The Deoxyribonucleases of Escherichia coli

V. ON THE SPECIFICITY OF EXONUCLEASE I (PHOSPHODIESTERASE)*

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Exonuclease I from Escherichia coli (1) (previously designated as E. coli phosphodiesterase (2)), shows a high degree of selectivity for denatured or single stranded deoxyribonucleic acid. This enzyme attacks at the 3' hydroxyl end of a polydeoxyribonucleotide and liberates 5'-mononucleotides in a stepwise manner until the terminal dinucleotide, which is not attacked. Because of these properties and the low level of endonuclease activity in even relatively impure preparations, it has come into widespread use in studies of DNA and polydeoxyribonucleotide structure (3-6).

We have purified exonuclease I to a stage approximately 10-fold beyond that reported earlier in order to determine whether a more purified enzyme might be a correspondingly more sensitive reagent for discriminating between native and denatured DNA. This has, in fact, proved to be the case.

Another aim of this investigation was to examine further the specificity of exonuclease I with the use of oligonucleotides of defined structure and sequence. More specifically, we wished to establish whether attack by this enzyme began exclusively at the 3'-hydroxyl end of a deoxyribonucleotide or whether attack began at the opposite end of the chain (5'-phosphoryl or 5' hydroxyl end) occurred as well. In addition, we wished to examine the effect of substitution of the 3'-hydroxyl group of an oligonucleotide on the rate at which it was attacked by exonuclease I.

These studies have revealed that exonuclease I attacks only at the 3'-hydroxyl end of a deoxyribonucleotide and that a free 3'-hydroxyl group is essential for enzymatic activity.

EXPERIMENTAL PROCEDURE

Materials

5'-Nucleotidase was purified from Crotaulus adamantaneus venom and endonuclease I was purified from E. coli as described previously (7). The alkaline phosphatase from E. coli (8) was purchased from the Worthington Biochemical Corporation (Lot 6004). This particular batch of enzyme was free of detectable DNase activity (7). Microcrocule nuclease (9, 10) was the gift of Dr. Charles A. Dekker and was assayed as described by Richardson and Kornberg (11). The DNA phosphatase-sensitive to total phosphorus.

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phosphorus in the product was 0.17 (theoretical, 0.17), and the recovery was 67%.

Antiserum to E. coli endonuclease I was prepared by treating a rabbit with an injection of 3 ml of an emulsion containing equal volumes of endonuclease I (3.3 mg of Amberlite IRC-50 fraction protein) and Freund's adjuvant, prepared in the VirTis homogenizer. The injection was repeated after 4 weeks. Two weeks after the second injection, the rabbit was bled by cardiac puncture and the serum was collected. The serum was diluted 5-fold with a solution containing 0.15 M NaCl and 0.01 M potassium phosphate buffer, pH 7.0, and heated for 1 hour at 70° before use.

DEAE-cellulose, 0.06 meq per g, was purchased from Brown Company, Berlin, New Hampshire. Hydroxylapatite (Hyapatite-C) was purchased from the Clarkson Chemical Company, Williamsport, Pennsylvania.

Crystalline bovine plasma albumin was the product of the Armour Laboratories, Chicago.

Concentrations of DNA and dAT polymer are expressed as equivalents of nucleotide phosphorus, unless otherwise indicated. Concentrations of oligonucleotides are expressed as moles of oligonucleotide.

Methods

Assay of Exonuclease I—The assay used routinely in enzyme purification measures the conversion of 32P-labeled, heat-denatured DNA to perchloric acid-soluble fragments. The incubation mixture (0.30 ml) contained 20 μmoles of glycine buffer, pH 9.5, 2 μmoles of MgCl2, 0.5 μmole of 2-mercaptoethanol, 20 μmoles of denatured DNA, and 0.05 to 0.3 unit of enzyme (in 0.02 ml). Dilutions of the enzyme were made in a solution composed of 0.1% crystalline bovine plasma albumin, 0.25 m ammonium sulfate, and 0.05 M Tris buffer, pH 7.5. The reaction mixture was incubated at 37° for 30 minutes; then 0.2 ml of cold "carrier" (calf thymus DNA, 2.5 mg per ml) and 0.5 ml of cold 0.3 M perchloric acid were added. After 5 minutes at 0°, the resulting precipitate was removed by centrifugation for 3 minutes at 10,000 × g, and 0.2 ml of the supernatant fluid was pipetted into a planchet. One drop each of 1 N KOH and a 0.1% solution of sodium lauryl sulfate were added directly to the planchet; the solution was taken to dryness and the radioactivity was determined.

The supernatant fluids obtained from control incubations with enzyme omitted contained 0.5 to 1.0% of the added radioactivity. One unit of enzyme is defined as the amount catalyzing the production of 10 μmoles of acid-soluble 32P in 30 minutes. The radioactivity made acid-soluble was proportional to enzyme concentration at levels of from 0.05 to 0.30 unit.

Assay of exonuclease I by measurement of the liberation of 5' mononucleotides from deoxyribosyl-oligonucleotides was carried out as follows. The reaction mixture (0.15 ml) contained 10 μmoles of glycine buffer, pH 9.5, 1 μmole of MgCl2, 0.25 μmole of 2-mercaptoethanol, 0.02 to 0.15 μmole of oligonucleotide, and 2 to 40 units of enzyme (in 0.01 ml). Dilutions of the enzyme were made in the diluent described above. The reaction mixture was incubated at 37°, and aliquots (usually 0.05 ml) were removed at 0, 15, and 30 minutes after addition of enzyme and assayed for mononucleotide content with 5'-nucleotidase or E. coli alkaline phosphatase (BAP). The reaction mixtures (0.15 ml) contained 10 μmoles of glycine buffer, pH 9.5, 1 μmole of MgCl2, 0.25 μmole of 2-mercaptoethanol, 15 units of enzyme, and either 0.115 μmole of d-pTpTpT or 0.113 μmole of d-TpTpTp. The progress of the hydrolysis was followed as described under "Methods." When alkaline phosphatase was used to measure mononucleotide formation, 95% of the phosphorus of d-pTpTpT and d-TpTpTp is converted to Pi even in the absence of added exonuclease. This blank value was subtracted from each of the experimental points. There was essentially no blank value when 5'-nucleotidase was used, since the nucleotidase does not attack a phosphomonoester group terminating an oligonucleotide.

from plots according to Lineweaver and Burk (22). Protein was determined by the procedure of Lowry et al. (23). Phosphatase was determined by the method of Chen, Toribara, and Warner (24) as modified by Ames and Dubin (25). Starch gel electrophoresis was carried out according to Smithies (26).

Results

Purification of Enzyme

A side fraction discarded during large scale purification of the DNA polymerase from E. coli (27) had a relatively high content of exonuclease I (Table I) and provided a plentiful and convenient starting material. This fraction was obtained by adjusting the autolyzed streptomycin eluate with ammonium sulfate to 42.5% of saturation and collecting the precipitate. Further purification of the enzyme involved a refractionation with ammonium sulfate and chromatography on columns of DEAE-cellulose and hydroxylapatite (Table I). All operations were carried out at 0–4°.

Ammonium Sulfate Refractionation—To 1 liter of polymerase ammonium sulfate I (in 0.02 M potassium phosphate, pH 7.2) were added 140 g of solid ammonium sulfate with stirring. After standing for 10 minutes, the precipitate was collected by centrifugation for 10 minutes at 15,000 × g and suspended in 150 ml of 0.02 M potassium phosphate buffer, pH 7.4. The ammonium sulfate I fractions contain variable amounts of residual ammonium sulfate. It was therefore necessary to carry out a small scale ammonium sulfate refractionation for each batch in order to determine the precise level of ammonium sulfate required for optimal purification.
insoluble residue was removed by centrifugation for 30 minutes at 15,000 \( \times \) g. The volume at this point was 170 ml (ammonium sulfate IR).

**DEAE-cellulose Chromatography**—A column of DEAE-cellulose, 12 \( \times \) 2.2 cm, was prepared and washed with 1000 ml of 0.02 M potassium phosphate buffer, pH 7.4, containing 10\(^{-3}\) M 2-mercaptoethanol. Ammonium sulfate IR (40 ml), previously dialyzed against 4 liters of this buffer for 12 to 15 hours, was added to the column at the rate of 30 ml per hour and washed into the column with a small amount of buffer. A linear gradient of elution was applied with 0.1 and 0.5 M potassium phosphate, pH 7.4 (containing 10\(^{-3}\) M 2-mercaptoethanol), as limiting concentrations; 200 ml of each buffer were used and the flow rate was maintained at 60 ml per hour. The peak of enzyme activity appeared approximately midway through the gradient. The best purification and most reproducible fractionation have been obtained under these conditions. Attempts to scale up the DEAE-cellulose chromatography by employing larger columns or by processing larger amounts of protein on a column of the same size have invariably led to reduced yields and fractions of lower specific activity. 

Four separate DEAE-cellulose chromatograms were therefore required to process the 170 ml of ammonium sulfate IR. The column elutes containing enzyme of specific activity of 4500 or greater were pooled (320 ml) and stored at 0\(^\circ\).

**Hydroxylapatite Chromatography**—A column of hydroxylapatite, 15 \( \times \) 2.2 cm, was equilibrated with 1500 ml of 0.05 M potassium phosphate buffer, pH 6.4, containing 10\(^{-3}\) M 2-mercaptoethanol. The DEAE-cellulose fraction (200 ml) previously dialyzed against 4 liters of this buffer for 12 to 15 hours, was added to the column at the rate of 45 ml per hour and washed into the column with 225 ml. The dialyzed DEAE-cellulose fraction was applied to the column at the rate of 0.30 ml per hour and washed into the column with 10 ml of the equilibration buffer. A linear gradient of elution was applied with 0.05 and 0.25 M potassium phosphate, pH 6.5 (containing 10\(^{-3}\) M 2-mercaptoethanol), as limiting concentrations; 250 ml of each buffer were used, and the flow rate was maintained at 72 ml per hour. The peak of activity appeared approximately midway through the gradient. The specific activity of the enzyme fractions throughout the entire peak of activity was constant with a value of approximately 15,000. Two hydroxylapatite chromatograms were required to process the 320 ml of DEAE-cellulose fraction. 

**Concentration of Hydroxylapatite Fraction**—To 270 ml of hydroxylapatite fraction were added 90 g of solid ammonium sulfate with stirring. After standing for 20 minutes at 0\(^\circ\), the suspension was centrifuged for 60 minutes at 15,000 \( \times \) g. The precipitate was taken up in 3.8 ml of 0.05 M Tris buffer, pH 7.5. A small amount of insoluble residue was removed by centrifugation for 5 minutes at 15,000 \( \times \) g.

The enzyme preparation obtained in this way, although purified about 1400 fold, appeared to be physically heterogeneous. In the ultracentrifuge the preparation revealed one major and two major boundaries. Electrophoresis in starch gels at pH 8.8 (Tris-citrate buffer) produced four bands, two major and two minor. Only one of the major bands possessed enzymatic activity.

The DEAE-cellulose fraction, when concentrated by precipitation with ammonium sulfate, retained essentially all of its activity upon storage at \(-12^\circ\) for periods as long as 2 years, even after repeated freezing and thawing. The concentrated hydroxylapatite fraction was found to be less stable and lost approximately 50% of its activity within 1 month when stored under these conditions. Unless otherwise indicated, all the studies described were carried out with the hydroxylapatite fraction.

**Hydrolysis of Denatured DNA**

**Relative Specificity for Denatured versus Native DNA**—Exonuclease I attacked native DNA at a rate approximately 40,000 times less than the rate observed with DNA which had been heat-denatured. The limit of hydrolysis reached with three different preparations of native *E. coli* DNA ranged from 0.5 to 1.6% of the total nucleotide (Fig. 2). The products formed in this way could be largely accounted for as deoxyribonucleoside 5'-monophosphates. In an experiment in which 0.75% of the \(^{32}P\) of the DNA was converted to an acid-soluble form, more than 90% of the acid-soluble \(^{32}P\) was susceptible to the action of *E. coli* alkaline phosphatase or 5'-nucleotidase.

The rate at which the susceptible fraction of native DNA is attacked is 40,000 times less than that found with denatured DNA, and the rate of hydrolysis of denatured DNA is not at all diminished by a 10-fold excess of native DNA. It is therefore unlikely that this fraction is composed solely of unattached fragments of denatured DNA present in the native DNA preparation. The susceptible fraction may rather be frayed, non-hydrogen-bonded stretches in the DNA double helix, perhaps formed during the isolation procedure; the much diminished rate at which the presumptive non-hydrogen-bonded stretches are attacked might be attributable to the relatively poor binding by exonuclease I of an essentially double helical DNA structure.

The finding that the acid-soluble hydrolysis products formed are mononucleotides rather than oligonucleotides suggests that the limited action of the purified exonuclease I on native DNA is not the result of a contaminating endonuclease. Further evidence is provided by experiments in which direct tests of exonuclease activity were performed. Thus, when 0.14 \( \mu \) mole of *E. coli* DNA was incubated with 400 units of exonuclease I for 3 hours under conditions optimal for assay of exonuclease I (see "Methods"), no decrease in specific viscosity of the DNA solution was detectable. In a more sensitive assay for endonuclease

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
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</thead>
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<tr>
<td>Blender fraction</td>
<td>171</td>
<td>6.8 ( \times ) 10^5</td>
<td>15.9</td>
</tr>
<tr>
<td>Streptomycin eluate</td>
<td>347</td>
<td>3.5 ( \times ) 10^4</td>
<td>11.8</td>
</tr>
<tr>
<td>Autolyzed streptomycin eluate</td>
<td>370</td>
<td>3.7 ( \times ) 10^4</td>
<td>1.6</td>
</tr>
<tr>
<td>Ammonium sulfate IR</td>
<td>3,430</td>
<td>3.4 ( \times ) 10^3</td>
<td>16.0</td>
</tr>
<tr>
<td>Pooled hydroxyapatite</td>
<td>115,000</td>
<td>4.6 ( \times ) 10^3</td>
<td>9.2</td>
</tr>
</tbody>
</table>
activity, 0.06 μmole of DNA from Hemophilus influenza (28) was incubated for 2 hours with levels of exonuclease I of up to 400 units. No significant decrease in transforming activity was detectable with resistance to either streptomycin or chloramphenicol as markers. Similarly, incubation of 0.02 μmole of DNA from phage λ (29) with 20 units of exonuclease I for 30 minutes caused no decrease in transforming activity; in the presence of 200 units, a 40% decrease in activity was observed. In a control experiment, incubation of 0.02 μmole of DNA with 0.003 unit of endonuclease I for 30 minutes under conditions which are optimal for endonuclease I activity (7) produced an 85% decrease in transforming activity.

Extent of Hydrolysis of Denatured DNA—Hydrolysis of heat-denatured E. coli DNA proceeded at a rapid rate until approximately 50% of the DNA had been degraded; the rate then fell off rapidly and the limit of hydrolysis, while variable and depending upon the particular DNA preparation used, reached a level of only 75 to 90% (Fig. 3). A similar result was obtained with E. coli DNA which had been denatured by alkali treatment and with heat-denatured DNA from phage T7. Addition of the DNA phosphatase (11) to the incubation mixture at relatively low levels significantly increased the extent of hydrolysis (from 75 to 90%) (Fig. 4). With very high concentrations of enzyme (200- to 300-fold excess) and prolonged periods of incubation, limits up to 96% have been observed even in the absence of added DNA phosphatase. This result is probably attributable to traces of DNA phosphatase which persist in the exonuclease I preparation. Assay of the hydroxylapatite fraction for DNA phosphatase showed it to contain a low but experimentally significant level of this enzyme (0.003% of the exonuclease I activity).

Oligonucleotides terminated by a 3'-phosphomonoester group are not attacked by exonuclease I (Fig. 1), so that the increased extent of hydrolysis observed in the presence of the DNA phosphatase is reasonably accounted for by the removal by the phosphatase of 3'-phosphomonoester groups present in the denatured DNA, permitting the chains bearing such groups to undergo hydrolysis.

We are indebted to Drs. Vernon Bode and A. D. Kaiser of the Department of Biochemistry, Stanford University, for performing these assays.

The extent of hydrolysis of denatured DNA by exonuclease I was also markedly increased by addition of levels of endonuclease I which are themselves insufficient to produce acid-soluble products from the DNA (Fig. 5).

The effect of endonuclease I in facilitating the rapid quantitative hydrolysis of denatured DNA might be visualized in at least two ways. First, an endonucleolytic cleavage producing a 3'-hydroxyl group in a polydeoxyribonucleotide chain terminated by a 3'-phosphoryl group should make the part with the newly created 3'-hydroxyl terminus susceptible to the action of exonuclease I. The closer the endonucleolytic scission to the 3'-phosphoryl terminus, the greater the fraction of the chain which becomes susceptible to the exonuclease. Second, mixtures of single stranded polydeoxyribonucleotide chains produced as a result of denaturation of DNA might be expected to form some intra- and interstrand hydrogen bonds. When the hydrogen-bonded regions are located at or very near the 3'-hydroxyl end
FIG. 4. The effect of DNA phosphatase on the extent of hydrolysis of heat-denatured DNA by exonuclease I. Two series of identical reaction mixtures were set up, containing 20 μmoles of glycine buffer, pH 9.5, 2 μmoles of MgCl₂, 0.5 μmole of 2-mercaptoethanol, 10 μmoles of 32P-labeled, denatured E. coli DNA, and 10 units of exonuclease I (hydroxylapatite fraction). To one series of reaction mixtures was added sufficient DNA phosphatase to convert approximately 1% of the DNA to acid-soluble products as a result of its exonuclease activity (30). The second series served as a control lacking the DNA phosphatase. The remainder of the experiment was carried out as described for Fig. 3.

of these chains, almost the entire polynucleotide should be made insusceptible to exonuclease I. One or a few endonucleolytic scissions at these hydrogen-bonded points could suffice to dissociate the chains, making them susceptible to the action of exonuclease I.

With preparations of exonuclease I less purified than the hydroxylapatite fraction (DEAE-cellulose), hydrolysis of denatured DNA proceeded rapidly to completion (2). Since the DEAE-cellulose fraction did not differ significantly in its DNA phosphatase content from the hydroxylapatite fraction, this result is probably attributable to the presence of traces of endonuclease I in the less purified fraction. Thus, incubation of 0.02 μmole of DNA from phage λ with 20 units of DEAE-cellulose fraction for 30 minutes resulted in a 78% decrease in transforming activity. No such decrease was observed with the hydroxylapatite fraction (see above). Additional evidence which suggests that endonuclease I is indeed present in the DEAE-cellulose fraction is provided by an experiment in which hydrolysis of heat-denatured DNA by this fraction was observed in the presence of S-RNA from E. coli. (S-RNA is a potent inhibitor of endonuclease I (31).) Under these conditions, the initial rate decreased by approximately 50% and the kinetics of hydrolysis resembled that obtained with the hydroxylapatite fraction (Fig. 6). A similar result was obtained when antiserum prepared against purified endonuclease I was used in place of S-RNA.

Rate of Hydrolysis of Denatured DNA—The rate at which a denatured DNA is hydrolyzed by exonuclease I depended to some degree on the manner in which denaturation was achieved. Moreover, the rate of hydrolysis was markedly stimulated by the addition of the DNA phosphatase (Table II). Since this stimulation, at least in the case of the heat-denatured DNA, was observed under conditions of saturating substrate concentration, this result would imply that 3'-phosphoryl groups terminating long chain oligonucleotides not only prevent hydrolysis of such chains by exonuclease I, but are inhibitors of the hydrolysis of the normally susceptible chains terminated by 3'-hydroxyl groups. Supporting this notion is the observation that treatment of E. coli DNA with micrococcal nuclease sufficient to produce a 58% drop in viscosity, followed by heat denaturation of the DNA, resulted in a 70% decrease in the rate at which the treated DNA was attacked. Since the exonuclease I preparations available contain DNA phosphatase activity, whose effect would be to remove some of the 3'-phosphoryl groups introduced into the DNA by the micrococcal nuclease, the actual extent of inhibition might even exceed the 70% which was observed.

Hydrolysis of Oligonucleotides

Rate and Extent of Hydrolysis of Oligonucleotides—Dinucleoside diphosphates and dinucleoside monophosphates are not attacked by exonuclease I (2). On the other hand, oligonucleotides containing more than 2 nucleotide residues were attacked at rates of similar magnitude in the presence of 20 units of DNA phosphatase (Table II). A similar result was obtained when antiserum prepared against purified endonuclease I was used in place of S-RNA. The rate at which a denatured DNA is hydrolyzed by exonuclease I depended to some degree on the manner in which denaturation was achieved. Moreover, the rate of hydrolysis was markedly stimulated by the addition of the DNA phosphatase (Table II). Since this stimulation, at least in the case of the heat-denatured DNA, was observed under conditions of saturating substrate concentration, this result would imply that 3'-phosphoryl groups terminating long chain oligonucleotides not only prevent hydrolysis of such chains by exonuclease I, but are inhibitors of the hydrolysis of the normally susceptible chains terminated by 3'-hydroxyl groups. Supporting this notion is the observation that treatment of E. coli DNA with micrococcal nuclease sufficient to produce a 58% drop in viscosity, followed by heat denaturation of the DNA, resulted in a 70% decrease in the rate at which the treated DNA was attacked. Since the exonuclease I preparations available contain DNA phosphatase activity, whose effect would be to remove some of the 3'-phosphoryl groups introduced into the DNA by the micrococcal nuclease, the actual extent of inhibition might even exceed the 70% which was observed.

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which exceed those found for denatured DNA. The concentration of substrate at which half-maximal velocity was developed ($K_m$) was, however, considerably greater for the oligonucleotides than for the denatured DNA (Table III). A similar result has been observed for exonuclease II from $E$. coli (21).

With venom phosphodiesterase, removal of the 5'-phosphomonoester group results in a 20- to 30-fold decrease in the rate at which the oligonucleotide is attacked (32, 33). By contrast, removal of the terminal phosphate from $d$-TpTpTpT actually resulted in a 2-fold increase in the maximal velocity found with exonuclease I. In the case of the pentanucleotide, there was no significant enhancement in rate as a result of the removal of the terminal phosphate group.

The deoxythymidine trinucleotide bearing a 3'-phosphomonoester grouping was completely resistant to the action of exonuclease I (Fig. 1). Removal of the phosphoryl group by means of $E$. coli alkaline phosphatase produced d-TpTpT which was attacked at the same rate as when it was generated by phosphatase action on $d$-TpTpTpT. The 3'-phosphoryl-terminated trihydromylate, even when added in a 3-fold excess, did not significantly inhibit hydrolysis of the corresponding trinucleotide with a 3'-hydroxyl group. This result contrasts with the effects noted above, in which the presence of 3'-phosphomonoester groups terminating long chain polydeoxyribonucleotides was found to be distinctly inhibitory to exonuclease I in its attack on polynucleotides bearing 3'-hydroxyl groups. In view of the very large differences in the apparent $K_m$ values between a long chain polydeoxyribonucleotide and, for example, a trinucleotide, the lack of inhibition by d-TpTpTpT may simply be due to the inability of exonuclease I to bind a trinucleotide with a 3'-phosphoryl group, to even a very slight extent.

Falaschi, Adler, and Khobana (34) have found that oligonucleotides in which the terminal 3'-hydroxyl group is acetylated are not attacked by exonuclease I, nor are they inhibitors of the enzyme in its attack on substrates which are normally susceptible.

### Table II

**Effect of DNA phosphatase on rate of hydrolysis of denatured $E$. coli DNA by exonuclease I**

Heat denaturation was performed as indicated, at a DNA concentration of 0.03 µmole per ml. After the heating period, the samples were cooled rapidly in an ice bath. Alkaline denaturation was carried out by treating 0.2 ml of DNA (1.85 µmoles per ml) with 0.01 ml of 1 N KOH for 30 minutes at room temperature. The pH of the solution was then adjusted to 8.6 by the addition of 0.032 ml of 1 M Tris buffer, pH 7.2. Formamide denaturation was carried out by diluting the DNA (1.85 µmoles per ml) 20-fold with a 99% solution of formamide. After standing for 90 minutes at room temperature, the solution was dialyzed for 18 hours at 2° against 1000 volumes of 0.02 M NaCl. Assays were performed as described under "Methods." Where indicated, an amount of DNA phosphatase was added which converted approximately 1% of the DNA to acid-soluble products as a result of its exonuclease activity (30). This value was subtracted from that obtained when both phosphatase and exonuclease I were present in the incubation mixture.

<table>
<thead>
<tr>
<th>Denaturation method</th>
<th>Rate of hydrolysis (conversion to acid-soluble form)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without DNA phosphatase</td>
</tr>
<tr>
<td>10 minutes, 100°, 0.02 M NaCl</td>
<td>1.2</td>
</tr>
<tr>
<td>10 minutes, 95°, 0.1 M NaCl</td>
<td>0.0</td>
</tr>
<tr>
<td>pH &gt; 12</td>
<td>0.8</td>
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<tr>
<td>Formamide</td>
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### Table III

**Kinetic parameters for hydrolysis of deoxyribo-oligonucleotides by exonuclease I**

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<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
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<tbody>
<tr>
<td>$d$-TpTpTpT</td>
<td>$2.5 \times 10^{-3}$</td>
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</tr>
<tr>
<td>$d$-TpTpT</td>
<td>$1.3 \times 10^{-3}$</td>
<td>512</td>
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<tr>
<td>$d$-TpTpTpTpT</td>
<td>$1.4 \times 10^{-3}$</td>
<td>470</td>
</tr>
<tr>
<td>$d$-TpTpTpT</td>
<td>$1.3 \times 10^{-3}$</td>
<td>528</td>
</tr>
<tr>
<td>Heat-denatured DNA</td>
<td>$6.2 \times 10^{-3}$</td>
<td>141</td>
</tr>
</tbody>
</table>

* These values are expressed per mole of polynucleotide. In the case of the denatured DNA, an average chain length of $10^4$ nucleotide residues is assumed.

† Micromoles of mononucleotide formed per 30 minutes per mg of protein.
Fig. 7. Kinetics of hydrolysis of d-pTpTpTpTpT and its 3'-O-acetyl derivative by exonuclease I. The reaction mixtures were made up as described under Fig. 1; 0.014 µmole of d-pTpTpTpTpTpT ((pT)₆) was incubated with 2.2 units of enzyme, and 0.016 µmole of the 3'-O-acetyl derivative ((pT)₆-OAc) was incubated with 11 units of enzyme. In the experiment testing the possible inhibition of hydrolysis of d-pTpTpTpTpT by the acetylated derivative, 0.032 µmole of the latter compound was added to the reaction mixture containing 0.014 µmole of d-pTpTpTpTpTpT. The progress of the reaction was monitored with E. coli alkaline phosphatase.

Hydrolysis of dAT Polymer

The dAT polymer can be degraded to 5'-mononucleotides by exonuclease I without prior denaturation (37). The rates at which various preparations of dAT are degraded by the hydroxylapatite fraction are variable, ranging from 0.6 to 10% the rate found with heat-denatured DNA. Moreover, differences in rate have been noted between the hydroxylapatite

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate hydrolyzed</th>
<th>Products recovered</th>
<th>d-pT:pTpT ratio</th>
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<td></td>
<td>µmole</td>
<td>µmole</td>
<td></td>
</tr>
<tr>
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<td>0.13</td>
<td>&lt;0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>0.13</td>
<td>&lt;0.02</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Table IV

Products of hydrolysis of d-pTpTpTpTpT by exonuclease I

The reaction mixture (0.117 ml) contained, in addition to substrate, 5 µmoles of glycine buffer, pH 9.5, 0.5 µmole of MgCl₂, 0.2 µmole of 2-mercaptoethanol, and 150 units of enzyme. After incubation for 4 hours at 37°C, the mixture was transferred quantitatively to Whatman No. 3MM paper, together with the appropriate reference standards. The products were separated by descending chromatography in the 1-propanol-ammonia-water system of Hanes and Isherwood (36) for 17 hours. After development of the chromatogram, the nucleotide areas were visualized with a Mineralight lamp and cut out together with adjacent areas of equal size, which served as blanks. In addition, the areas of the chromatogram where d-pTpT and deoxythymidine (d-T) would be expected to appear, as well as the appropriate blanks, were cut out. The nucleotides were eluted with 3.0 ml of 0.01 N HCl overnight at room temperature, and their extinction was measured at 260 nm.
and the DEAE-cellulose fractions. For example, with one preparation of dAT polymer, its rate of hydrolysis by the hydroxylapatite fraction was 1.7% of that found with heat-denatured DNA; its rate of hydrolysis by the DEAE-cellulose fraction was 4.7% of that observed with the denatured DNA.

In contrast to heat-denatured DNA, in which the ratio of activity in pH 9.5 glycine buffer to that in pH 7.4 phosphate buffer was approximately 4, the ratio found with dAT polymer was about 20. At both pH 7.4 and 9.5, however, the dAT polymer could be degraded completely.

Since the dAT polymer is so similar to naturally occurring DNA by the physicochemical criteria which have been applied (13, 38), the susceptibility of the polymer to the action of exonuclease I is difficult to explain. It may have its basis in the relatively low temperature at which the adenine-thymine hydrogen bond pair dissociates. Thus, given a dAT preparation with frayed, non-hydrogen-bonded ends (the degree of fraying may vary from one preparation to the next), hydrolysis can begin at these ends, and with successive cleavage of phosphodiester bonds the two strands of the molecule can dissociate, forming non-hydrogen-bonded stretches susceptible to further enzymatic attack. Consistent with this notion is the observation (Fig. 8) that while the dAT polymer is attacked by exonuclease I approximately 50 times more rapidly at 37° than at 23°, the rate observed with denatured DNA is only 5 times greater at 37° than at 23°. Also of interest in this regard is the observation of Radding, Josse, and Kornberg (39) that the enzymatically synthesized dGdC polymer, containing only the guanine-cytosine bond the two strands of the molecule can dissociate, forming non-hydrogen-bonded stretches susceptible to further enzymatic attack. Consistent with this notion is the observation (Fig. 8) that while the dAT polymer is attacked by exonuclease I approximately 50 times more rapidly at 37° than at 23°, the rate observed with denatured DNA is only 5 times greater at 37° than at 23°. Also of interest in this regard is the observation of Radding, Josse, and Kornberg (39) that the enzymatically synthesized dGdC polymer, containing only the guanine-cytosine bond pair, which melts at a high temperature, is essentially insusceptible to the action of exonuclease I.

**Lack of Capacity to Hydrolyze Polyribonucleotides**

The hydroxylapatite fraction of exonuclease I appears to have only very slight activity on polyribonucleotides. Thus, incubation of 25 μg of 32P-labeled tobacco mosaic virus RNA (approximately 80 μmoles of phosphorus) with 100 units of enzyme for 1 hour under standard assay conditions resulted in the conversion of 5.4% of the 32P of the RNA to an acid-soluble form. When 1000 units of enzyme were added, 33.3% of the 32P became acid-soluble. Incubation of 60 μg of RNA with 2400 units of enzyme at 0° for 1 hour resulted in a 62% loss in its infectivity.

On the other hand, Ofengand, Dieckmann, and Berg (15) observed that S-RNA from E. coli was resistant to the action of exonuclease I. In confirmation of this result, we have observed that S-RNA (0.85 μmole of phosphorus) was incubated with 150 units of exonuclease I under the standard assay conditions for 3 hours, there was no loss in activity when the treated RNA was subsequently tested as an acceptor of methionine in the reaction catalyzed by the methionyl-RNA synthetase (40). An assay of this kind is an extremely sensitive measure of ribonuclease activity, since removal of the terminal adenosine residue or a single endonucleolytic break in the RNA would be sufficient to destroy its amino acid acceptor activity completely (41). Since a low but significant level of activity can be observed with RNA from tobacco mosaic virus which probably exists as a random coil in solution, the resistance of S-RNA to attack by exonuclease I may be related to its ordered conformation (42).

**Discussion**

Although E. coli exonuclease I has been purified about 1400-fold, it is still physically heterogeneous. In spite of this heterogeneity, tests of this enzyme for other nucleolytic activities have shown it to be essentially free of the other deoxyribonucleases thus far identified in E. coli. The only exception appears to be the E. coli DNA phosphatase-exonuclease, which is present to the extent of 0.003% of the exonuclease I activity.

The studies reported here, which have utilized a more highly purified enzyme preparation than that used previously, have in essence confirmed the earlier description of the specificity of exonuclease I. The enzyme initiates its attack exclusively at the 3'-hydroxyl end of a single stranded polynucleotide and successively liberates deoxyribonucleoside 5'-monophosphates, but leaves the terminal dinucleotide intact.

In view of this specificity and its nucleolytic homogeneity, exonuclease I would appear to be a suitable reagent for the study of the secondary structure and end groups of DNA. An obvious difficulty in this regard is the isolation of DNAs which are themselves homogeneous. Thus, DNA from phage λ, φX174, or T7, while probably consisting of a single molecular species in vivo (29, 3, 43), may well undergo shear or enzymatic breakage during isolation, so that the preparations available for enzymatic examination may in fact be markedly heterogeneous (44). Nevertheless, procedures have been developed (29, 43, 45) which permit isolation of DNAs whose structures would seem to be relatively intact even when judged by highly sensitive criteria (43, 45, 46).

Given a linear DNA molecule whose sequence of nucleotides is unbroken, it should be possible, therefore, to isolate two terminal dinucleotides, one from each of the two chains of the DNA double helix. An analysis of this kind could yield significant information about the ends of the DNA molecule. For example, it might be possible to determine whether these terminal residues bear free 5'-hydroxyl groups or whether this group is substituted by a mono- or perhaps even a triphosphate grouping.

Exonuclease I preparations less purified than that described here have already been used successfully for the selective hydrolysis of denatured fragments in a population of native DNA molecules. They have also been used for the removal of single stranded regions within DNA molecules (4). With the more purified enzyme these operations may be carried out with assurance that few if any endonucleolytic scissions would be introduced into the native DNA as a result of exposure to the exonuclease.

**Summary**

Exonuclease I (phosphodiesterase) from Escherichia coli has been further purified to a level of approximately 1400-fold. The purified enzyme is still physically inhomogeneous. Tests of its enzymatic properties have demonstrated that it is free of other deoxyribonuclease activities, with the exception of a trace of the deoxyribonucleic acid phosphatase-exonuclease.

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7 We are grateful to Dr. A. Burness of the Virus Laboratory, University of California, Berkeley, for carrying out these experiments.

8 Dr. Paul Berg, personal communication.
With the purified enzyme, native DNA is attacked at a rate about 40,000 times less than the rate at which heat-denatured DNA is attacked. This low rate with native DNA is probably due to the action of exonuclease I on single stranded regions within the DNA molecules used.

The specificity of exonuclease I is characterized by a stepwise attack beginning exclusively at the 3'-hydroxyl terminus of a single stranded polydeoxyribonucleotide chain, producing deoxyribonucleoside 5'-monophosphates and a dinucleoside diphosphate from those chains with 5'-phosphoryl groups, and deoxyribonucleoside 5'-monophosphates and a dinucleoside monophosphate from those chains lacking a 5'-terminal phospho-grouping.

The following features of the specificity of exonuclease I have also been established.
1. Chains in which the terminal 3'-hydroxyl group is blocked by a phosphoryl or acetyl group are resistant to the action of exonuclease I.
2. Polynucleotides with terminal 3'-phosphoryl groups are inhibitors of the enzyme in its attack on oligonucleotides with terminal 3'-hydroxyl groups; small polynucleotides (3 residues) with 3'-phosphoryl groups are not.
3. Oligonucleotides with 3 to 6 residues are attacked at a greater rate than denatured DNA, but the observed $K_m$ values are about 10$^6$ times greater than for denatured DNA.

REFERENCES

The Deoxyribonucleases of *Escherichia coli* : V. ON THE SPECIFICITY OF EXONUCLEASE I (PHOSPHODIESTERASE)

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