Exonucleolytic Degradation of High Molecular Weight Deoxyribonucleic Acid and Ribonucleic Acid to Nucleoside 3'-Phosphates by a Nuclease from Bacillus subtilis*

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SUMMARY

A Ca++-dependent extracellular nuclease which degrades high molecular weight native and denatured deoxyribonucleic acid and ribonucleic acid completely to nucleoside 3'-monophosphates has been partially purified from culture fluids of Bacillus subtilis. The enzyme is easily prepared and completely stable to storage for over a year. Degradation of DNA and RNA is predominantly exonucleolytic. The approximate ratio of exo- to endonucleolytic scissions by the enzyme of native phage λ DNA is 2 x 10^4, of single stranded "circular" M13 phage DNA it is 10^2, and of 23S Escherichia coli ribosomal RNA it is 10^3. Hydrolysis of oligoribonucleotides terminated with a 3'-phosphate group occurs from the 3' end. Hydrolysis of oligoribonucleotides lacking a 3'-phosphate is 5- to 20-fold slower and is not exclusively from either the 3' or the 5' end. Denatured DNA appears to be degraded from the 5' end of the chain but a simultaneous attack from the 3' terminus cannot be excluded.

An exonuclease capable of degrading polynucleotides exclusively from the 5' end of the polymer would be a valuable reagent in studies on the structure and function of deoxyribonucleic acid and ribonucleic acid. Spleen phosphodiesterase (1) has in fact been shown by Raszell and Klurman (2) to degrade oligonucleotides from their 5'-hydroxyl end; however, it attacks high molecular weight polynucleotides at a relatively low rate, if at all, and, in its current state of purification, contains significant levels of endonuclease activity (3, 4). A similar situation exists with respect to the phosphodiesterase from Lactobacillus acidophilus (5).

In a preliminary report from this laboratory, a Ca++-dependent extracellular nuclease from Bacillus subtilis was described which appeared to catalyze an exonucleolytic degradation of heat-denatured DNA starting at the 5' end (6). More recently, Okazaki, Okazaki, and Sakabe (7) described a similar but physically distinct nuclease from B. subtilis which also catalyzed the exonucleolytic degradation of denatured DNA from the 5' terminus of the molecule. Surprisingly, however, attack on native DNA was from the 3' terminus.

In this report, details of the purification and characterization of the B. subtilis enzyme are presented. In confirmation of our earlier communication (6), degradation of native and denatured DNA and RNA is predominantly exonucleolytic and proceeds rapidly to completion. Hydrolysis of denatured DNA appears to be initiated at the 5' terminus of the chain; however, a simultaneous attack from the 3' terminus cannot be excluded. Studies with small oligoribonucleotides of defined sequence have indicated that those containing a 3'-phosphoryl group are degraded from the 3' terminus. On the other hand, attack of oligonucleotides lacking a 3' phosphate does not occur exclusively from either the 3' or 5' end. No such effect of the presence or absence of a 3'-phosphate group is observed with an RNA of moderately high molecular weight (transfer RNA).

These findings taken with those of Okazaki et al. (7) suggest that the initial site of attack by the B. subtilis nuclease may differ depending upon the presence or absence of a phospho monoester group and the molecular weight and secondary structure of its substrate. They further indicate the necessity for caution in extrapolating to large polynucleotides findings derived from studies of the action of exonucleases on small oligonucleotide substrates.

EXPERIMENTAL PROCEDURE

DNA and RNA concentrations are expressed as equivalents of nucleotide phosphorus.

Materials

Veal infusion broth and yeast extract were purchased from Difco Laboratories, Inc.

1 On chromatography of the total extracellular DNase activity of B. subtilis cultures on DEAE-cellulose, two peaks of apparently identical activity are obtained (I. M. Kerr and I. R. Lehman, unpublished results); see also Fig. 1. The enzyme fraction characterized by Okazaki et al. (7) was derived from material corresponding to Peak I (Fig. 1), and that described here and by Kerr, Pratt, and Lehman (6) was derived from material corresponding to Peak II.
Escherichia coli DNA labeled with ³²P was prepared as described previously (8); denaturation was accomplished by heating the DNA at 100° for 10 min in 0.02 m NaCl at a concentration of 1 µ mole per ml, followed by rapid cooling at 0°. Salmon sperm DNA (grade A) was purchased from Calbiochem. Phage λ DNA with or without a ³²P label was prepared as described by Kaiser and Hogness (9). Alkaline-denatured E. coli and phage λ DNAs were prepared by exposure of the DNA for 15 to 30 min at room temperature to sufficient 0.05 m KOH to yield a pH >12. One equivalent of HCl and 0.1 volume of 1.0 m glycine-NaOH buffer, pH 9.35, were then added rapidly at 0° to a final pH of 9.4. T4 phage DNA was isolated as described previously (10). ᵃ⁻ Labelled and nonlabelled T7 phage DNAs were prepared by a modification (11) of the method of Richardson, Inman, and Kornberg (12). M13 phage DNA was obtained by phenol-sodium dodecyl sulfate extraction of phage grown as described by Salivar, Tzagoloff, and Pratt (13), and it was purified by differential centrifugation. Intact M13 DNA circles were isolated by centrifugation through 22,000 rpm in a 5 to 20% sucrose gradient containing 0.1 m Tris-HCl buffer, pH 8.0, for 36 hours at 4° in the SW 25 rotor of the Spinco model L ultracentrifuge. ᵃ⁻ Labelled M13 DNA was prepared from phage grown on E. coli 5274, a thymine auxotroph obtained from Dr. E. Lederberg. Growth was for 10 hours at 37° in 2X medium (14) in the presence of 5 µCi of ᵃ⁻-deoxythymidine (Nuclear-Chicago) per liter at a concentration of 10 µg per ml. The intact ᵃ⁻-labeled M13 DNA circles were isolated as described for the unlabeled DNA. DNA from phage ϕX174 was the kind gift of Dr. R. L. Sinamseiner.

E. coli tRNA was isolated as described by Zitaj (15). E. coli ribosomal RNA used in the routine assay of RNase activity was prepared according to Littauer and Eisenberg (16). The ribosomal RNA used in the analytical ultracentrifugation studies was isolated from E. coli ribosomes by extraction with phenol-sodium dodecyl sulfate. The 23 S component was isolated by centrifugation through a 5 to 20% sucrose gradient containing 0.1 m KCl, 0.01 m Tris-HCl buffer, pH 8.0, 1 mM EDTA, and 0.005% sodium dodecyl sulfate; centrifugation was for 15 hours at 22,000 rpm in the SW 24 rotor of the Spinco model L ultracentrifuge maintained at 4°.

Crystalline pancreatic RNAse, snake venom phosphodiesterase, and E. coli alkaline phosphatase were purchased from Worthington. ᵃ⁻-Nucleotidase was purified from Crotales adamanteus venom as described previously (17). E. coli exonuclease I (DEAE-cellulose fraction) and the Neurospora crassa nuclease (hydroxylapatite fraction) were prepared as described by Richardson et al. (20). Polynucleotide phosphorylase from Micrococcus lysodeikticus (21) was the kind gift of Dr. M. Bretschneider and Mr. I. Soll.

All reference mononucleotides were purchased from Calbiochem. Oligodeoxyribonucleotides were synthesized and characterized as previously described (18).

Oligonucleotides of the type (Ap)nUp and (Ap)mU were prepared by exhaustive pancreatic RNAse digestion of poly (A, U) which had been synthesized with polynucleotide phosphorylase (21) in a reaction mixture containing ADP and UDP in the ratio 3:2. Riboadenylate oligonucleotides ((pA)n) were prepared by controlled digestion of poly A (K⁺ salt, Calbiochem) with the N. crassa endonuclease. The individual oligonucleotide components were isolated by paper chromatography sequentially in the ethanolic-ammonium acetate and 1-propanol-ammonia-water systems (see "Methods"). As required, the 3'-phosphate group was removed by treatment with E. coli alkaline phosphatase. The base composition, sequence, and purity of the A- and U-containing oligonucleotides were confirmed by 1-propanol-ammonia-water chromatography of alkaline digests of the phosphatase-treated oligonucleotides. Ap and U, in the expected ratios, were the only chromatographically detectable products. Estimation of phosphatase-sensitive phosphate before and after alkaline hydrolysis provided confirmation of chain length.

DEAE-cellulose (0.96 meq per g) was purchased from Brown Company, Berlin, New Hampshire and Sephadex G-200 from Pharmacia, Uppsala, Sweden. Crystalline bovine plasma albumin was the product of the Armour Laboratories.

Dialysis tubing was routinely treated with a solution of 5% sodium carbonate containing 0.01 m EDTA for 30 min at 60°. It was then rinsed with distilled water and stored in 70% ethanol.

Methods

Enzyme Assays—One unit of DNase or RNase activity is defined as the amount catalyzing the production of 10 µmole of acid-soluble nucleotide in 30 min at 37°.

For the assay of DNase activity with denatured DNA as substrate, the reaction mixture (0.3 ml) contained enzyme, 67 µm denatured ³²P-labeled E. coli DNA, 67 mM glycine-NaOH buffer, pH 9.4, and 1.7 mm CaCl₂. With native DNA as substrate, the reaction mixture was the same except for the replacement of the glycine-NaOH buffer with Tris-HCl buffer, pH 8.0. The reaction mixtures were incubated for 30 min at 37° and assayed for the production of acid-soluble ³²P as described previously (8). Under the conditions of the assay, release of acid-soluble ³²P was linear over the range of 0.002 to 0.2 unit of enzyme.

For the assay of RNase activity, the incubation mixture (0.3 ml) contained 0.37 mm ribosomal RNA, 67 mm glycine-NaOH buffer, pH 9.4, 1.7 mm CaCl₂, and sufficient enzyme for 30 to 60% digestion of the RNA. Incubations were for 30 min at 37°. The release of acid-soluble nucleotide was determined spectrophotometrically as described by Linn and Lehman (19).

Phosphatase activity was assayed by the release of ³²P not adsorbable to Norit (17) from a mixture of the four ³²P-labeled deoxyribonucleoside 3' monophosphates produced by the digestion of ³²P-labeled denatured E. coli DNA with the B. subtilis nuclease described here. Each assay (0.3 ml) contained 67 mm Tris-HCl buffer, pH 8.0, 1.7 mm CaCl₂, 33 µm deoxyribonucleoside 3' monophosphates, and 0.01 to 0.2 unit of enzyme. One unit of phosphatase is defined as the amount which will catalyze the release of 10 µmole of P₁ in 30 min at 37°.

Sedimentation Analysis—Sedimentation analyses were performed with a Spinco model L ultracentrifuge equipped with an ultraviolet light optical system. Zone sedimentation of DNA and RNA was performed in 0.1 m NaOH, 0.9 m NaCl, 1 mm EDTA, or in 1.0 m NaCl, 0.01 m Tris-HCl, pH 8.0, 1 mm EDTA as described by Studier (22). Estimates of the degree of degradation of the DNA or RNA were made by measuring the proportion of trailing material in tracings made with a Joyce-Loebl densitometer.
Analysis of Products of Digestion of DNA, RNA, and Oligonucleotides—The susceptibility of digestion products to E. coli alkaline phosphatase or to snake venom 5’-nucleotidase was determined by measuring the release of phosphate which did not adsorb to Norit (17). Further characterization of the products of digestion was carried out by paper chromatography or electrophoresis on Whatman No. 3MM paper (W. and R. Balston, Ltd., England). Two descending paper chromatographic systems were used, the 1-propanol-ammonia-water system of Hanes and Isherwood (23) and the ethanol-1 M ammonium acetate system of Thach and Doty (24). In the case of the latter, ammonium acetate could be conveniently removed by rinsing the dried paper in ethanol at the end of the run. Paper electrophoresis was carried out in 0.05 M or 0.02 M sodium citrate buffer, pH 3.3 (25), either at room temperature at a potential of 1150 volts or at 4° at a potential of 5000 volts. Nucleotides were eluted from the paper by ascending chromatography in water. Quantitative estimation of the nucleotide concentration in the eluates was performed spectrophotometrically, the optical density at 260 nm of the eluates being corrected for recoveries determined with suitable standards. The ratio $A_{260}:A_{280}$ provided a check on adequate correction for the “paper” blanks and purity of the eluates. Recoveries obtained by this method were normally in excess of 80%. In the case of $^{32}$P-labeled nucleotides, radioactivity was measured either with a Vanguard autoscanner 8000 chromatogram scanner or by direct counting of the individual spots cut out from the paper in a low background Nuclear-Chicago gas flow counter. In each case recoveries were corrected with suitable standards.

RNA and oligoribonucleotides were degraded to mononucleotides by treatment for 18 hours at 37° with 0.3 x KOH. Protein was determined by the method of Lowry et al. (26); where necessary the protein was first precipitated at 0° by the addition of trichloroacetic acid to a final concentration of 4.7%. Inorganic phosphate estimations were by the method of Chen, Toribara, and Warner (27) as modified by Ames and Dubin (28). A Zeiss PMQ II spectrophotometer was used for all optical measurements.

RESULTS

Purification of Enzyme

All procedures were carried out at 0-4° unless otherwise indicated. MnCl₂ was added to all buffers at a final concentration of 1 mM.

Growth of Bacteria—A 30-liter culture of B. subtilis SB19 was grown with vigorous aeration at 30° in a Biogen apparatus (American Sterilizer Company). The culture medium contained 25 g of veal infusion broth and 5 g of yeast extract per liter. Antifoam (Dow-Corning Antifoam A) was added as required. After 24 hours the culture was harvested, the cells were removed by centrifugation, and the culture fluid (Fraction I) was immediately subjected to ammonium sulfate precipitation.

Concentration with Ammonium Sulfate—Finely powdered ammonium sulfate (600 g per liter) was added to Fraction I over a period of 2 hours with constant stirring. The stirring was continued for an additional 2 hours until all the ammonium sulfate had dissolved. Upon standing, the precipitate separated into two layers; one of these rose to the surface, while the other settled to the bottom (the specific activities of the two layers as assayed on denatured DNA were identical). The time required for separation varied between 18 and 48 hours. The bulk of the liquid between the two layers was siphoned off; the precipitate was collected by centrifugation for 10 min at 10,000 x g and was taken up in 1 liter of 0.05 M Tris-HCl buffer, pH 8.0. The resulting slurry was stirred overnight and centrifuged for 30 min at 10,000 x g; the supernatant fluid was collected as Fraction II.

Acetone—Acetone fractionation was carried out at 0° on four 250 ml-batches of Fraction II. To each batch 300 ml of acetone were added as rapidly as possible with constant stirring, and the mixture was centrifuged immediately for 10 min at 10,000 x g. The precipitate from each 250-ml batch was taken up in 25 ml of 0.05 M sodium acetate buffer, pH 5.1. The suspension was thoroughly mixed with a loosely fitting Dounce homogenizer and was centrifuged for 30 min at 20,000 x g. The precipitate was discarded, and the supernatant solution was collected as Fraction III.

Ammonium Sulfate—To 90 ml of Fraction III were added 22 g of finely powdered ammonium sulfate; the mixture was stirred for 30 min and was centrifuged for 30 min at 140,000 x g; the precipitate was then discarded. An additional 140 mg of ammonium sulfate were added per ml of the supernatant fluid. The mixture was stirred for 1 hour and centrifuged for 30 min at 140,000 x g. The pellet was resuspended in 30 ml of 0.05 M Tris-HCl buffer, pH 8.0, and centrifuged for 30 min at 140,000 x g. The supernatant fluid was collected as Fraction IV.

DEAE-cellulose Chromatography—A DEAE-cellulose column, 2.5 x 11 cm, was equilibrated with 0.01 M Tris-maleate buffer, pH 7.1. One-half (15 ml) of Fraction IV, which had been dialyzed overnight against 1.5 liters of the buffer, was applied to the column, which was then washed with 200 ml of buffer. Elution was begun with 465 ml of 0.075 M Tris-maleate, pH 7.1, at a flow rate of 60 ml per hour; then a linear gradient (1,000 ml) of 0.075 M to 0.2 M Tris-maleate, pH 7.1, was applied. The flow rate was initially 40 ml per hour but gradually fell to 30 ml per hour; 10-ml fractions were collected. Under these conditions the nuclease activity separated into two components. The first was eluted on the stepwise increase of buffer concentration from 0.01 M to 0.075 M; the second component appeared when the salt content of the gradient reached a concentration of 0.112 M (Fig. 1). Total recovery of nuclease activity on both DNA and RNA was normally 100%. Peak I (Fractions 14 to 19, Fig. 1) accounted for 40%; and Peak II (Fractions 65 to 85, Fig. 1) for 30% of the activity. The remaining activity, corresponding to intermediate or trailing fractions, was discarded.

In trial experiments a chromatographic pattern of this type was obtained reproducibly with starting material from different batches of 24-hour and 48-hour culture fluid. Each peak of activity retained its distinctive elution pattern on rechromatography on a second DEAE-cellulose column. Moreover, application to Fraction IV of procedures designed to remove nucleic acids, i.e. phase separation (29) or streptomycin sulfate precipitation, had no effect on its chromatographic behavior.

Unless otherwise stated all of the studies reported below were carried out with material derived from Peak II.

Concentration of DEAE-cellulose Peak II—Fractions 65 to 85 (Fig. 1) from the DEAE-cellulose column were pooled (184 ml) and dialyzed for 4 hours against 4 liters of 0.025 M Tris-maleate, and

* The amount of acetone or ammonium sulfate (Step IV) required for optimum fractionation is not reproducible from preparation to preparation and should be determined by trial experiment.
pH 7.1. The dialyzed material was immediately applied over a 6.5-hour period to a DEAE-cellulose column, 1 × 2.5 cm, which had been equilibrated with the same buffer. Essentially all (99%) of the activity was eluted with 0.25 M Tris-maleate, pH 7.1, in a volume of 3.7 ml (Fraction V).

Fractionation on Sephadex G-200—A column, 2 × 85 cm was prepared with 10 g of Sephadex G-200 which had been equilibrated with 750 ml of 0.025 M Tris-maleate, pH 7.1. Fraction V, 3.5 ml, was applied after the column had been washed with an additional 1 liter of this buffer. Elution was carried out with 0.025 M Tris-maleate, pH 7.1. A flow rate of 5 to 6 ml per hour was maintained throughout and fractions were collected hourly. The activities on native and denatured DNA and RNA eluted as a single sharp peak in Fractions 24 to 29. Purification with respect to protein was slight, but the phosphatase contamination was reduced by a factor of 10. Although over-all recovery of nuclease activity was only 60% in the preparation described in Table I, in other runs as much as 95% of the activity was recovered in an identical peak fraction. The nuclease as eluted from the column is very unstable and should be concentrated immediately.

Concentration of Sephadex Fraction on DEAE-cellulose—The pooled Sephadex fractions were applied directly to a DEAE-cellulose column, 1 × 2.5 cm, which had been equilibrated with

In one purification, activity on native DNA was preferentially lost prior to concentration. However, after concentration and subsequent storage at 0° or −20°, the activity was restored over a period of several months; it finally regained the same level, relative to the activity with denatured DNA, as is shown for Fraction VI (Table I).

### Table I

<table>
<thead>
<tr>
<th>Fraction and step</th>
<th>Total activity [a]</th>
<th>Specific activity [b]</th>
<th>Ratio of activity with denatured DNA to native DNA</th>
<th>Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units x 10⁻¹</td>
<td>units per mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Culture fluid</td>
<td>938</td>
<td>211</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. Concentrated culture fluid</td>
<td>800</td>
<td>330</td>
<td>4.5</td>
<td>1.0</td>
</tr>
<tr>
<td>III. Acetone</td>
<td>582</td>
<td>700</td>
<td>3.6</td>
<td>1.2</td>
</tr>
<tr>
<td>IV. Ammonium sulfate</td>
<td>336</td>
<td>700</td>
<td>3.8</td>
<td>1.0</td>
</tr>
<tr>
<td>V. DEAE-cellulose Peak I</td>
<td>132</td>
<td>3,080</td>
<td>4.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Peak II (concentrated)</td>
<td>99</td>
<td>6,600</td>
<td>3.8</td>
<td>2.1</td>
</tr>
<tr>
<td>VI. Sephadex G-200</td>
<td>51</td>
<td>15,000</td>
<td>4.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*a Steps V and VI were carried out on one-half of the material corresponding to Fraction IV. The values listed for these steps have, therefore, been corrected by a factor of 2.
*b Activity assayed with denatured DNA as substrate.
*c The specific activities given here are those for trichloracetic acid-precipitable protein. Assay of total protein gave specific activities of 4.2, 8, 3, and 700 for Fractions I to IV, respectively.
Properties of Enzyme

Stability—Enzyme activity as measured with denatured DNA or RNA as substrate was stable to storage for over a year at 0° or -20°. The preparation could be thawed and refrozen several times without significant loss of activity. The activity as measured with native DNA was less stable; after storage at -20° for 9 months, a 2-fold decrease in activity was observed.

pH Optima and Metal Ion Requirement—Enzyme activity was optimal at pH 8.5 with native DNA and at pH 9.5 with denatured DNA and RNA. Activity with all three substrates was very low (<3%) in the absence of added Ca++ and was totally eliminated by the addition of 10⁻⁹ M EDTA. Activity was completely restored on addition of Ca++, but not of Mg++ or Mn++, in excess of the added EDTA.

Inhibition of DNase Activity by RNA—Although detailed competition studies with native and denatured DNA and RNA have not been performed, it is clear that RNA is an inhibitor of DNase activity. For example, the addition of 2 and 10 μmoles of unlabeled E. coli ribosomal RNA to standard reaction mixtures containing 2 μmoles of 32P-labeled denatured E. coli DNA resulted in 36 and 64% inhibition, respectively, of the DNase activity.

Phosphatase Activity—The ratio of phosphatase activity to nuclease activity (denatured DNA substrate) under conditions optimal for each was approximately 1:4000 (Table II). Assay of phosphatase under conditions optimal for nuclease activity reduced this ratio to approximately 1:6000. The phosphatase activity on other nucleotide substrates, however, was up to 10-fold greater than that observed with deoxyribonucleoside 3'-phosphates, suggesting that several phosphatases with differing specificities may be present in the enzyme preparation.

Studies with both native and denatured λ DNA in which the 5'-phosphoryl group is labeled with 32P (30) have shown that the enzyme does not possess appreciable DNA phosphatase activity (31), at least with respect to these substrates. Moreover, studies with tRNA bearing a 3'-phosphoryl group (32) indicated that the enzyme is incapable of removing the monooester phosphate from molecules of this type.

Activity with Native DNA Substrates—No significant difference was observed in activity of the enzyme with denatured DNA from E. coli, with phage λ or M13 as substrate. In contrast, the activity observed with native DNA depended upon the particular DNA substrate used. For example, with a sample of enzyme having an activity on denatured E. coli DNA of 2100 units per ml, values of 500, 400, and 250 units per ml were obtained for the activity with 32P-labeled native E. coli DNA, 3H-labeled λ DNA, and 3H-labeled T7 DNA, respectively.

Characterization of Activity on DNA

Products of Digestion of DNA—Degradation of E. coli DNA by the B. subtilis nuclease yielded nucleoside 3'-monophosphates exclusively (>99%) (6). Glucosylated T4 DNA was only partially degraded, a limit being reached when 14% of the DNA had been converted to acid-soluble material.

Mode and Site of Attack—The complete degradation of DNA to mononucleotides observed with this enzyme suggested an exonucleolytic mode of attack. More definitive evidence was provided by experiments in which the products at various stages in the digestion of native and denatured DNA were assayed for susceptibility to alkaline phosphatase as an index of mononucleotide content. In the case of denatured DNA, when 4, 7, and 15% of the DNA had been digested, more than 97% of the acid-soluble products were mononucleotides. A similar result was obtained with native DNA between 8 and 49% digestion. Studies in which the products of enzyme action on M13 phage DNA and λ phage DNA were analyzed in the analytical ultracentrifuge confirmed that attack is predominantly exonucleolytic (see below).

With denatured DNA as substrate, degradation appeared to proceed from the 5' terminus of the polynucleotide chain. With the use of DNA labeled with a 32P-labeled d'-nucleotide at its 3' terminus (33), the relative rates of release of total acid-soluble nucleotide and either acid-soluble 32P or 32P susceptible to alkaline phosphatase were compared for the B. subtilis nuclease and E. coli exonuclease I (Fig. 2). The latter enzyme is known to degrade denatured DNA exonucleolytically, liberating deoxyribonucleoside 5'-phosphates from the 3'-hydroxyl terminus (18). As shown in Fig. 2, at a time when, for example, 10% of the DNA had been made acid-soluble by the action of exonuclease I, about 50% of the 32P-labeled 3'-terminal residue had been released as mononucleotides (as judged by their susceptibility to alkaline phosphatase). At a comparable stage of digestion of

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphatase</th>
<th>Ratio of activity with denatured DNA to phosphatase *</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-dCMP</td>
<td>12.0*</td>
<td>3750</td>
</tr>
<tr>
<td>3'-dTMP</td>
<td>8.5</td>
<td>5300</td>
</tr>
<tr>
<td>5'-dAMP</td>
<td>112.0</td>
<td>400</td>
</tr>
<tr>
<td>5'-dTMP</td>
<td>73.0</td>
<td>620</td>
</tr>
<tr>
<td>d-(pT)₂</td>
<td>87.0</td>
<td>520</td>
</tr>
<tr>
<td>3'-AMP</td>
<td>73.0</td>
<td>620</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>10.0</td>
<td>4500</td>
</tr>
<tr>
<td>(pA)₄</td>
<td>85.0</td>
<td>530</td>
</tr>
</tbody>
</table>

* The sample of Fraction VI used had been further concentrated by a second DEAE-cellulose step to yield material with an activity of 45,000 units per ml, assayed with denatured DNA as substrate.

**3'-dCMP represents a mixture of the four deoxyribonucleoside 3'-phosphates prepared as described in "Methods."

* A value of 7.5 units per ml was obtained under conditions optimal for nuclease activity with denatured DNA as substrate.

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0.025 m Tris-maleate, pH 7.1. More than 95% of the activity applied to the column was eluted with 0.5 m buffer in a total volume of 3.2 ml (Fraction VI).

A summary of the purification is given in Table I. Fraction VI was used for all the studies to be described.
the DNA by the \textit{B. subtilis} nuclease essentially no \textsuperscript{32}P-labeled mononucleotides had been liberated. The results obtained when release of total acid-soluble \textsuperscript{32}P was measured, although less impressive, exclude as a predominant mechanism the initial removal by the \textit{B. subtilis} enzyme of a dinucleoside monophosphate (X$^5_p$C in this case) from the 5'-hydroxyl end, followed by sequential degradation from the 3'-phosphoryl group so produced.

\textbf{Activity on Oligodeoxyribonucleotides}—Digestion of the oligodeoxyribonucleotide d-TpTpT was complete and the linear rate of hydrolysis continued in the region of digestion of the residual dinucleoside monophosphate. Release of d-Tp, d-TpT, and d-T was in quantitative agreement with digestion from the 5' terminus (Table III). No other products, either 5'-nucleotidase-sensitive material or dinucleoside diphosphates (d-pTpT and d-TpTp), could be detected.

Digestion of d(pT)\textsubscript{n} occurred at approximately 50% of the rate observed with d-TpTpT, indicating a slight inhibitory effect by the 5'-phosphate. Hydrolysis, however, was not dependent upon removal of the 5'-phosphate, since the rate of appearance of P\textsubscript{i} was considerably less than the rate of hydrolysis of the oligonucleotide as a whole. Thus the release of 112, 275, and 475 mmoles of mononucleotide was accompanied by the liberation of only 23, 50, and 106 mmoles, respectively, of P\textsubscript{i}.

![Fig. 2. Release of nucleotides from the 3'-hydroxyl end of denatured DNA by \textit{E. coli} exonuclease I and by the \textit{B. subtilis} nuclease. The 3'-terminal residues of \textit{E. coli} DNA were labeled by incubation of the DNA with \textit{E. coli} DNA polymerase in the presence of a single \textsuperscript{32}P-labeled deoxyribonucleoside triphosphate (dCTP) (33). The DNA was denatured with alkali prior to digestion. Digestion with \textit{E. coli} exonuclease I was performed as described by Lehman and Nussbaum (18). Digestion with the \textit{B. subtilis} nuclease was carried out under the conditions optimal for denatured DNA. The release of P\textsubscript{i} by alkaline phosphatase was assayed as described by Lehman, Roussos, and Pratt (17), except that after incubation with the phosphatase residual DNA from the original digest was removed by precipitation with 0.175 N perchloric acid prior to Norit treatment. The proportion of DNA rendered acid-soluble (abscessus) was based on the absorption at 260 nm of the total perchloric acid-soluble fraction at different time points in digestion compared with that of a 100% digest of the DNA.](image)

\textbf{Table III}

<table>
<thead>
<tr>
<th>Experiment and d-TpTpT digested</th>
<th>d-TpT</th>
<th>d-Tp</th>
<th>d-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. 277 mmoles</td>
<td>208</td>
<td>345</td>
<td>69</td>
</tr>
<tr>
<td>II. 869 mmoles</td>
<td>570</td>
<td>1210</td>
<td>205</td>
</tr>
</tbody>
</table>

* Calculated from initial and recovered amounts.

\textbf{Characterization of Activity on RNA}

Degradation of \textit{E. coli} ribosomal RNA was optimal at pH 9.5 and showed a requirement for Ca\textsuperscript{2+}, properties which clearly distinguish this RNase activity from that isolated from culture fluids of \textit{B. subtilis} by Nishimura (34).

Like DNA, RNA was attacked in a predominantly exonucleolytic fashion with the liberation of nucleoside 3'-monophosphates. On extensive digestion of \textit{E. coli} ribosomal RNA, 5 to 8% of the RNA phosphate was released as P\textsubscript{i} (as a result of contaminating phosphatase activity); the remainder was released as mononucleotide, as shown by its complete susceptibility to alkaline phosphatase. Ribonucleoside 3'-phosphates were the only products which could be detected on paper chromatographic analysis of such digests. Similarly at 5, 10, 19, and 100% digestion of poly A, 3'-AMP was the only product observed on paper chromatography of the digests under conditions in which di- to pentanucleotides would have been detected readily.

\textbf{Activity on Oligoribonucleotides}—The \textit{B. subtilis} nuclease degraded oligoribonucleotides completely to ribonucleoside 3'-monophosphates. In the case of oligonucleotides with a terminal 3'-phosphate group such as ApApUp and ApApApUp, degradation was exonucleolytic from the 3'-phosphate and occurred at a rate comparable to that observed with \textit{E. coli} ribosomal RNA (Table IV). The results of experiments in which the products of digestion of ApApUp and ApApApUp were analyzed after paper chromatographic fractionation are given in Table V. In the case of digestion of ApApUp, the ratio of Ap to Up released was <1, and ApAp rather than ApUp was the only dinucleotide which could be detected. Again with ApApApUp the products expected of exonucleolytic digestion from the 5' terminus, i.e. ApApUp and ApUp, were not found (although the possible presence of small amounts of ApApUp cannot be eliminated). Furthermore, in both digests the Up released was, to within experimental error, equal to the ApApUp or ApApApUp degraded.

The rate of attack of oligonucleotides lacking a terminal phosphate group or possessing a 5'-phosphate was comparatively slow (Table IV). Moreover, the mixture of products obtained on digestion of such substrates as ApApU and ApApApU was complex and can only be tentatively interpreted.
Because of the contrast in the results obtained with 3'-phosphate-terminated molecules, 10, 20, 30, and 60 min; in the case of tRNA-CpCp, samples were taken at 0, 30, and 60 min, and, after addition of a further 15 units of enzyme, at 90, 120, and 240 min. For digestion of tRNA-CpCp by the B. subtilis nuclease, the reaction mixture (1.0 ml) contained 0.1 M Tris-HCl buffer, pH 8.5, 2 mM CaCl₂, 0.25 mM MgCl₂, and 30 units (56 µg of protein) of enzyme; incubation was at 37°. When tRNA-CpCp was used as substrate, samples were taken at 0, 3, 6, 10, 30, and 60 min; in the case of tRNA-CpCpa, samples were taken at 0, 30, and 60 min, and, after addition of a further 15 units of enzyme, at 90, 120, and 240 min. For digestion of tRNA-CpCpa by the B. subtilis nuclease, the reaction mixture (1.0 ml) contained 0.1 M Tris-HCl buffer, pH 8.5, 2 mM CaCl₂, 0.3 mM 32P-labeled tRNA, and 40 units (RNase) of enzyme. Samples were taken after 0, 5, 10, 15, 20, 40, 60, and 120 min at 37°. For digestion of tRNA-CpCpa by the B. subtilis nuclease, the reaction mixture (1.0 ml) contained 0.1 M Tris-HCl buffer, pH 8.5, 2 mM CaCl₂, 0.25 mM 32P-labeled tRNA, and 20 units (RNase) of enzyme. Samples were taken at 0, 5, 10, 20, 30, and 60 min. All samples were assayed for acid-soluble 32P adsorbable to Norit as described in “Methods.”

Table IV
Rates of hydrolysis of RNA and oligoribonucleotides by B. subtilis nuclease

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of hydrolysis %</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli ribosomal RNA</td>
<td>100</td>
</tr>
<tr>
<td>E. coli tRNA-CpCpA</td>
<td>80</td>
</tr>
<tr>
<td>E. coli tRNA-CpCpa</td>
<td>80</td>
</tr>
<tr>
<td>ApApUp</td>
<td>100</td>
</tr>
<tr>
<td>ApApUp</td>
<td>80</td>
</tr>
<tr>
<td>(pA)₄</td>
<td>15</td>
</tr>
</tbody>
</table>

Table V

Incubations were as described for the assay of RNase activity. Digestion of ApApUp was performed in a reaction mixture containing 3 units (RNase) of enzyme and 116 mmoles of ApApUp. Incubation was for 30 and 90 min. In the case of ApApApUp, 98 mmole of ApApUp was digested for 60 min in the presence of 6 units of enzyme. Chromatographic analysis of the products was carried out as described in “Methods.” Where necessary, the composition of the eluates from the initial chromatograms was confirmed by a second chromatographic analysis after treatment with alkaline phosphatase or alkaline hydrolysis or both.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate digested</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmoles</td>
<td>mmoles</td>
</tr>
<tr>
<td>ApApUp</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>ApApU</td>
<td>70</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
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<td></td>
<td>158</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
</tr>
</tbody>
</table>

* Calculated from initial and recovered amounts.

Fig. 3. Release of nucleotides from the 3'-end of tRNA-CpCpa and tRNA-CpCpa by snake venom phosphodiesterase (SVPD) and by the B. subtilis nuclease. Snake venom phosphodiesterase digestion was carried out in a reaction mixture (1.0 ml) containing 0.1 mM 32P-labeled tRNA, 0.1 mM Tris-HCl buffer, pH 8.0, 10 mM MgCl₂, and 30 units (56 µg of protein) of enzyme; incubation was at 37°. When tRNA-CpCpa was used as substrate, samples were taken at 0, 3, 6, 10, 30, and 60 min; in the case of tRNA-CpCpa, samples were taken at 0, 30, and 60 min, and, after addition of a further 15 units of enzyme, at 90, 120, and 240 min. For digestion of tRNA-CpCpa by the B. subtilis nuclease, the reaction mixture (1.0 ml) contained 0.1 mM Tris-HCl buffer, pH 8.5, 2 mM CaCl₂, 0.3 mM 32P-labeled tRNA, and 40 units (RNase) of enzyme. Samples were taken after 0, 5, 10, 15, 20, 40, 60, and 120 min at 37°. For digestion of tRNA-CpCpa by the B. subtilis nuclease, the reaction mixture (1.0 ml) contained 0.1 mM Tris-HCl buffer, pH 8.5, 2 mM CaCl₂, 0.25 mM 32P-labeled tRNA, and 20 units (RNase) of enzyme. Samples were taken at 0, 5, 10, 20, 30, and 60 min. All samples were assayed for acid-soluble 32P adsorbable to Norit as described in “Methods.”

The 5'-phosphate group was not removed from the tRNA in these experiments.
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rations of the _B. subtilis_ nuclease (see below), only a positive result similar to that obtained with the venom diesterase would have been definitive. However, unless one postulates that under conditions of substrate excess, nondissociable enzyme-substrate complexes are formed (progressively releasing mononucleotides from the same polynucleotide), it is suggested by the clear-cut contrast between the results obtained with venom diesterase (despite its endonuclease content) and with the _B. subtilis_ nuclease that degradation of tRNA by the latter does not occur in a simple exonucleolytic manner from the 3’ terminus.

**Examination of Endonuclease Activity**

Analytical ultracentrifuge analyses have confirmed the exonucleolytic attack suggested by the examination of the products of digestion of DNA and RNA. They have also shown, however, that Fraction VI contains a low but significant level of endonuclease activity.

Assay of endonuclease activity with single stranded DNA as substrate was routinely carried out with the single stranded circular DNA of phage M13 (36). Alkaline zone sedimentation of the DNA after pretreatment with increasing amounts of enzyme provided results of the type shown in Fig. 4. Linear molecules produced by a single endonucleolytic scission can be clearly identified as a shoulder on the slower side of the more rapidly sedimenting peak of intact circles. After treatment with sufficient enzyme to convert 0.5, 2.5, 5.5, and 9.5% of the DNA to acid-soluble material, 4.8, 17.4, 26.4, and 57.5%, respectively, of the M13 DNA circles were cleaved. From these data, maximum values ranging from 700 to 1 to 1400 to 1 were calculated for the ratio of exo- to endonucleolytic scissions introduced by the enzyme. A similar result was obtained with _φX174_ DNA as substrate.

Endonuclease activity with a native DNA substrate was assayed with DNA from phage λ. Again, alkaline zone sedimentation was used and endonucleolytic scissions in single strands of the DNA were measured. Treatment with sufficient enzyme to convert 5, 10, and 15% of the DNA to acid-soluble material resulted in the introduction of single strand breaks into 11, 18, and 23%, respectively, of the DNA molecules. From these values a ratio of about 20,000 to 1 for the exo- to endonucleolytic scissions was obtained. Separate experiments involving neutral zone sedimentation of native T7 phage DNA, which had been pretreated with various amounts of enzyme, gave ratios of about 60,000 to 1 for exonucleolytic cleavage to endonucleolytic double strand breaks.

_E. coli_ ribosomal RNA (23 S) was also analyzed in the analytical ultracentrifuge after digestion with the _B. subtilis_ nuclease. On treatment with increasing amounts of nuclease no detectable loss of 23 S material was observed up to enzyme concentrations sufficient for conversion of 5% of the RNA to acid-soluble material. However, on incubation with enough enzyme for 10% conversion, essentially no intact 23 S RNA remained. This “all-or-none” effect may have been a consequence of the secondary structure of ribosomal RNA (37) so that several endonucleolytic scissions were required before breakdown of the molecule occurred. If one assumes that in the assay in which 10% digestion occurred all molecules were attacked endonucleolytically and that four endonucleolytic breaks were required per molecule for breakdown to occur, a value for the ratio of exo- to endonucleolytic scissions of approximately 100 is obtained.

**DISCUSSION**

The partially purified _B. subtilis_ enzyme (or enzyme complex) described here is capable of a predominantly exonucleolytic degradation of native and denatured DNA and RNA completely to nucleoside 3’-monophosphates. Phosphatase activity with deoxyribonucleoside 3’-monophosphates as substrate has been reduced to a negligible level; the enzyme is relatively easily prepared, and completely stable to storage for over a year. It may, therefore, be used in preference to the micrococcal nuclease-
spleen phosphodiesterase combination previously required for the degradation of DNA to deoxyribonucleoside 3'-phosphates. It furthermore provides a convenient enzymatic reagent for the complete degradation of RNA to 3'-mononucleotides.

Degradation of oligonucleotides bearing a 3'-phosphate is from the 3' terminus. On the other hand, the results with ApAp-ApU suggest that in the absence of a 3'-phosphate group simple exonucleolytic degradation from the 3' end is negligible whereas degradation from the 5' end occurs to a significant extent. A phenomenon has been observed with spleen phosphodiesterase, in which brief treatment of oligonucleotides of the type d-Cp(Xp)Gp yielded d-Gp initially rather than d-Cp, the first mononucleotide to be anticipated upon hydrolysis of d-Cp(Xp)Gp (2).

With high molecular weight substrates the results seem equally, if not more, complex. No clear-cut result was obtained with tRNA. In the case of denatured DNA, although endonuclease contamination makes the exclusion of alternative mechanisms impossible, the results presented here as well as previously (6) and those of Okazaki et al. (7) indicate the 5' terminus to be the initial site of exonucleolytic attack. On the other hand, Okazaki et al. have shown that with their enzyme preparation the initial attack on native DNA occurs at the 3' terminus. However, unless it is assumed a priori that the enzyme can only degrade a polynucleotide chain in one direction, i.e., exclusively from either the 3' or the 5' terminus, a mechanism involving attack at significant rates from both ends of the molecule is difficult to exclude.

It should be emphasized that in the case of the enzyme fraction described here, despite co-chromatography and electrophoresis of the activities on native and denatured DNA and RNA, there is conclusive evidence that they reside in a single protein. Certainly, if degradation of denatured DNA does indeed occur from the 5' to the 3' terminus and of oligoribonucleotides from the 3' to the 5' terminus of the molecule, it is simpler to conceive of these chemically different reactions being carried out by different enzymes. Further studies with more highly purified enzyme fractions could settle this question and that of the "contaminating" endonuclease.

REFERENCES

34. NISHIMURA, S., Biochim. Biophys. Acta, 45, 10 (1960).