thus yielded two further characteristics of
the enzyme which were not obvious from the assays of acid-solubility: namely, the stimulation of the rate of hydrolysis of small oligonucleotides by a terminal phosphate group, and the dependence of reaction rate upon size of the substrate. The failure of the enzyme to degrade the smaller fragments presumably reflects a low rate of reaction rather than a complete resistance to enzymatic attack, since exhaustive degradation of DNA (see below) yields all possible mononucleotides and only a small proportion of dinucleotides.

**Identification of Products of Digestion of Denatured DNA**

As depicted in Fig. 3, hydrolysis of denatured DNA as judged by the formation of acid-soluble products proceeded in two phases, an initially rapid phase reaching 60 to 70% conversion of the DNA to acid-soluble products and a second, slower phase in which the DNA could become more than 98% acid-soluble. A more complicated pattern of digestion was revealed, however, when the susceptibility of products of 5'-nucleotidase or bacterial alkaline phosphatase was used as the criterion for the extent of the digestion (Table VI). It was found that at the point where digestion at the initial rate was complete (about 70% acid-soluble products), approximately 4.5% of the phosphate of the digest was sensitive to 5'-nucleotidase, a level relatively independent of the amount of enzyme used. Then a secondary phase of digestion, corresponding to the secondary phase as judged by tests of acid solubility, was observed; in this case, the level of phosphate sensitive to 5'-nucleotidase was dependent upon the amount of enzyme present. Finally, a third phase was noted, considerably slower than the first two, wherein the susceptibility of the products to 5'-nucleotidase exceeded 80% after prolonged (overnight) incubations.

To lessen the possibility that the contaminating nuclease (see above) was responsible for some of the hydrolysis observed (particularly during the third phase of digestion), conditions were used which might tend to minimize the action of this enzyme. Incubation at 55°C actually increased the extent of digestion of the DNA (Table VI). Incubations at pH 8.2 or in 0.004 M 2-mercaptoethanol under standard assay conditions yielded digests which did not differ significantly from Experiment 1 in Table VI. Incubation in the presence of 2-mercaptoethanol at 55°C resulted in inactivation of the enzyme.

**Table VI**

Susceptibility to alkaline phosphatase and 5'-nucleotidase of products of digestion of DNA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme added</th>
<th>Time of incubation</th>
<th>Phosphatase-sensitive products</th>
<th>5'-Nucleotidase-sensitive products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/μmole substrate</td>
<td>hrs</td>
<td>% total</td>
<td>% total</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>4.5</td>
<td>58 (61)</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>1</td>
<td>27 (33)</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>1</td>
<td>52</td>
<td>82 (82)</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>2</td>
<td>72</td>
<td>92 (95)</td>
</tr>
</tbody>
</table>

The values in parentheses were obtained after further incubation for 1 hour with an additional 25 units of enzyme per μmole of substrate.

The values in parentheses were obtained after further incubation for 4 hours with an additional 5 units of enzyme per μmole of substrate.

The values in parentheses were obtained after further incubation for 5 hours with an additional 16 units of enzyme per μmole of substrate.

The use of 2-mercaptoethanol was complicated by the observation that, when high levels of enzyme were used, it was necessary to raise the concentration of the 2-mercaptoethanol above 0.004 M in order to observe any effect on the degradation of either native or denatured DNA. The effect of 2-mercaptoethanol appeared to depend upon the ratio of the quantity of 2-mercaptoethanol to the...

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**Fig. 4. Hydrolysis of deoxythymidine oligonucleotides.** The reaction mixtures ranged in volume from 25 to 125 μl and contained 0.1 M Tris, pH 7.5, 0.01 M MgCl₂, 0.25 to 0.30 pmole of oligonucleotide deoxythymidine, and 1.0 unit of enzyme per pmole of substrate. After 30 minutes at 37°C, the reaction mixtures were transferred quantitatively to Whatman No. 3MM paper and chromatographed for 53 to 58 hours as described under "Methods." The column at the left depicts the positions of known oligonucleotides; the other columns show the products formed from the oligonucleotides after treatment with the enzyme. In all cases, controls with enzyme omitted showed no detectable degradation. Dotted lines indicate the existence of trace amounts of products. Chromatographic characteristics of the various oligonucleotides reflected the number of charged phosphates of the molecule. Thus (pT)₄ acetate (OAc) and T(pT)₃ show the same behavior as does (pT)₄; T(pT)₃ chromatographs in the same manner as does (pT)₄, and T(pT)₃ migrates like (pT)₄. As would be expected, (pT)₄p moves at a somewhat slower rate than (pT)₄.
Identification of products of digestion of denatured DNA

Reaction mixtures for Experiments 1, 4, and 5 contained (in 0.3 ml) 24 mmoles of 32P-labeled denatured E. coli DNA, 3 μmoles of MgCl₂, and 30 μmoles of Tris buffer (pH 7.5 for Experiments 1 and 5 and pH 8.2 for Experiment 4); 1.2 μmoles of 2-mercaptoethanol were added in Experiment 5. Reaction mixtures for Experiments 2 and 3 were the same as those described for Experiments 2 and 5, respectively, in Table VI. In Experiments 1 through 3, after incubation at 37°C for the times indicated, the digests were quantitatively applied to Whatman No. 3MM paper, chromatographed in the 1-propanol-ammonia-water system, and then subjected to paper electrophoresis as described under "Methods." In Experiments 4 and 5, following incubation, 0.2 ml of each digest was applied to paper along with 0.1 μmole of each of the four deoxyribonucleoside 5'-monophosphates and chromatographed. The amounts of 32P in the products were determined directly as described under "Methods," except for the ">mononucleotide" values of Experiments 4 and 5, which were determined by the difference between the total 32P applied to the paper and that appearing in the mononucleotide fractions.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme added</th>
<th>Time of incubation</th>
<th>Products</th>
<th>% total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/μmole substrate</td>
<td>hrs</td>
<td>dCMP</td>
<td>dAMP</td>
</tr>
<tr>
<td>1</td>
<td>0.4</td>
<td>1</td>
<td>0.3</td>
<td>0.4</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>1</td>
<td>3.5</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>35^c</td>
<td>2</td>
<td>4.8</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>24</td>
<td>15.5</td>
<td>20.0</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>3</td>
<td>0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

^a Values for components larger than dinucleotides are somewhat arbitrary, since guanine-containing oligonucleotides would be retarded compared to those without guanine.
^b Composed of at least six major components.
^c Initially, 35 units per μmole of substrate were present; after 20 hours an additional 5 units per μmole were added.
^d Composed of two major dinucleotide and one major trinucleotide component.

Examination of nucleotides formed in the course of hydrolysis showed that throughout all phases of the digestion of denatured E. coli DNA, the most obvious characteristics of the digests were a large preponderance of dGMP and a relatively small amount of dCMP (Table VII). This pattern did not alter appreciably until essentially all of the guanine residues within the DNA were converted to dGMP, after which the relative amounts of dAMP and dTMP in the mononucleotide fraction increased. The deoxycytidine residues within the DNA were the last to appear as mononucleotides; when more than 90% of the DNA had been converted to mononucleotides, the small core of di- and trinucleotides remaining was composed mainly of deoxycytidine residues.

The disproportionately large amount of dGMP produced, particularly early in the course of digestion, suggested that perhaps the enzyme could under certain conditions be made specific for these residues. However, incubation at pH 8.2 or in 0.004 M 2-mercaptoethanol, conditions which virtually eliminated the contaminating nuclease, again had little effect on the pattern of the digests (Table VII).

The mononucleotides formed very early during digestion with relatively small amounts of enzyme were also examined (Fig. 5). Although at all stages in the digestion there was a large predominance of dGMP and a relatively small amount of dCMP, there was no point at which dGMP was the sole mononucleotide formed. It must be concluded, therefore, that the enzyme has a distinct preference for deoxyguanosine residues within the polydeoxyribonucleotide chain, but that this preference is not absolute.

Measurement of the course of digestion by an examination of oligonucleotide formation (Table VII) was in good agreement with the values obtained by measurement of formation of alkaline phosphatase- and 5'-nucleotidase-sensitive phosphate (Table VI). Furthermore, these studies confirmed the endonucleolytic mode of attack suggested by the analysis of the products formed by hydrolysis of the deoxycytidine oligonucleotides. Finally, it would appear that the distribution of products and the over-all course of hydrolysis observed were due primarily and perhaps exclusively to the enzyme described here; there was little if any contribution by the contaminating nuclease. The diminishing rates of hydrolysis observed were therefore most likely due to the decreased susceptibility to the nuclease of the progressively smaller fragments produced as hydrolysis proceeded.

Hydrolysis of Single Stranded Regions of Double Stranded DNA

The fact that the enzyme appeared to be an endonuclease specific for single stranded DNA suggested that it might be able...
to remove single stranded regions from double stranded DNA. Such regions can be formed with the use of E. coli exonuclease III, an enzyme which catalyzes the stepwise hydrolysis of the DNA starting from the 3'-hydroxyl ends of the chains leaving the complementary 5'-terminal ends as single strands attached to the undegraded native region (17, 27). Further treatment of the DNA with the \( N. \) crassa nuclease should then remove the residual single stranded 5'-terminal ends, leaving a shortened double stranded molecule.

Native \(^{32}P\)-labeled T7 phage DNA was treated with varying amounts of exonuclease III so that the extent of hydrolysis ranged from 0.08 to 15% of the total nucleotide equivalents removed. The former value is equivalent to an average of about 25 nucleotides removed from each strand of the native DNA (on the assumption of a molecular weight of about \( 19 \times 10^6 \)) (28). In every case, further treatment of the DNA with the \( N. \) crassa nuclease resulted in an amount of material made acid-soluble which was comparable to that removed by the treatment with exonuclease III (Fig. 6). The value of the slope (0.86) is consistent with the fact that under conditions used in this experiment, single stranded DNA is degraded by the enzyme to the point where 88% of the products are acid-soluble. Within a mean experimental error of about 10 nucleotides per DNA strand, these results indicate that the \( N. \) crassa enzyme is able to remove a number of nucleotides equal to that initially removed by exonuclease III.

That these results were not dependent upon the amount of \( N. \) crassa enzyme added was shown by an experiment in which native DNA was hydrolyzed approximately 2% by exonuclease III, then incubated with amounts of \( N. \) crassa enzyme ranging from 1 to 4 times the level used above. Although the blank
sRNA was attacked at about 40% the rate of ribosomal RNA.

For both substrates, in the presence of 0.01 mM magnesium ion, sRNA from E. coli was hydrolyzed at about 70% the rate of ribosomal RNA, compared to 1.6% found for the rate of hydrolysis of dAT or native DNA versus denatured DNA. It thus appears that the ribonuclease activity of the N. crassa nuclease shows the same high degree of selectivity for random coil polynucleotides as does its deoxyribonuclease activity.

The mononucleotides were shown to contain 5' phosphomonoester groups by their susceptibility to the action of 5'-nucleotidase and by their chromatographic behavior in the 1-propanol-ammonia-water system, in which nucleoside 3' and 5'-monophosphates are easily resolved. Thus, the pattern of products formed from ribosomal RNA by the N. crassa nuclease was qualitatively the same as that from DNA. Both contain nucleoside 5'-monophosphates among which there is a large predominance of the guanosine nucleotide and a low level of cytidine nucleotide. Furthermore, both RNA and DNA require large excesses of enzyme in order that a high proportion of mononucleotides and small oligonucleotides be formed.

A similar qualitative analysis of digests of sRNA showed patterns which did not differ greatly from those obtained with ribosomal RNA.

**Hydrolysis of RNA**

As shown in the preceding paper (1), the N. crassa nuclease will attack ribosomal RNA at approximately the same rate as denatured DNA. Moreover, the conditions which were optimal for hydrolysis of RNA were generally those giving maximal rates of hydrolysis of denatured DNA. The conclusion reached was that a single enzyme is responsible for both activities.

Hydrolysis of sRNA—At 37° and in the absence of added cations, sRNA from E. coli was hydrolyzed at about 70% the rate of ribosomal RNA, the dependence upon pH being the same for both substrates. In the presence of 0.01 mM magnesium ion, sRNA was attacked at about 40% the rate of ribosomal RNA.

At 27°, sRNA was hydrolyzed at one-tenth the rate observed at 37°; addition of 0.01 mM Mg++ inhibited the rate at 27° by about 50%. At 19°, sRNA was hydrolyzed at less than 1% the rate observed at 37°. At 27°, the initial rate of hydrolysis diminished after about 20% of the substrate had been converted to acid-soluble products, but there was no indication that any discrete "limit" short of complete hydrolysis existed. The rate of hydrolysis of sRNA at 19° was one-seventh the rate found for denatured DNA.

**Analysis of Products of Digestion of RNA**—The products of a digest of ribosomal RNA were analyzed in the same manner as the products of digestion of DNA. Incubation of the RNA for 60 minutes with 14 units of enzyme per pmole of substrate yielded a digest containing 23% GMP, 5.9% UMP, 16.5% AMP, and 1.7% CMP; the remainder of the digest was composed mainly of di-, tri-, tetra-, and pentanucleotides. Incubation of the RNA with 1.4 units of enzyme per pmole of substrate for 30 minutes yielded a digest containing no detectable (less than 0.5%) CMP, a trace of UMP, and significant amounts of AMP and GMP. The remainder of the digest was mainly in the form of tetra-, penta-, and larger oligonucleotides.

The mononucleotides were shown to contain 5' phosphomonoester groups by their susceptibility to the action of 5'-nucleotidase and by their chromatographic behavior in the 1-propanol-ammonia-water system, in which nucleoside 3' and 5'-monophosphates are easily resolved. Thus, the pattern of products formed from ribosomal RNA by the N. crassa nuclease was qualitatively the same as that from DNA. Both contain nucleoside 5'-monophosphates among which there is a large predominance of the guanosine nucleotide and a low level of cytidine nucleotide. Furthermore, both RNA and DNA require large excesses of enzyme in order that a high proportion of mononucleotides and small oligonucleotides be formed.

A similar qualitative analysis of digests of sRNA showed patterns which did not differ greatly from those obtained with ribosomal RNA.

**Discussion**

The nuclease described here and in the preceding paper constitutes a complementary enzyme to the exonuclease I (phosphodiesterase) from E. coli. Both enzymes show an almost complete specificity for polynucleotides lacking an ordered structure, and it appears that in both instances the low level of activity observed with native DNA as substrate to some extent reflects the action of these enzymes on denatured or single stranded regions within native DNA molecules. The important difference between these two enzymes which permits them to complement each other as reagents for studying polynucleotide structure is their mode of attack; the E. coli enzyme is strictly
an exonuclease, while the \textit{N. crassa} nuclease is clearly an endo-
nuclease. The \textit{N. crassa} enzyme is, therefore, similar to the 
enzyme isolated from \textit{Amanita muscaria} by Healy, Stollar, Simon, and 
Levine (29). It is an endonuclease which is highly selective for 
denatured or single stranded DNA.

The enzyme preparation in its current state of purification 
still contains detectable levels of another activity which is able 
to attack native DNA; however, the observed hydrolysis of 
native DNA appears to be largely, if not entirely, due to a con-
taminating enzyme (which can be differentially inactivated) 
rather than to an intrinsic property of the enzyme described 
here.

The observed inhibition of nuclease activity by relatively low 
concentrations of thiols is not without precedent; similar effects 
were noted for venom diesterase by Razzell and Khorana (20). 
The inhibition might be due either to reduction of peculiarly 
susceptible disulfide bridges within the enzyme, or alternatively 
to binding by the thiol of a cation (presumably Co$^{2+}$). The 
stimulatory effect of oxidized glutathione noted previously (1) 
supports the former alternative, but the extreme sensitivity of 
the enzyme to EDTA (1) suggests that metal binding might be 
involved.

The results of the study of enzyme action upon defined oligo-
nucleotides resembled in many respects a similar study of pan-
creatic DNase by Ralph, Smith, and Khorana (30). Thus, under the 
conditions used, the \textit{N. crassa} nuclease attacked hexanucleo-
tides bearing terminal 3'-phosphates, but not smaller homologues;
pancreatic DNase was reported to attack tetranucleotides bear-
ing terminal 5'-phosphates, but not smaller homologues. Fur-
thermore, both enzymes preferentially cleaved internal bonds, 
producing relatively few mono- and dinucleotides. Finally, the 
observation that \textit{d(pT)}$_3$ was attacked at about the same rate 
as \textit{d(pT)}$_n$, is analogous to the observation made by Ralph, Smith, 
and Khorana (30) and by Vanek and Laskowski (31) that a 
3'-phosphomonoester group simulates a phosphodiester bond for 
pancreatic DNase action.

The resistance to the \textit{N. crassa} nuclease of oligonucleotides 
without terminal phosphate groups is difficult to explain in view 
of our inability to observe similar effects when DNA treated 
with a large excess of phosphatase was used as substrate. One 
must assume that the absence of a terminal phosphate group is 
inhibitory only in the case of smaller, acid-soluble substrates. 
It would indeed be surprising if the presence of a terminal phos-
phate group on a substrate as long as a DNA chain could in-
fluence the rate of endonucleolytic attack on the molecule unless 
degradation proceeded stepwise from the end of the molecule.
The action of pancreatic DNase on similar dephosphorylated 
oligonucleotides has not been reported.

Another interesting feature of the \textit{N. crassa} nuclease is its 
relatively high degree of specificity for diester bonds involving 
guanosine or deoxyguanosine residues. During the initial stages 
of hydrolysis of denatured DNA, the level of deoxyguanosine 
5'-phosphate was about 3-fold greater than the sum of the other 
three deoxyribonucleotides. The same result was noted in di-
gests of ribosomal RNA.

There are at least two possible explanations for this result. 
One is that the \textit{N. crassa} nuclease preparation contains both an 
endonuclease and a nonspecific exonuclease component. The 
endonucleolytic attack would invariably produce oligonucleo-
tides terminated by deoxyguanylate, while the presumed exo-
nucleolytic component would then attack these oligonucleotides 
to yield first deoxyguanosine 5'-phosphate and then, as the exo-
nucleolytic attack proceeded, the other mononucleotides. The 
net effect of such a mechanism would be to produce a relatively 
high proportion of deoxyguanylate early in the course of hydroly-
sis which would gradually diminish in the later phases. A 
major difficulty with this hypothesis is the disproportionately 
low level of deoxyctydylate 5'-phosphate formed, particularly 
during the very early phases of hydrolysis. Since nearest neigh-
bor frequency analyses of \textit{E. coli} DNA (32) have shown the 
diminution sequence, \textit{pCpG}, to be rather common (6.7%), it 
would, therefore, be necessary to impose additional limitations 
on the nucleotie specificity, i.e. that attack to produce deoxy-
guanylate-terminated oligonucleotides occur at a very low rate 
when the residue immediately following deoxyguanosine in the 
polyribonucleotide chain happens to be deoxyctydylate, or, alter-
atively, that deoxyctydylate residues be relatively resistant to 
the exonucleolytic component of the nuclease preparation. 
A second difficulty with this hypothesis is the apparent absence 
of significant exonuclease activity in the purified nuclease prep-
olation. Thus, hydrolysis of deoxythymidylate oligonucleotides by 
the enzyme did not, within the sensitivity of the chromatographic 
assay employed, produce any mononucleotides; the products 
formed from the larger oligonucleotides being the tetramer, the 
trimer, and dimer. On the other hand, the observation that 
with sufficient enzyme and long periods of incubation (20 to 24 
hours) DNA can in fact be reduced almost entirely to a mixture 
of mononucleotides might indicate the presence of an exonuclease 
the level of deoxyctydylate early in digestion suggest that 
diester bonds involving these residues are relatively resistant to 
hydrolysis.

As a second model to explain the preponderance of deoxy-
guanylate among the products of the early phases of digestion, 
one might propose that the enzyme is characterized exclusively 
by an endonucleolytic mechanism which has a high degree of 
preference, but not absolute specificity, for deoxyguanosine resi-
dues within the polynucleotide. Since all four mononucleotides 
are found among the products of digestion, one would then 
postulate that cleavage can take place at diester bonds involving 
any of the residues, and that the relative frequencies of the 
mononucleotides found simply reflect the relative susceptibility 
of these bonds to the endonuclease. The extremely slow, but 
steady, rate of formation of mononucleotides at the end of a 
digestion would not be due to a contaminating exonuclease, but 
rather to the slow hydrolysis of polynucleotides which have 
limited susceptibility because of (a) their small size and (b) their 
content of relatively insusceptible bonds. The observations 
that at the limit of digestion the oligonucleotides remaining 
are extremely rich in deoxyctydylate and that there is a disproporti-
ately low level of deoxyctydylate early in digestion suggest that 
diester bonds involving these residues are relatively resistant to 
hydrolysis.

In contrast to the extensive literature which describes the 
genetics of \textit{N. crassa}, very few studies of nucleic metabolism of 
the organism have appeared. A notable exception is the study 
of Suskind and Bonner (33), in which these authors described a 
ribonuclease in mycelia from this organism. The relationship, 
if any, of their nuclease to the one described here is not clear.

Thus, while their enzyme was also maximally active at pH 7.5 
and at 55\(^\circ\), Suskind and Bonner found maximal activity at 
concentrations of potassium phosphate which resulted in virtually 
complete inhibition of the enzyme described here.

Several attempts have been made to demonstrate genetic 
transformation in \textit{N. crassa}. Although two of these had been
partially successful (34, 35), high frequencies of “transformants” have not as yet been obtained. It is possible that at least part of the difficulty may be due to the extremely high nuclease activity in extracts of N. crassa, a factor which might prevent the isolation of fully native DNA. The information derived from the present study, as well as studies concerned with the other nucleases of this organism, should be helpful in achieving a reproducible transformation system for N. crassa. Thus the enzyme described here is strongly inhibited by relatively low concentrations of EDTA, 2-mercaptoethanol, or phosphate buffer. Purification of the other nuclease or nucleases of this organism may yield similarly effective measures for their inhibition.

SUMMARY

The specificity and mode of attack of oligonucleotides and polynucleotides by the Neurospora crassa nuclease were examined. Those studies have established the following points.

The purified enzyme preparation attacks native deoxyribonucleic acid at 2% the rate of denatured deoxyribonucleic acid. Hydrolysis of native DNA is due largely to the activity of a contaminating nuclease which can be removed by a variety of treatments, including incubation at 55° or exposure to thiols. After such treatment, native DNA is attacked at less than 0.1% the rate found with denatured DNA. Similar results were observed with synthetic polynucleotides (ribo- and deoxyribo-) in the helical and random coil forms.

The action of the enzyme is predominantly, and may be exclusively, endonucleolytic in character; it will, therefore, remove single stranded regions from double stranded DNA.

Oligonucleotides composed of x numbers of residues of deoxythymidine 5'-phosphate (d(pT)x) are attacked at a significant rate for x ≥ 6. d(pT)4p and d(pT)x acetate are degraded at a rapid rate, but DT(pT)x, DT(pT)x, and DT(pT)x are relatively unreactive. The stimulatory effect of terminal phosphate groups cannot be detected with larger DNA substrates in which formation of acid-soluble fragments is the criterion for extent of degradation.

Analysis of the products of digestion indicates that the enzyme has a distinct preference, but not absolute specificity, for guanosine or deoxyguanosine residues within a polynucleotide.

REFERENCES

An Endonuclease from \textit{Neurospora crassa} Specific for Polynucleotides Lacking an Ordered Structure: II. STUDIES OF ENZYME SPECIFICITY
Stuart Linn and I. R. Lehman


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