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

II. STUDIES OF ENZYME SPECIFICITY*

Stuart Linn and I. R. Lehman

From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California

(Received for publication, September 25, 1964)

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 by the method of Kay, Simmone, and Doumes (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 Zubay (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 1.0 M KOH (final pH, 12.0 to 12.5) and incubating this mixture for 30 minutes at room temperature; 0.082 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 30% formamide in about a 3-fold concentration of the DNA.

Synthetic Polynucleotides—The copolymer dAT was prepared as described by Schachman et al. (8); the homopolymer dGdC was prepared according to Radding et al. (9); the homopolymers dIdC, dG, dC, and dI were prepared as described by Inman (10). Polyriboadenylate was purchased from the California Corporation for Biochemical Research, and the copolymer rAU (11) was a generous gift of Dr. M. Chamberlin. Ultracentrifugal analysis of the rAU as described by Chamberlin et al. (11) showed it to have an s20,w of 7.6 in 1 M NaCl. Before use, all polymers were dialyzed exhaustively against 0.02 M NaCl-0.001 M Tris, pH 7.5, to remove any EDTA which might have been present.

Nucleotides—All reference mononucleotides were purchased from the California Corporation for Biochemical Research. dAMP was prepared as described previously (12). The oligonucleotides d(pT)6, d(pT)7, d(pT)10, d(pT)3, and d(pT)10 and d(pT)3 were prepared as described by Khorana and Vizsolyi (13) and Turner and Khorana (14). De(dT)3 with a terminal 3'-acetyl group ((pT)3-acetate) was prepared as described previously (15); dephosphorylations of d(pT)6, d(pT)7, and d(pT)10 to form dT(pT)6, dT(pT)7, and dT(pT)10, respectively, were carried out with E. coli alkaline phosphatase as described previously (16).

Enzymes—The concentrated hydroxylapatite fraction of the N. crassa nuclease was used in all experiments to be described. E. coli exonuclease III (phosphocellulose pervaorate) was prepared according to the method of Richardson and Kornberg (17). The conditions used in the experiments to be described were not optimal for the activity of exonuclease III, and consequently the amount of enzyme to be used for each digestion had to be determined empirically. Alkaline phosphatase from E. coli (Lot 6123A), purchased from the Worthington Biochemical Corporation and assayed as previously described (18). It was found to have negligible diesterase activity (0.004% of its monoesterase activity) as measured by hydrolysis of the together; dG, dC, and dI, the homopolymers of deoxyguanylate, deoxythymidylate, and deoxyinosinate, respectively, bonding not specified; rAU, an alternating copolymer of adenylate and uridylicate; d(pT)x, refers to an oligonucleotide composed of x numbers of residues of deoxythymidine 5'-phosphate; d(pT)x-acetate is the hexamer, (pT)x-acetate, in which the 3'-hydroxyl group has been acetylated; dT(pT)y, dT(pT)z, and dT(pT)x refer to thymidine oligonucleotides containing 6, 7, and 8 residues, and lacking a terminal phosphomonoester group; dT(pT)y is the thymidine pentamer with both 3'- and 5'-phosphomonoester groups. sRNA is used to designate soluble ribonucleic acid.

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

1 The abbreviations used are: dAT, an alternating copolymer of deoxyadenylate and deoxythymidylate; dGdC, a polymer consisting of homopolymers of deoxyguanylate and deoxythymidylate, hydrogen-bonded together; dIdC, a polymer consisting of homopolymers of deoxyinosinate and deoxythymidylate, hydrogen-bonded together; dG, dC, and dI, the homopolymers of deoxyguanylate, deoxythymidylate, and deoxyinosinate, respectively, bonding not specified; rAU, an alternating copolymer of adenylate and uridylicate; d(pT)x, refers to an oligonucleotide composed of x numbers of residues of deoxythymidine 5'-phosphate; d(pT)x-acetate is the hexamer, (pT)x-acetate, in which the 3'-hydroxyl group has been acetylated; dT(pT)y, dT(pT)z, and dT(pT)x refer to thymidine oligonucleotides containing 6, 7, and 8 residues, and lacking a terminal phosphomonoester group; dT(pT)y 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.

1294
p-nitrophenyl ester of thymidine 5'-phosphate (20). 5'-Nucleotidase from venom of Bothrops atrox (21) was a generous gift of Dr. M. Laskowski, Sr., and was assayed by the method of Heppel and Hilmoe (22). One unit is defined as that amount of enzyme which forms 1 μmole of inorganic phosphate per hour under these conditions. The diesterase activity of the preparation as determined by hydrolysis of p-nitrophenylthymidine 5'-phosphate was less than 0.1% of the monoesterase activity. No hydrolysis of deoxyadenosine 3'-phosphate by the enzyme could be detected.

Unless otherwise noted, all concentrations of substrate are expressed per μmole of nucleotide.

Methods

Enzyme Assays—Assays of the N. crassa nuclease with 32P-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 μmolecs 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 perchloric 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 μm and 280 μm was determined by reading against a blank containing no enzyme with a Zeiss PMG II spectrophotometer. The blank was also read against distilled water in order to determine that the untreated substrate was totally acid-precipitable. The A₆₆₀ for the acid-soluble fragments produced from DNA was assumed to be 10.0, and the A₆₂₈₀ for the products formed from synthetic polynucleotides was assumed to be equal to that of its constituent mononucleotides at acid pH. The A₆₆₀: A₆₂₈₀ was determined in order to check against extraneous ultraviolet absorption and, in the case of the synthetic polynucleotides, 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 32P 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 32P which did not adsorb to Norit as described earlier (10). Blanks 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 μmolecs of degraded 32P-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 32P 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. A₁₆₅₀: A₂₈₀ and A₂₆₅₀: A₃₀₀ 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 dAMP 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 Autoscanner 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 Micronil 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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Denatured DNA</td>
<td>Native DNA²</td>
</tr>
<tr>
<td>----</td>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>7.5</td>
<td>Mg⁺⁺</td>
<td>1000 15 (16)</td>
</tr>
<tr>
<td>5.5</td>
<td>Mg⁺⁺</td>
<td>128 18 (23)</td>
</tr>
<tr>
<td>8.2</td>
<td>Mg⁺⁺</td>
<td>1180 10</td>
</tr>
<tr>
<td>7.5</td>
<td>Mn⁺⁺</td>
<td>200 23</td>
</tr>
<tr>
<td>5.5</td>
<td>Mn⁺⁺</td>
<td>111 23</td>
</tr>
</tbody>
</table>

* Values in parentheses were obtained with 0.44 unit of enzyme.

² 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-chloronemecurobenzoate, 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

---

3 Units of enzyme refer to the activity measured with denatured E. coli DNA as substrate (Assay A of the preceding paper (1)).
DNA had occurred yielding fragments that were not acid-soluble error (less than 0.1 y0 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 time. Moreover, at pH 8.2, even in the absence of 2-mercapto-ethanol, degradation of the native DNA was markedly reduced products was observed. With 0.02 unit of enzyme in the absence of 2-mercaptoethanol for 11 hours, essentially no formation of acid-soluble double stranded molecules to be intact.

3. 2-Mercaptoethanol added...
4. pH 8.2..

The possibility that limited endonucleolytic action upon native DNA had occurred yielding fragments that were not acid-soluble homogeneous species, was used as substrate. When native mP-labeled T7 DNA was incubated under usual assay conditions with 0.02 unit of enzyme in the presence of 0.004 x 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% the rate found with denatured DNA).

The possibility that limited endonucleolytic action upon native DNA had occurred yielding fragments that were not acid-soluble crassa nuclease is not significantly different from the rate observed with E. coli DNA (see below).

* When the DNA was examined by zone sedimentation as described in "Methods," it was found to contain a large number of scissions, presumably as a consequence of mP decay.

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

was also considered. The intactness of the single strands of native DNA after exposure to the nuclease was examined by observing sedimentation of the DNA in 0.9 m NaCl-0.1 m NaOH (Table III). After incubation of native phage λ DNA with the enzyme in the absence of 2-mercaptoethanol, essentially no single strands remained intact, suggesting that a relatively large number of endonucleolytic cleavages had occurred. However, in the presence of 0.004 m 2-mercaptoethanol, approximately 60% of those strands which were intact before exposure to the enzyme remained so after treatment, the amount of enzyme used being capable of degrading an equivalent amount of denatured 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 DNA, more than 20 units of enzyme per pmole of substrate was 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).


**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>% extent of hydrolysis 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>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 $\lambda$</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>

**TABLE V**

**Hydrolysis of synthetic polydeoxynucleotides**

Assays were performed as described under "Methods" with variations as indicated. Composition of the products was determined by analysis of the ultraviolet spectrum; in the case of $d$IdC the absorption of hypoxanthine was used in place of inosine, since the spectrum of the products formed from $d$1 was that of hypoxanthine. The rate of hydrolysis of E. coli DNA under these conditions is given in Table I.

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 $\lambda$ 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 $\lambda$ phage DNAs was due to renaturation after heating.

**Rate of Hydrolysis of Synthetic Polydeoxynucleotides**

As might be anticipated from the resistance of native DNA to the nuclease, the rate at which enzymatically synthesized polydeoxynucleotides were attacked was related to the extent to which these polymers possessed an ordered structure (Table V). Thus $d$C in the presence or absence of magnesium ion and $d$I 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, $d$AT and $d$I under conditions in which the helical forms predominate were degraded at the low rates characteristic of native DNA, while $d$G and $d$GdC, which possess highly ordered structures, appeared to be totally resistant to the nuclease.

At 37° in the absence of magnesium ion, $d$IdC 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 $d$I as compared with $d$C was reflected in a predominance of deoxynucleosine nucleotides in the acid-soluble products. At 27°, where the structure of $d$IdC 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, $d$IdC was attacked at an extremely low rate. In this case the hydrolysis products were composed predominantly of deoxycytidine nucleotides, in agreement with the greater susceptibility of the $d$C as compared with $d$I under these conditions.

**Hydrolysis of Deoxycytidine Oligonucleotides**

In order to determine the effect of chain length on enzymatic rate, a series of deoxycytidine oligonucleotides terminated by a 5'-phosphomonoester group was exposed for 30 minutes to 1 unit of enzyme per mmole of deoxycytidine and then examined for degradation by paper chromatography (Fig. 4). The trimer remained undegraded under these conditions, while the pentamer exhibited 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 phosphatase 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.

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

Table VI

<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></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>1</td>
<td>58 (64)a</td>
<td>27 (33)a</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>1</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>18</td>
<td>72 (77)c</td>
<td>92 (95)c</td>
</tr>
</tbody>
</table>

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

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

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

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.

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
Identification of products of digestion of denatured DNA

Reaction mixtures for Experiments 1, 4, and 5 contained (in 0.3 ml) 24 μmoles of 32P-labeled denatured E. coli DNA, 3 μmoles of MgCl₂, and 90 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 μ mole of each of the four deoxycytidylate 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>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>1</td>
<td>3.5</td>
<td>4.9</td>
</tr>
<tr>
<td>3</td>
<td>35†</td>
<td>2</td>
<td>4.8</td>
<td>10.2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>24</td>
<td>15.5</td>
<td>20.0</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>1</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>3</td>
<td>0.3</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 deoxythymidine 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 32P-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 x 106) (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

![Fig. 5. Formation of mononucleotides during the course of digestion of denatured DNA. The reaction mixture (2.5 ml) contained 220 μmoles of Tris, pH 8.2, 25 μmoles of MgCl2, 300 μmoles of denatured 32P-labeled E. coli DNA, and 0.2 unit of enzyme. After incubation at 37° for the times indicated, 0.15-ml samples were removed for determination of acid-soluble 32P, and 0.15 ml was spotted on paper along with 0.1 μ mole of each of the four deoxyribonucleotide 5'-monophosphates to act as "carriers." Determinations of acid-soluble 32P and identification of the mononucleotides were carried out as described under "Methods." The levels of acid-soluble 32P at 0, 30, 60, 120, 240, and 480 minutes were 0.1, 0.3, 3.2, 8.4, 80.1, 85.7, and 90.6% of the total, respectively. Addition of another 0.2 unit of enzyme to the reaction mixture which remained, followed by further incubation for 1 hour, showed no significant change in the relative amount of the individual nucleotides, although the levels of all four of the mononucleotides rose by about 15%.

![Fig. 6. Effect of pretreatment of T7 DNA with E. coli exonuclease III on its susceptibility to the N. crassa nuclease. The results shown are derived from several different experiments. Duplicate reaction mixtures (0.55 ml), containing 51 μmoles of 32P-labeled T7 DNA, 60 μmoles of Tris, pH 7.5, 0.6 μ mole of MgCl2, 2.4 μ moles of 2-mercaptoethanol, and varying levels of exonuclease III producing the extents of digestion indicated, were incubated for 30 minutes at 37°. They were then heated for 10 minutes at 55° in order to inactivate the exonuclease III, and 5 μ moles of MgCl2 (in 0.05 ml) were added to each mixture. To one of each of the duplicate reaction mixtures was added 0.05 unit of N. crassa nuclease in 0.01 ml, and to the other mixture 0.01 ml of distilled water, and both reaction mixtures were incubated for an additional 30 minutes at 37°. The mixtures were then chilled, 0.2 ml of carrier DNA and 0.25 ml of 0.7 N perchloric acid were added, and acid-soluble 32P was determined as described under "Methods." The absence gives the level of acid-soluble 32P in those reaction mixtures to which distilled water had been added for the second 37° incubation; the ordinate gives the difference between the percentage made acid-soluble in mixtures incubated with both enzymes and the percentage made acid-soluble in mixtures incubated with exonuclease III only. The values found for samples treated only with exonuclease III have been corrected for blanks in which exonuclease III had also been replaced by distilled water. This correction (six determinations) was found to be 0.033 ± 0.005%. The values found for the samples to which both enzymes were added were corrected for a control to which the N. crassa enzyme was added, but from which the exonuclease III was omitted. This control, which provided a correction for any denatured DNA in the preparation of native DNA, gradually increased as the preparation aged from 0.01 to 0.18%, presumably as a result of 32P decay. Measurement of inorganic 32P formed as a result of the phosphatase activity of exonuclease III as described by Richardson and Kornberg (17) showed it to be negligible (0.001%).]
sRNA was attacked at about 40% the rate of ribosomal RNA for both substrates. In the presence of 0.01 M magnesium ion, the dependence upon pH being the same except that which was added (Fig. 7). The reaction for the blank, the additional degradation due to the N. crassa nuclease was independent of the level of this enzyme contaminant in the purified N. crassa preparation, after correction for the blank, the additional degradation due to the N. crassa nuclease was independent of the level of this enzyme which was added (Fig. 7).

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 M 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 M 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. Hydrolysis of Synthetic RNA Polymers—In the absence of magnesium ion, polyadenyl acid, a single stranded polymer, was hydrolyzed at 2.6 times the rate of ribosomal RNA. On the other hand, rAU, a double stranded polyribonucleotide, was degraded at about 15% the rate of ribosomal RNA, a value similar to that found for the relative rate of hydrolysis of native versus denatured DNA in the absence of magnesium ion.

In the presence of magnesium ion, rAU was degraded at less than 2.5% 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.

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 is 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 *N. crassa* nuclease is clearly an endonuclease. The *N. crassa* enzyme is, therefore, similar to the enzyme isolated from lamb brain by Haldy, Stollas, 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 contaminating 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 Co²⁺). 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 oligonucleotides resembled in many respects a similar study of pancreatic DNase by Ralph, Smith, and Khorana (30). Thus, under the conditions used, the *N. crassa* nuclease attacked hexanucleotides bearing terminal 3'-phosphates, but not smaller homologues; pancreatic DNase was reported to attack tetranucleotides bearing terminal 5'-phosphates, but not smaller homologues. Furthermore, both enzymes preferentially cleaved internal bonds, producing relatively few mono- and dinucleotides. Finally, the observation that d(pT)a was attacked at about the same rate as d(pT)a, is analogous to the observation made by Ralph, Smith, and Khorana (30) and by Vavrouk and Laskowski (31) that a 3'-phosphomonoester group simulates a phosphodiester bond for pancreatic DNase action. The resistance to the *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 phosphate group on a substrate as long as a DNA chain could influence 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 *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 digests of ribosomal RNA.

There are at least two possible explanations for this result. One is that the *N. crassa* nuclease preparation contains both an endonuclease and a nonspecific exonuclease component. The endonucleolytic attack would invariably produce oligonucleotides terminated by deoxyguanylate, while the presumed exonucleolytic component would then attack these oligonucleotides to yield first deoxyguanosine 5'-phosphate and then, as the exonucleolytic attack proceeded, the other mononucleotides. The net effect of such a nuclease would be to produce a relatively high proportion of deoxyguanylate early in the course of hydrolysis which would gradually diminish in the later phases. A major difficulty with this hypothesis is the disproportionately low level of deoxythymidine 5'-phosphate formed, particularly during the very early phases of hydrolysis. Since nearest neighbor frequency analyses of *E. coli* DNA (32) have shown the dinucleotide sequence, pCpG, to be rather common (6.7%), it would, therefore, be necessary to impose additional limitations on the nucleolytic specificity, i.e. that attack to produce deoxyguanylate-terminated oligonucleotides occur at a very low rate when the residue immediately following deoxyguanosine in the polynucleotide chain happens to be deoxythymidine, or, alternatively, that deoxythymidylate residues are 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 preparation. Thus, hydrolysis of deoxythymidylic 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 at very low levels.

As a second model to explain the preponderance of deoxyguanylate 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 residues 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 deoxyguanylic and that there is a disproportionately low level of deoxythymidylate 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 *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.
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 deoxyribo- nucleic 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)_6, d(pT)_8, and dT(pT) acetate are degraded at a rapid rate, but DT(pT)_n, DT(pT)_6, and DT(pT)_8 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