An Exonuclease Induced by Bacteriophage \( \lambda \)

I. PREPARATION OF THE CRYSTALLINE ENZYME*

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SUMMARY

The deoxyribonucleic acid exonuclease induced by bacteriophage \( \lambda \) has been obtained in crystalline and physically homogeneous form. The purified preparation is free of detectable contamination by the endonuclease I, DNA polymerase, DNA phosphatase-exonuclease, alkaline phosphatase, 5'-nucleotidase, and ribonuclease activities known to be present in Escherichia coli; it may contain a trace (0.01%) of exonuclease I.

In 1962, Weissbach and Korn discovered a new deoxyribonucleic acid exonuclease in induced lysogen of the temperate bacteriophage \( \lambda \) (1, 2). Subsequent studies have shown that, whenever normal vegetative phage development proceeds, \( \lambda \) exonuclease is synthesized (3-5). A cell lysogenic for phage \( \lambda \) does not produce the enzyme when its prophage is repressed, but upon induction of vegetative phage growth the enzyme appears. Infection of a sensitive cell by phage \( \lambda \) also leads to vegetative growth and to synthesis of \( \lambda \) exonuclease. When, on the other hand, a \( \lambda \) lysogen is superinfected with wild type \( \lambda \), the growth of the superinfecting phage is repressed and the enzyme is not produced. When the superinfecting phage is sensitive to repression by the prophage, the superinfecting phage can grow and synthesis of \( \lambda \) exonuclease takes place. The enzyme appears after induction of a \( \lambda \) lysogen at a time closely following the time at which induction has become irreversible, as judged by disappearance of the ability to re-establish the repression system by superinfection with the noninducible mutant \( \lambda_{\text{ind}} \). Its appearance is thus one of the earliest detectable events in vegetative phage multiplication.

Among defective mutants of phage \( \lambda \), two classes have been observed which display altered phenotypic expression of \( \lambda \) exonuclease following induction. The first type, located in the N cistron of the \( \lambda \) genome (6), induces no detectable enzyme (7, 8). Mutants of this class also produce little or no phage-specific DNA, no phage lysozyme, and no tail antigen (9-12). It has been suggested that in these mutants the expression of early phage functions may have suffered a general block (11). The second type of defective mutant, typified by \( \lambda_{711} \), produces abnormally high levels of \( \lambda \) exonuclease (13) and makes no phage DNA or lysozyme. Synthesis of the enzyme by induced \( \lambda_{711} \) lysogens continues for several hours, in contrast to the cessation of synthesis which is observed late in the growth cycle of wild type \( \lambda \). This suggests that a control mechanism may be impaired in these mutants as well. Finally, several other defective mutants produce the enzyme but little viral DNA or lysozyme (7).

The physiological role of \( \lambda \) exonuclease is not known. The kinetics of its appearance following induction or infection indicate that it is an early function (3, 10). The alterations of its phenotypic expression in the defective mutants support this notion. Since viral DNA synthesis also begins early in the growth cycle and since \( \lambda \) exonuclease is active on DNA in vitro, it is possible that the enzyme plays some part in \( \lambda \) DNA replication. We sought to characterize \( \lambda \) exonuclease in the hope that knowledge of its catalytic properties would provide some insight into its physiological role. Although Korn and Weissbach had achieved extensive purification of \( \lambda \) exonuclease (2, 14), their most highly purified fractions still contained DNA phosphatase activity and DNA polymerase activity. Our first goal was thus to purify the enzyme further in order to determine whether it could be freed of known Escherichia coli enzymatic activities related to DNA synthesis and breakdown. This report describes the purification and crystallization of \( \lambda \) exonuclease from an induced lysogen of \( \lambda_{711} \) and an evaluation of the physical and catalytic purity of this enzyme preparation. The following paper (15) will describe the rather unusual exonucleolytic reaction catalyzed by the enzyme.

EXPERIMENTAL PROCEDURE

Materials

Unlabeled deoxyribonucleoside triphosphates were purchased from Calbiochem. \( \alpha^{32}\text{P}\)-labeled dTTP was purchased from International Chemical and Nuclear Corporation, City of Industry, California, and was further purified according to the procedure of Lehman et al. (16). Streptomycin sulfate (Lot 3B-5607) was the generous gift of Merck. Polyethylene glycol (Carbowax 6000) was purchased from Union Carbide Corporation, New York; Dextran 500 (Lot To 852) from Pharmacia, Uppsala, Sweden; crystalline bovine plasma albumin from the Armour Pharmaceutical Company; DEAE-cellulose (Cellex D) from BioRad Laboratories, Richmond, California; phosphocellulose (P-70, Lot 178) from Whatman; and Bacto-tryptone from Difco.
M-9 minimal medium (17) was modified to contain the additional components: 0.5 g of NaCl per liter and 0.2 g (instead of 0.15 g) of MgSO₄ per liter.

Tris-EDTA buffer was 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

**Bacteria and Phage—**E. coli K-12 strains used were: W3350 (λcigw) (18), transmitted by Dr. R. L. Baldwin; W3101 gal⁻, previously described (19); and T11 (λT11), the kind gift of Dr. C. M. Radding (13). A mutant of strain W3101 resistant to phage T1 was isolated by plating with an excess of phage T1; a resistant colony, designated W3101/1,5, was isolated and purified. The defective λ strain, λcigw, was transferred to W3101/1,5 by ultraviolet induction of T11 (λT11) and superinfection with λcigw ac described by Radding (13). The resulting lysogen, W3101 (λT11)/1,5, was used as a source of λ exonuclease in the purification described below.

Bacteriophages λ⁺ and λcigw were described previously (20). Lysates of phage λcigw were produced by temperature induction of W3350 (λcigw) at a density of 3 to 4 x 10⁶ cells per ml, and phage were purified as previously described (21). Stocks of bacteriophage T7 were prepared as described by Richardson, Inman, and Kornberg (22), except that M-9 glucose minimal medium was used. Following two cycles of low and high speed centrifugation, the phage were banded in a nonequilibrium CsCl density gradient (23), produced by layering in a tube successive 1.5-ml volumes of CsCl solutions of density 1.4, 1.5, and then 1.3 g per ml, followed by 1.0 ml of phage suspension in 1.0 M NaCl 0.01 M Tris HCl, pH 8.0. After centrifugation for 1 hour at 35,000 rpm in the SW50 rotor of the Spinco model L ultracentrifuge, the phage band was collected and dialyzed against 0.1 M Tris HCl, pH 8.0-0.1 M NaCl.

**Enzymes—**E. coli endonuclease I (24), exonuclease I (25, 26) exonuclease III (27), and DNA polymerase (28) were purified and assayed as previously described, as was Bacillus subtilis nuclease (29). Micrococcal endonuclease from Micrococcus pyogenes was the gift of Dr. C. A. Dekker.

Antiserum to λ exonuclease was produced by intraperitoneal injection into a rabbit of 160 µg of Fraction VI enzyme in Freund’s adjuvant, followed by secondary injections of 20 µg of enzyme given intravenously on Days 30 and 40. Bleeding was done by heart puncture on Day 52. Blood was permitted to clot at 25° for 6 hours, then overnight at 5°; it was then centrifuged for 5 min at 1000 x g. The serum was diluted 5-fold with 0.15 M KCl-0.01 M potassium phosphate, pH 7.0, and heated at 70° for 45 min to inactivate nucleases.

**Nucleic Acids—**DNA was isolated from bacteriophages M13, T7, and λcigw by phenol extraction as previously described for the DNA of phages λdg and λ⁺ (29). Carrier DNA was either salmon sperm DNA purchased from Calbiochem or calf thymus DNA isolated according to the method of Kay, Simmons, and Downe (30); it was dissolved in 0.02 M NaCl at a concentration of 2.5 mg per ml. ³²P-Labeled E. coli DNA, 10⁶ cpm per µmole, was prepared as previously described (25). ³²P-Phosphoryl-terminated ³²P-DNA was prepared according to the procedure of Richardson and Kornberg (27). dAT copolymer ² was prepared as described by Schachman et al. (31). Heat-denatured ³²P-labeled E. coli DNA was digested with B. subtilis nuclease ³ or with E. coli exonuclease I (25) to produce ³²P-labeled deoxyribonucleotide 3'- or 5'-monophosphates, respectively. Following acid precipitation to remove undigested material, mononucleotides were recovered from the supernatant fluid by adsorption to and elution from Norit. Ribosomal RNA was isolated by phenol extraction of E. coli ribosomes (32). All concentrations of polynucleotides are expressed in terms of moles of mononucleotide.

**Methods**

**Assay of λ Exonuclease—**The assay measured the conversion of native ³²P-labeled E. coli DNA to acid-soluble products. The standard assay mixture (0.3 ml) contained 67 mM glycine-KOH, pH 9.4, 2.5 mM MgCl₂, 67 µM ³²P-labeled E. coli DNA, and 0.02 ml of enzyme, added last. After a 30 min incubation at 37°, tubes were chilled to 0°. Acid-soluble ³²P was determined as described previously (33).

One unit of λ exonuclease is defined as that amount of enzyme which renders 10 µmole of DNA acid soluble in 30 min under standard assay conditions. The range of linearity in the assay varied from one preparation of E. coli DNA to another and was checked for each preparation. In general, the assay was linear over the range of 0.02 to 0.2 unit of enzyme. A variation in apparent activity of as much as 2-fold was observed when a given enzyme preparation was assayed with different DNA preparations. To minimize variability within a given preparation, the DNA was divided into small aliquots after isolation, quickly frozen at -70°, and stored at -20°; once thawed the DNA was not refrozen.

**Other Methods—**The radioactivity of ³²P-labeled samples was determined in an end window gas flow counter; in some cases a low background counter was used. Analytical ultracentrifugation was performed with a Spinco model L ultracentrifuge equipped with ultraviolet absorption optics, schlieren optics, and RTIC temperature control. Photographs were traced with a Joyce-Loebl photodensitometer. The percentage of single-strand breaks in bacteriophage DNA preparations was estimated by alkaline zone sedimentation (34, 35), and was taken as 100 the ratio of the height of material trailing behind the main zone to the maximum height of the zone. For example, from the tracing in Fig. 3e we estimated 30% single-strand breaks for that particular DNA preparation.

Preparative ultracentrifugation was done in a Spinco model L ultracentrifuge, except where otherwise noted. Protein was assayed by the method of Lowry et al. (36), with bovine plasma albumin as standard. Optical density was measured in a Zeiss PMQ II spectrophotometer. The pH of all buffers was measured at 25° and at a concentration of 0.05 M.

**RESULTS AND DISCUSSION**

**Purification of Enzyme**

λ exonuclease was purified by a method derived from the procedure of Korn and Weissbach (2). A plentiful source of enzyme was provided by Radding’s discovery (13) that a defective λ mutant, λT11, induces the production of greatly enhanced levels of enzyme. A derivative of E. coli K-12 lysogenic for phage λT11 was prepared and designated W3101 (λT11)/1,5. Under the conditions of induction and subsequent growth de-
Purification of Χ exonuclease

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>9.5</td>
<td>360 (100)*</td>
<td>30</td>
</tr>
<tr>
<td>II. Streptomycin eluate</td>
<td>7.8</td>
<td>2,230</td>
<td>82</td>
</tr>
<tr>
<td>III. Phase partition</td>
<td>6.5</td>
<td>2,620</td>
<td>69</td>
</tr>
<tr>
<td>IV. Dialysis eluate</td>
<td>5.2</td>
<td>6,170</td>
<td>55</td>
</tr>
<tr>
<td>V. DEAE-cellulose chromatography</td>
<td>2.9</td>
<td>11,400</td>
<td>31</td>
</tr>
<tr>
<td>VI. Phosphocellulose chromatography</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total enzyme recovered</td>
<td>2.15</td>
<td>49,000</td>
<td>23</td>
</tr>
<tr>
<td>Peak fraction</td>
<td>0.87</td>
<td>49,000*</td>
<td>9.2</td>
</tr>
</tbody>
</table>

* The yield of the crude extract was set at 100%.

Specific activity of the final preparation ranged between 24,000 and 49,000 units per mg of protein, depending upon the preparation of 32P-labeled E. coli DNA used for assay. The specific activities of the other fractions varied in proportion.

scribed below, the specific enzymatic activity of a crude extract made from this strain was 30 times greater than that observed in a crude extract of W3101 (λ+)/1-5 made 70 min after ultraviolet induction.

Growth of Cells and Induction of Enzyme—A culture of W3101 (λR)1-5 was grown at 37°C with forced aeration in the Biogen apparatus (American Sterilizer Company), with the use of M-9 minimal medium with glucose (4 g per liter) as a carbon source. At a density of 107 cells per ml the culture was induced by irradiation with a dose of ultraviolet light previously determined to give maximum enzyme production (four 20-sec pulses, separated by 40-sec intervals, in our apparatus). After induction, 0.1 volume of the gradient was 1000 ml, and 0.02 M NaCl-0.02 M Tris-HCl, pH 7.4; the diluted material was immediately applied to the column at a flow rate of 4 ml per min. The column was washed with 200 ml of 0.15 M NaCl-0.02 M Tris-HCl, pH 7.4, then eluted with a linear gradient of NaCl from 0.15 to 0.35 M. The total volume of the gradient was 1000 ml, and 0.02 M Tris-HCl, pH 7.4, was present throughout. Fractions of 20 ml were collected; the enzyme was eluted as a single peak between 2.6 and 4.7 column volumes. Fractions with a specific activity representing a purification greater than 1.6-fold over Fraction IV were pooled to yield Fraction V.

Fraction VI, Phosphocellulose Chromatography—A column of phosphocellulose (Whatman P-70), 16 cm × 8.6 cm2, was washed with 500 ml of 1 mM EDTA, 0.05 M potassium phosphate, pH 7.0, and 0.01 M β-mercaptoethanol, and then with 2 liters of 0.01 M potassium phosphate, pH 7.0, and 0.01 M β-mercaptoethanol. Fraction V, 280 ml, was dialyzed for 4 hours each against two successive 8-liter portions of 0.01 M potassium phosphate, pH 7.0, and 0.01 M β-mercaptoethanol, and the dialyzed solution was applied to the column at a flow rate of 4 ml per min. The column was washed with 290 ml of the same buffer; a stepwise wash then carried out with successive 250-ml volumes of 0.05 M NaCl, 0.10 M NaCl, and, finally, 0.25 M NaCl; 0.01 M potassium phosphate, pH 7.0, and 0.01 M β-mercaptoethanol were present throughout. The enzyme was eluted promptly with the 0.25 M

I were added 115 ml of a cold, freshly prepared 5% solution of streptomycin sulfate over a period of 30 min with continuous stirring. The resulting suspension was stirred for 30 min, then centrifuged for 10 min at 19,000 × g; the supernatant fluid was discarded. The precipitate was dissolved in 800 ml of 1 M NaCl-0.05 M Tris-HCl, pH 7.4. The solution was clarified by centrifugation at 12,000 × g for 10 min to yield Fraction II, a small amount of yellow precipitate being discarded.

Fraction III, Phase Partition-To 600 ml of Fraction II were added 230 g of NaCl, 258 ml of a 30% (w/w) solution of polyethylene glycol (Carbowax 6000), and 92 ml of a 20% (w/w) solution of Dextran 500 (Pharmacia) (37). The resulting suspension was stirred for 2 hours and then centrifuged for 5 min at 12,000 × g. The clear upper phase, containing polyethylene glycol and protein, was collected as Fraction III; the turbid lower phase, containing dextran and nucleic acids, was discarded.

Fraction IV, Dialysis and Elution of Precipitate—Fraction III (950 ml) was dialyzed in 30-ml portions for 4 hours against 25 liters of 0.02 M Tris-HCl, pH 7.4, then overnight against an additional 25 liters of the same buffer. Dialysis resulted in the formation of a heavy white precipitate containing the enzyme and gave an approximate doubling of the volume.

The suspension was centrifuged for 5 min at 12,000 × g, and the supernatant fluid was discarded. The precipitate was gently resuspended at room temperature in 190 ml of 0.2 M NaCl-0.02 M Tris-HCl, pH 7.4, with the aid of a loose fitting glass pestle, and gently homogenized for 5 min at room temperature. The suspension was then centrifuged for 5 min at 5°C and 12,000 × g, and the precipitate was discarded. The clear supernatant fluid was Fraction IV.

Fraction V, DEAE-cellulose Chromatography—A column of DEAE-cellulose (Bio-Rad Cellulose D), 30 cm × 3.8 cm2, was washed with 1000 ml of 0.15 M NaCl-0.02 M Tris-HCl, pH 7.4. To 180 ml of Fraction IV were added 60 ml of 0.02 M Tris-HCl, pH 7.4; the diluted material was immediately applied to the column at a flow rate of 4 ml per min. The column was washed with 200 ml of 0.15 M NaCl-0.02 M Tris-HCl, pH 7.4, then eluted with a linear gradient of NaCl from 0.15 to 0.35 M. The total volume of the gradient was 1000 ml, and 0.02 M Tris-HCl, pH 7.4, was present throughout. Fractions of 20 ml were collected; the enzyme was eluted as a single peak between 2.6 and 4.7 column volumes. Fractions with a specific activity representing a purification greater than 1.6-fold over Fraction IV were pooled to yield Fraction V.
NaCl; 5-ml fractions were collected. Enzyme fractions containing more than 1 mg of protein per ml were dialyzed against 0.01 M potassium phosphate, pH 7.0. Side fractions of the same specific activity and containing more than 50 μg of protein per ml were pooled and concentrated by pressure dialysis (27) to a protein concentration of 4 to 5 mg per ml, then dialyzed against 0.01 M potassium phosphate, pH 7.0. The dialyzed fractions represent Fraction VI. The final preparation was purified 89-fold over the crude extract and represented a recovery of 23% of the initial activity (Table I). Fraction VI was used in all the studies to be reported here and in the following paper (15).

Crystallization of Enzyme—Fraction VI, at a protein concentration of 4 mg per ml, was dialyzed against 100 volumes of a solution of ammonium sulfate, previously adjusted to pH 7.0 and containing 0.01 M potassium phosphate, pH 7.0. The initial concentration of ammonium sulfate was 25% of saturation at 5°; it was increased by 1% of saturation every 12 hours by the addition of saturated ammonium sulfate solution at pH 7.0, until a faint flocculent suspension appeared (at approximately 31% of saturation). This gradually grew denser and acquired the silky sheen characteristic of crystalline protein preparations in suspension. After several days the ammonium sulfate concentration was increased by 1% of saturation, and 3 days later the suspension was taken from the dialysis bag and stored at 5°. The crystals contained more than 90% of the enzymatic activity.

Stability—A preparation of the enzyme which had been stored in solution for 1 year at −20° and had been thawed twice displayed >90% of the specific activity of a freshly isolated preparation. Little loss of activity was observed upon storage for 2 months at 0° as well. Repeated freezing and thawing led to some precipitation of protein and consequent loss of activity. Heating to 60° for 5 min in the absence of substrate completely (>99%) inactivated the enzyme.

Optimum Reaction Conditions

pH Optimum—The enzyme displayed optimal activity at pH 9.2 to 9.5. At pH values of 7.0, 8.0, 10.0, and 10.2, the rates were 18%, 35%, 35%, and 5%, respectively, of maximal.

Metal Ion Requirement—A divalent cation (Mg++ or Mn++) was required for enzymatic activity. The optimal Mg++ concentration was 1.0 to 2.5 μM; at 0.05 μM, 0.25 μM, and 20 μM, the rates were 13%, 72%, and 19%, respectively, of that at 1 μM. In the absence of Mg++, with or without EDTA (10−4 M), less than 1% of the activity at 2.5 μM Mg++ was observed. In the presence of 2.5 × 10−3 M Mn++, the rate was 30% of that observed with Mg++. Of other metal cations tested (Ca++, Cd++, Co++, Cu++, Fe++, Ni++, and Zn++), none satisfied the requirement (<5% of the rate with Mg++).

Inhibitors—Salt at relatively low concentrations inhibited the enzyme. Assays performed in the presence of 0.05 M, 0.1 M, and 0.2 M KCl showed 31%, 83%, and 99% inhibition, respectively, of the activity in the absence of KCl. A stronger inhibition was afforded by Na+ ion; with glycine-NaOH in place of glycine-KOH, 0.025 M, 0.05 M, and 0.1 M NaCl produced 52%, 82%, and 99% inhibition, respectively, of the activity in the absence of NaCl. A strong inhibition by low levels of p-chloro-
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mother liquor, and the starting material displayed specific activities of 25,000, 23,000, and 25,000, respectively.

Upon high speed sedimentation of λ exonuclease in the analytical ultracentrifuge, a single sedimenting component was observed (Fig. 2), with an $s_{20,w}$ of 5.0 S. At a protein concentration of 10 mg per ml, a very small peak of rapidly sedimenting (30 S) material was seen which represented perhaps 1% of the total. The broadness of the peak in these photographs may again be the result of aggregation.

By all these criteria, the enzyme preparation appears to be at least 90% pure.

Catalytic Purity

The purified λ exonuclease preparation was assayed for the presence of contaminating enzyme activities. Assays for several

FIG. 2. Sedimentation diagram of purified λ exonuclease. Schlieren patterns showing the sedimentation of λ exonuclease, Fraction VI, at 2.7 mg per ml in 0.1 M KCl-10 mM potassium phosphate, pH 6.5. A 2° filled Epon double-sector cell was used, the rotor speed was 59,780 rpm, and the temperature was 5°. Left, 6 min after speed was reached, phase plate angle, 70°. Right, 70 min after speed was reached, phase plate angle, 60°.

Photographs were taken at 16, 18, 20, and 18 min, respectively after speed was reached, and were traced on a Joyce-Loebl photodensitometer. The spike at the left in each tracing is the meniscus; sedimentation is from left to right. Tracings are numbered to correspond to the reaction mixtures described above. In Tracings e and f, the sedimentation conditions were the same as for Tracings a through d, except that the bulk solution was 0.9 M NaCl-0.1 M NaOH-1 mM EDTA. Tracings e and f correspond to Samples a and b, respectively. Photographs were taken 17 and 19 min, respectively, after speed was reached. Since endonuclease I produces double-strand breaks (35), zone sedimentation in alkali is a less sensitive criterion for its presence and so was not performed on Samples e and d.

mercuribenzoate has also been reported (14). Unlike endonuclease I, the enzyme was not inhibited by soluble RNA at a concentration of $10^{-4}$ M.

Physical Purity

The state of physical purity of the enzyme was judged by several criteria. High voltage electrophoresis on starch gel (38) (Fig. 1.4) resulted in the appearance of a single zone of protein. Polyacrylamide gel electrophoresis (39, 40) (Fig. 1B) revealed a single major component and, in addition, a very faint trailing component which represents, at most, 5% of the protein. The broadness of the zones in both systems may be due to a rapidly reversible aggregation which the enzyme appears to undergo (41).

When the enzyme was crystallized under conditions which left 5% of the activity in the mother liquor, the washed crystals, the

nucleases known to be present in E. coli were carried out, but none was found at detectable levels, with the possible exception of a trace of exonuclease I.

Endonuclease I—The activity of λ exonuclease is inhibited by the presence of relatively low concentrations of NaCl (see above). In 0.2 M NaCl, for example, λ exonuclease exhibits <1% of its maximum activity, while endonuclease I (24) shows 40% of its maximum activity. Thus high levels of enzyme protein can be assayed for the presence of endonuclease I without degradation of the substrate by the λ exonuclease activity. Zone sedimentation of a homogeneous DNA (34, 35) serves as a sensitive measure of endonuclease activity, inasmuch as the product of endonucleolytic cleavage sediment more slowly than intact DNA and can be detected as material trailing behind the main zone. The product of treatment of T7 phage DNA by λ exonuclease, by E. coli endonuclease I, or by both enzymes was examined by zone

Fig. 3. Zone sedimentation of T7 phage DNA before and after treatment with λ exonuclease, endonuclease I, or both enzymes. Incubation mixtures (0.3 ml) contained 0.2 M NaCl, 67 mM Tris-HCl, pH 8.0, 1.0 mM MgCl$_2$, and 67 μM T7 phage DNA. Sample a, no enzyme; Sample b, λ exonuclease, 4.4 μg (215 units); Sample c, endonuclease I, 0.0064 unit; Sample d, both enzymes, at respective levels indicated in Samples b and c. Incubations were for 30 min at 37° and were terminated by the addition of 0.5 μmoles of EDTA, pH 7.0. The reaction mixtures were then dialyzed overnight against 400 volumes of Tris-EDTA buffer. Tracings a through d, zone sedimentation was performed as described by Studier (35), with the use of 1.0 M NaCl-10 mM Tris-HCl, pH 8.0-1 mM EDTA as bulk solution; the rotor speed was 35,000 rpm.

Photographs were taken at 16, 18, 20, and 18 min, respectively after speed was reached, and were traced on a Joyce-Loebl photodensitometer. The spike at the left in each tracing is the meniscus; sedimentation is from left to right. Tracings are numbered to correspond to the reaction mixtures described above. In Tracings e and f, the sedimentation conditions were the same as for Tracings a through d, except that the bulk solution was 0.9 M NaCl-0.1 M NaOH-1 mM EDTA. Tracings e and f correspond to Samples a and b, respectively. Photographs were taken 17 and 19 min, respectively, after speed was reached. Since endonuclease I produces double-strand breaks (35), zone sedimentation in alkali is a less sensitive criterion for its presence and so was not performed on Samples e and d.
sedimentation at pH 8.0 for assessment of double-strand scissions (Fig. 3, Tracings a to d), and in alkali for the detection of single-strand breaks (Fig. 3, Tracings e and f). No increase in the amount of material trailing behind the main zone was observed upon treatment of T7 DNA with 215 units of the λ exonuclease preparation (compare Tracings a and b, and Tracings c and d). In control experiments, treatment of T7 DNA with as little as 0.0064 unit of endonuclease I gave rise to fragmented material sedimenting behind the position of intact molecules; this activity was not inhibited by the presence of λ exonuclease (compare Tracings c and d). On the assumption that scission of 10% of the strands is detectible by this assay (such a level would be detected above background variations in the densitometer tracings) and that attack by endonuclease I is random, it can be calculated that the λ exonuclease preparation produces less than 1 endonucleolytic cleavage per 3 × 10^6 endonucleolytic scissions.

Exonuclease I—Purified λ exonuclease displays a high degree of specificity toward native DNA as substrate (see following paper (15)) and therefore cannot be grossly contaminated by exonuclease I (25). To detect smaller amounts of exonuclease I activity, antiserum directed against λ exonuclease was allowed to react with the enzyme preparation. In control experiments, treatment with antiserum followed by centrifugation to remove antigen-antibody complexes did not inhibit the activity of exonuclease I, whether carried out in the presence or in the absence of the λ enzyme (Table II). Thus any exonuclease I present in the λ exonuclease preparation should be detectable, after antiserum treatment, as activity toward denatured DNA. Treatment of purified λ exonuclease for exonuclease I

**Table II**

Examination of purified λ exonuclease for exonuclease I

<table>
<thead>
<tr>
<th>Enzyme added</th>
<th>DNA substrate</th>
<th>Activity</th>
<th>Residual activity after antiserum treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without antiserum</td>
<td>With antiserum</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>munits/25 μl</td>
<td>munits/25 μl</td>
<td></td>
</tr>
<tr>
<td>Exonuclease I</td>
<td>Denatured</td>
<td>0.051^a</td>
<td>0.060</td>
</tr>
<tr>
<td>Exonuclease I + λ exonuclease</td>
<td>Denatured</td>
<td>0.233</td>
<td>0.081</td>
</tr>
<tr>
<td>λ exonuclease</td>
<td>Native</td>
<td>5.1^b</td>
<td>0.0014</td>
</tr>
<tr>
<td></td>
<td>Denatured</td>
<td>0.220</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

^a Exonuclease I was partially inactivated by incubation in the absence of serum; assay of the enzyme without prior incubation showed an activity of 0.003 unit.

^b The incubation mixture was diluted for assay in the supernatant fluid from the control incubation mixture; the presence of NaCl in this diluent partially inhibited the action of the enzyme, lowering the above figure from the expected value of 10.0 units.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>λ exonuclease</th>
<th>DNA phosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium phosphate, pH 7.0</td>
<td>0.1</td>
<td>(1.0)</td>
</tr>
<tr>
<td>Tris-HCl, pH 8.0</td>
<td>1.5</td>
<td>0.44</td>
</tr>
<tr>
<td>Glycine-KOH, pH 9.4</td>
<td>6.6</td>
<td>0.16</td>
</tr>
</tbody>
</table>

^a Expressed as units of phosphatase activity per unit of exonuclease activity at pH 9.4.

^b Expressed as activity relative to that at pH 7.0 (set at 1.0).

**Table III**

Experiment 1, examination of purified λ exonuclease for DNA phosphatase activity

Incubation mixtures (0.15 ml) contained 67 mm potassium phosphate, pH 7.0, 6.7 mm MgCl₂, 10 mm β-mercaptoethanol, and 0.17 mm 3'-phosphoryl-terminated ³²P-labeled DNA (micrococcal endonuclease digest) (27). Where indicated, 67 mm potassium phosphate was replaced by 67 mm Tris-HCl, pH 8.0, or 67 mm glycine-KOH, pH 9.4. After incubation for 30 min at 37°, Norit-nonsorbable ³²P was determined as described by Richardson and Kornberg (27).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Prior incubation with polynucleotide kinase</th>
<th>³²P1 released</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>0.032</td>
</tr>
<tr>
<td>Exonuclease III</td>
<td>+</td>
<td>0.123</td>
</tr>
<tr>
<td>Exonuclease III</td>
<td>+</td>
<td>0.124</td>
</tr>
<tr>
<td>λ exonuclease</td>
<td>+</td>
<td>0.030</td>
</tr>
</tbody>
</table>

^a One unit of exonuclease III releases 1.0 mmoles of orthophosphate in 30 min under conditions described for Experiment 1, Table III; one unit of λ exonuclease releases 10 mmoles of acid-soluble nucleotide in 30 min under standard assay conditions (pH 9.4).

^b Second (30-min) incubation was omitted.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Prior incubation with polynucleotide kinase</th>
<th>³²P1 released</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>0.032</td>
</tr>
<tr>
<td>Exonuclease III</td>
<td>+</td>
<td>0.123</td>
</tr>
<tr>
<td>Exonuclease III</td>
<td>+</td>
<td>0.124</td>
</tr>
<tr>
<td>λ exonuclease</td>
<td>+</td>
<td>0.030</td>
</tr>
</tbody>
</table>

**Table IV**

Experiment 2, examination of purified λ exonuclease for DNA phosphatase activity

Incubation mixtures (0.15 ml) contained 67 mm Tris-HCl, pH 8.0, 5 mm MgCl₂, 10 mm β-mercaptoethanol, 17 mm 3'-phosphoryl-terminated ³²P-labeled DNA; and 0.1 mm ATP. Where indicated, polynucleotide kinase, 0.5 unit, was added. Following incubation for 10 min at 37°, tubes were heated for 5 min at 60°, then chilled. Exonuclease III, 0.11 unit,* λ exonuclease, 22 units, was added as noted. After incubation for 30 min at 37°, Norit-nonsorbable ³²P was determined as described in Table III.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Prior incubation with polynucleotide kinase</th>
<th>³²P1 released</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>0.032</td>
</tr>
<tr>
<td>Exonuclease III</td>
<td>+</td>
<td>0.123</td>
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<tr>
<td>Exonuclease III</td>
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</tr>
<tr>
<td>λ exonuclease</td>
<td>+</td>
<td>0.030</td>
</tr>
</tbody>
</table>

* One unit of exonuclease III releases 1.0 mmoles of orthophosphate in 30 min under conditions described for Experiment 1, Table III; one unit of λ exonuclease releases 10 mmoles of acid-soluble nucleotide in 30 min under standard assay conditions (pH 9.4).

The activity toward native DNA, but 0.4% of that toward denatured DNA remained. This trace of activity toward denatured DNA represented approximately 1 part in 10⁶ of the activity toward native DNA before antiserum treatment. This level of activity may not be significant, since the actual radioactivity measured in the assay was only 30% above background and represented hydrolysis of only 0.57% of the substrate.

Exonuclease II (DNA Polymerase)—The exonuclease II activity of E. coli appears to be closely associated with DNA poly-
Exonuclease III (DNA Phosphatase)—The purified λ exonuclease preparation was assayed for the DNA phosphatase activity which appears to be associated with λ exonuclease III (27, 43). Under standard DNA phosphatase assay conditions (pH 7.0), λ exonuclease exhibited 1 part of DNA phosphatase activity per 1 x 10^8 parts of exonuclease (assayed at pH 9.4).

Two lines of evidence suggest that the actual level of exonuclease III is even less than 1 part in 10^9 and that the phosphatase activity observed may be intrinsic to λ exonuclease. First, the phosphatase activity of the enzyme preparation was higher at pH 9.4 than at pH 7.0, whereas purified exonuclease III is more active at pH 9.4 than at pH 7.4 (Table III). Second, pretreatment of the phosphatase assay substrate (a micrococcal endonuclease digest of 32P-labeled E. coli DNA) with ATP and polynucleotide kinase (44) resulted in a large stimulation of the phosphatase activity per 1 x 10^4 parts of exonuclease (assayed at pH 9.4).

Other Activities—λ exonuclease was examined for (a) ribonuclease activity (45) by assay on ribosomal RNA substrate, (b) alkaline phosphatase (46) by assay on 32P-labeled deoxyribonucleoside 3' monophosphates, and (c) 5'-nucleotidase (47) by assay on 32P-labeled deoxyribonucleoside 5'-monophosphates. None of these activities was detectable at levels of 1 hydrolytic cleavage per 5 x 10^5, per 2 x 10^6, and per 10^6 exonucleolytic scissions in Assays (a), (b), and (c), respectively.

Acknowledgment—We are indebted to Dr. Charles Radding for providing us with the hyperactive mutant λ₁₁. 

REFERENCES