Purification and Characterization of DNA Polymerase III from *Bacillus subtilis*

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DNA polymerase III from *Bacillus subtilis* has been purified about 4,500-fold. Disc gel electrophoresis of the purified fraction reveals a single major protein band which co-migrates with the polymerase activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the polymerase yields a single, 166,000 dalton band. The hydrodynamic properties of the enzyme are ionic strength-dependent. The average values from determinations in high and low salt are 7.6 S for the sedimentation coefficient and 52 Å for the Stokes radius. These two parameters indicate a molecular weight for the native enzyme of 160,000. Therefore, the enzyme appears to contain a single, long, polypeptide chain. The enzyme has no endonuclease activity but does have single strand specific exonuclease activity. Hydrolysis is initiated exclusively from the 3' terminus yielding 5' mononucleotides, and a dinucleotide is the limit of digestion. The exonuclease activity has an ionic strength dependence and pH optimum similar to that of the polymerase but appears to be more fastidious in its divalent metal requirement. The mode of attack by the enzyme is strictly distributive. The activity of the exonuclease decreases markedly with increasing substrate size. Two opposing mechanisms account quantitatively for this effect—intrinsic competitive inhibition by interior substrate nucleotides and increasing accessibility of the substrate terminus to the enzyme with increasing chain length. The polymerase synthesizes DNA in the 5'→3' direction and the apparent $K_m$ for each of the deoxyribonucleoside triphosphates is about 1 μM. The polymerase replicates RNA-primed, φX174 DNA in the presence of *Escherichia coli* elongation Factors I and II. In contrast to polymerase III, *B. subtilis* DNA polymerase II has no detectable nuclease activity.

*Bacillus subtilis* has three distinct DNA polymerases, designated as polymerases I, II, and III (1-3). Studies of DNA polymerase I mutants demonstrated that this enzyme acts in the repair of chromosomal damage (4, 5). The function of DNA polymerase II is not known. The central role of DNA polymerase III in replication of the chromosome was firmly established by the isolation of an arylhydrazinopyrimidine-resistant mutant whose purified DNA polymerase III was drug-resistant (6); the arylhydrazinopyrimidines, such as OHP(NH)$_2$Ura, selectively inhibit DNA polymerase III (7-9). Subsequently, several temperature-sensitive polymerase III mutants have been shown to fail to synthesize DNA at nonpermissive temperatures (10, 11). In addition to synthetic activity, DNA polymerase III catalyzes the hydrolysis and pyrophosphorolysis of DNA and the exchange of PP$_i$ with deoxyribonucleoside triphosphates (11, 12). The intrinsic nature of these activities is shown by their OHP(NH)$_2$Ura sensitivity, and the exonuclease and polymerase activities co-purify, have identical thermolability in enzyme purified from wild type and temperature-sensitive mutant cells, and are equally scavenged into the ternary complex with OHP(NH)$_2$Ura and DNA (11-13).

In order to gain insight into the mechanism of chromosomal replication, the enzymological properties of *B. subtilis* DNA polymerase III have been examined (2, 3, 12, 14). The enzyme has also been used to determine the mechanism of inhibition by the arylhydrazinopyrimidines with a molecular detail not yet achieved for any other DNA synthesis inhibitor (reviewed in Ref. 15). In this paper, we report the extensive purification of the enzyme to near homogeneity and some of its physical and enzymological properties. A quantitative analysis of the intrinsic exonuclease properties is also presented. Some of these results have been summarized recently (11, 13).

**MATERIALS AND METHODS**

* Growth of Bacteria—*Bacillus subtilis* DNA polymerase III was purified from the polymerase I-deficient strain BC26(F) (2). Bacteria were grown as described previously except the harvested cells were washed twice with "subtilis salts" before storage at -20° (2).

* Enzymes and Protein Standards—Bacterial alkaline phosphatase, pancreatic DNase I, snake venom phosphodiesterase, and *Micrococcus luteus* primer-independent polynucleotide phosphorylase were obtained from Worthington Biochemical Corp. Grade IV *Escherichia coli*

* OHP(NH)$_2$Ura: 6-(p-hydroxyphenylhydrazino)-uracil.

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β-galactosidase, crystallized bovine serum albumin, yeast alcohol dehydrogenase, beef liver catalase, yeast glucose-6-phosphate dehydrogenase, ovalbumin, and type II rabbit muscle lactic dehydrogenase were purchased from Sigma Chemical Co. and calf thymus DNA from Calbiochem-Behring. B. subtilis DNA polymerase III, the enzyme obtained at Step 5 in the polymerase III purification detailed below was first purified by phosphocellulose chromatography as has been described (2). Peak fractions were further purified by DNA-cellulose column chromatography as described in the legend to Fig. 15. The enzyme was concentrated by successive dialysis against polyethylene glycol (Carbowax 6000) and 50% glycerol buffers and stored at -20°C.

Chemicals—N-Ethylmaleimide, sodium dodecyl sulfate, Coomassie brilliant blue R, dithiothreitol, spermine, and GSH were obtained from Sigma Chemical Co. The spermidine was passed through Chelex 100 to minimize divalent metal ion contamination. Streptomycin sulfate was purchased from Nutritional Biochemicals Corp. Sephadex G 50, G 75, and G 200, Sepharose 4B, and DEAE Sephadex (A-25) were purchased from Pharmacia Fine Chemicals. DEAE-cellulose (DE11 and DE52), DEAE-cellulose paper (DE81), and phosphocellulose (PE11), and cellulose (CP11) were from Beece Angel. Hydroxylapatite (Bio-Gel HTP powder) and Chelex 100 were obtained from Bio-Rad. The antimicrobial agent 6-(p-hydroxyphenylazo)uracil was provided by Dr. Bernard Langley of Imperial Chemical Industries, Ltd., Macclesfield, England, and was chemically reduced immediately before use (7).

Nucleotides and Nucleic Acids—Tritium-labeled nucleoside triphosphates and 32P, were purchased from Schwarz BioResearch. Unlabeled nucleotide triphosphates were obtained from Sigma Chemical Co. and purified by DEAE-Sephadex chromatography before use. [-3H]dCTP and Na[32P]PPi were from New England Nuclear Corp. [35S]ATP (2 x 10⁶ cpm/pmol) was prepared by the method of Goedken and Wyman (17) and purified by DEAE-Sephadex chromatography. Unlabeled poly(dG), poly(U), and d(T)₉₋₁₅ were obtained from Collaborative Research. Poly(dA)y was synthesized enzymatically (18); [32P]dGTP and the deoxyoligonucleotide pG-C-T-T-C-C-G-A (19) were gifts of Dr. K. Agarwal. E. coli DNA was isolated by the method of Marmur (20) and col E, DNA was provided by Dr. A. Markovitz of the University of Chicago. The template-primer for DNA polymerase III was salmon testes DNA digested by pancreatic DNAse I to 3% acid-soluble A₂₆₀. Single-stranded, 3'-phosphohovorl-terminated DNA was produced by micrococcal nuclease treatment of E. coli [3H]DNA to 9% acid solubility followed by heating at 100° for 2 min. A portion was treated with bacterial alkaline phosphatase and phosphorylated control. Sonicated E. coli [3H]DNA was prepared by eight 30-s sonic bursts at 1.25 A with a MSE sonicator. Native E. coli [3H]DNA was separated from single-stranded DNA by chromatography with benzoylated, naphthoylated DEAE-cellulose (Gerhard Schiesinger Co.) by the procedure of Schlegel et al. (21). Single-stranded DNA was prepared by heat denaturing native DNA at 100°C for 2 min followed by rapid chilling in ice water. Minicircular [3H]DNA from E. coli 15 was prepared according to the method of Cozzarelli et al. (22). N4, T7, and φ1 phage DNA were extracted with sodium dodecyl sulfate and phenol from CsCl gradient-purified virions. The concentration of polynucleotides is expressed as nucleotide equivalents unless otherwise indicated.

DNA-cellulose was prepared by a modification of the method of Litman (23). Salmon testes DNA (225 mg, 1.5 mg/ml) which had been treated with DNase I as described above was mixed with 50 μl of CFF1 cellulose. The thick paste was air-dried overnight and then desiccated under vacuum for several hours. After grinding to a fine powder, 5-μl portions were suspended in 25 μl of absolute ethanol and, while gently stirring, exposed to a dose of 10⁴ ergs/mm² of ultraviolet light. Unbound DNA was removed by extensive washing with 10 μl Tris-HCl, pH 7.3, 0.05 M EDTA, and 1.0 M NaCl, and then with the same buffer without NaCl. About 50% of the initial DNA remained bound. The washed DNA-cellulose was stored as a dry powder at -20°C. Without the photochemical treatment, the DNA sometimes leaked off the cellulose during chromatography.

Preparation of Labeled Synthetic Polynucleotides—[3H]Poly(rU) was synthesized using polynucleotide phosphorylase and [3H]UDP (50 cpm/pmol) (24). The reaction was terminated by adding EDTA to 10 mM and by heating at 100°C, 5 min. Unreacted UDP was removed from the polymer by Sephadex G-50 filtration.

[3H]Poly(dT) was synthesized with terminal deoxynucleotidyltransferase (TdT) and [3H]dGTP (15 cpm/pmol) according to the method of Bollum (25). The average size of the product, measured as described below, was varied by adjustment of the concentration of the primer and dGTP. The reaction was monitored by spotting aliquots on strips of DEAE-cellulose (DE52) paper (2 x 5 cm). The strips were washed three times with 0.4 M ammonium formate, pH 8.0, twice with 40% ethanol, and once with diethyl ether, dried, and the radioactivity measured. About 60 to 80% of the monomer was incorporated. The polymers were purified by Sephadex G-75 filtration followed by dialysis, first against 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 1.0 M KCl, and then against the same buffer without KCl.

The polymer [3H]poly(dT),, was prepared analogously using [3H]dCTP (200 cpm/pmol) at 0.5 μM primer termini and 0.5 mM monomer, respectively. The reaction was terminated after about 10% of the [3H]dCTP had been incorporated by adding EDTA to 5 mM and heating at 100°C for 3 min. This product, like the three subsequent doubly labeled polymers, was purified by filtration through Sepharose 4B and concentrated against dry Sephadex C-200. The average length of the poly(dC) portion was calculated from the specific activities and final ratio of [3H] and [32P], the concentration of primer termini, and the average chain length of the poly(dT) primer.

[3H]Poly(dC),, was synthesized with [3H]dATP (20 cpm/pmol) at 0.5 μM primer termini and 1.0 mM monomer, respectively. The mixed polymer was purified after about 40% of the [3H]dATP had been incorporated.

To synthesize [5-32P,3H]poly(dT),, [3H]poly(dT),, and [α-32P]poly(dT) (200 cpm/pmol), 0.5 μmol, was treated with 2.5 units of bacterial alkaline phosphatase for 15 min at 37°C. The phosphatase was inactivated by adding EDTA and NaOH to 5 and 100 mM, respectively, and heating for 10 min at 37°C. After neutralization, the poly(dT) was phosphorylated using 60 μM [3H]ATP (4000 cpm/pmol) and T4 polynucleotide kinase by the method of Richardson (26). The kinase reaction was terminated by the addition of EDTA to 20 mM and heating at 100°C, 2 min, and the polymer was purified. A portion of this polymer was extended with unlabeled poly(dC) by the procedure described above for the synthesis of [3H]poly(dT),, and [α-32P]poly(dT). The oligonucleotides (about 10⁴ cpm/pmol) were purified by filtration through Sephadex G-50 and concentrated by freeze drying.

Chromatography and Electrophoresis—Chromatography and electrophoresis used Whatman No. 3MM paper. The electrophoresis buffer was 0.05 M sodium citrate, pH 5.5. The solvent for chromatography was isotonic acid/1 M NH₄OH/0.1 M EDTA, 100/80/1.8 (Solvent II).

Enzymatic Reactions—The standard assay for DNA polymerase III contained in a total volume of 0.1 ml: 60 mM Tris-HCl pH 7.5, 6.5 mM MgCl₂, either 3 mM 2-mercaptoethanol or 2 mM dithiothreitol, 0.3 mM dNase I-treated salmon testes DNA, 10% glycerol, 0.1 mg/ml of albumin, 40 μg each of dATP, dCTP, and dGTP, 10 μM [3H]dATP (1 to 5 x 10⁶ cpm/mmol), and 0.002 to 0.02 unit of polymerase. After a 10-min incubation at 30°C, the reaction was stopped by the addition of 0.1 ml of 0.4 mg/ml of salmon testes DNA, 0.1 M Na₂P₂O₇, 20 mM EDTA followed by 0.4 ml of 1.2 M perchloric acid, and 0.1 M Na₂HP₄. After 10 min at 0°C, the acid precipitates were collected on Whatman GF/C glass fiber filters, washed four times with 5 ml of cold 0.1 N HC1/0.1 M Na₂HP₂O₇, and once with 10 ml of 95% ethanol. After drying, the filters were counted in toluene-based scintillation fluid. One unit of polymerase catalyzes the conversion of 10 nmol of total nucleotide into an acid-insoluble product in 30 min at 30°C; unless otherwise indicated, the amount of polymerase III is given in these units and not the units of the intrinsic exonuclease.

The standard reaction mixture for assay of the polymerase-associated exonuclease contained in a total volume of 50 μl: 30 mM Tris-HCl, pH 7.5, 6.5 mM MgCl₂, 3 mM dithiothreitol, 10% glycerol, 0.1 mg/ml of albumin, 25 μg sonicated, heat-denatured E. coli [3H]DNA (1.6 x 10⁶ cpm/mmol), and 0.002 to 0.02 unit of enzyme. After a 30-min incubation at 30°C, the reaction was quenched on ice and 50 μl of 1.5
mg/ml of salmon testes DNA and 50 μl of 2.5 M perchloric acid were added. After a 10-min, 0°C incubation, the mixtures were centrifuged at 9000 x g for 10 min at 0°C, and a 75-μl aliquot of the supernatant was counted in Tl-ton-supplemented scintillation fluid. One unit of activity catalyzes the release of 10 nmol of total nucleotide in 30 min at 30°C. 

In reactions which used [3H]poly(dt) in place of E. coli [3H]DNA, 20 μl of 0.7% uranyl acetate were added with the carrier DNA and perchloric acid to increase the precipitation of the homopolymer.

Endonuclease activity was sometimes monitored by the decrease in radioactivity adsorbed to DE81 strips.

Homochromatography—Homochromatography of [5'3P]PPG -C-TPC-C-G-A digestion intermediates used the Kiel et al. (28) modification of the method of Brownlee and Sanger (29). Glass plates were coated with a 255-mm layer of Avicel microcrystalline cellulose (Brinkmann) to MN300 DEAE-cellulose (Macherey, Nagel, and Co.), 7.1. The nonadenyribonucleotide (16 μM) was digested in 6 μl of the standard exonuclease reaction mixture by 0.01 unit of polymerase III at 37°C for 30 min and the soluble radioactivity was measured by a scan of a stained polyacrylamide gel rather than by the usual procedure. The acid-precipitable radioactivity in the supernatant was measured by a scintillation counter. The acid-extracted radioactivity in the supernatant was measured by a scintillation counter. The acid-extracted radioactivity in the supernatant was measured by a scintillation counter.

Results

Purification of DNA Polymerase III

The results of a purification from 200 g of cells are summarized in Table I. All operations were performed at 4°C.

Step 1: Preparation of Extract—Two hundred grams of BC26(F) were suspended in 400 ml of 0.05 M Tris-HCl, pH 7.5, 5 mM GSH, 10 mM MgCl2, 0.5 mM EDTA, and 10% glycerol (v/v). The suspension was passed once through a French pressure cell press at 18,000 psi. The viscous lysate was centrifuged at 16,000 x g for 1 hour and the supernatant was further clarified by ultracentrifugation at 44,000 x g for 3 hours (Fraction I).

Step 2: Streptomycin Sulfate Precipitation—The high speed supernatant (Fraction I) was usually diluted with 3 volumes of a 5 mM GSH and 0.5 mM EDTA solution titrated to neutrality

Determination of Stokes Radius—The Stokes radius was estimated by gel filtration or 0.2 M sodium phosphate, pH 7.0, according to the method of Siegel and Mottly (34). For the higher salt values, a column of Sephadex G-200 (0.64 cm x 90 cm) was equilibrated with 10% glycerol, 14 mM 2-mercaptoethanol, 0.5 mM EDTA, and phosphate at 4°C. DNA polymerase III (0.2 unit, Fraction VII, N4 [T]-phage, [3H]thymidine, alcohol dehydrogenase (200 units), catalase, and 300 units, and alcohol dehydrogenase (200 units), catalase, and 300 units were added to the sample. Approximately 0.4-ml fractions were collected. Albumin and human hemoglobin were monitored by the absorbance at 280 nm and 280 nm, respectively, and catalase, polymerase III, β-galactosidase, and alcohol dehydrogenase were assayed.

Protein Determinations—Protein determinations (37) were made after precipitation with cold 3% trichloroacetic acid. The low protein concentrations in Fraction VII were measured by densitometry of a brilliant blue R-stained sodium dodecyl sulfate-polyacrylamide gel and by a microfluorometric assay (38) after removal of interfering glutathione by dialysis and Sephadex G-50 filtration. In all protein assays, albumin was the standard.

Determination of Chain Lengths of Polynucleotides—Chain length was measured by enzymic end group labeling (36) and by sedimentation velocity. In the former method, DNA (about 10 pmol of ends) was dephosphorylated by treatment with 7.5 μl of bacterial alkaline phosphatase at 37°C for 10 min. To inactivate the phosphatase, the pH was raised to 13 and 10 mM EDTA was added, and incubation was continued for 10 min at 80°C. The 5'-hydroxyl termini were then phosphorylated using [γ-3P]ATP and 12 units of DNase-free T4 polynucleotide kinase; the reaction was terminated when phosphorylation reached a plateau. The DNA was purified by Sephadex G-50 gel filtration and the chain length calculated from the optical density and radioactivity of the polymer. The second procedure used sedimentation through 5 to 20% sucrose gradients containing 0.3 mM NaOH, 0.8 M NaCl, and 0.5 mM EDTA. The Studier (39) equation was used to calculate molecular weight from s20,W. Internal standards were either N4, T7, or col E1 DNA depending on the size of the polymer.

Table I

<table>
<thead>
<tr>
<th>Protein Specific Activity</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>High speed supernatant</td>
<td>19,500</td>
<td>11,000</td>
<td>1.1</td>
</tr>
<tr>
<td>II. Streptomycin sulfate</td>
<td>9,700</td>
<td>2,400</td>
<td>4.0</td>
</tr>
<tr>
<td>III. DEAE-cellulose I</td>
<td>7,600</td>
<td>810</td>
<td>9.4</td>
</tr>
<tr>
<td>IV. Sephadex G-200</td>
<td>4,400</td>
<td>220</td>
<td>20</td>
</tr>
<tr>
<td>V. DEAE-cellulose II</td>
<td>3,900</td>
<td>59</td>
<td>66</td>
</tr>
<tr>
<td>VI. Phosphocellulose</td>
<td>1,500</td>
<td>3.7</td>
<td>410</td>
</tr>
<tr>
<td>VII. DNA-cellulose</td>
<td>320</td>
<td>0.06*</td>
<td>5,000</td>
</tr>
<tr>
<td>VIII. Hydrolysapto</td>
<td>1,050</td>
<td>0.52</td>
<td>2,000</td>
</tr>
</tbody>
</table>

*To conserve purified enzyme the protein concentration in this fraction was measured by a microfluorometric assay and by a scan of a stained polyacrylamide gel rather than by the usual procedure. The two determinations differed by 40% and the mean value is shown.
with 1 M Tris base. Sometimes a more extensive dilution was required for good precipitation of polymerase III by streptomycin. A 5% solution of streptomycin sulfate neutralized with Tris base was added over a period of 1 hour to a final concentration of 1.0%. After 20 min of stirring, the mixture was clarified by centrifugation at 9000 × g for 20 min. The supernatant, containing less than 5% of the total polymerase activity, was discarded. The precipitate was resuspended during 2 hours by gently stirring in 240 ml of 0.1 M potassium phosphate, pH 7.4, 5 mM GSH, 0.5 mM EDTA, and 20% glycerol. MgCl₂ was then added to 2 mM (Fraction II).

Step 3: DEAE-cellulose Chromatography I—Fraction II was diluted with 1:2.5 with 5 mM GSH, pH 7.0, and applied to a column of DE11 (52 cm² × 23 cm), equilibrated with 0.05 M potassium phosphate, pH 7.4, 5 mM GSH, 0.5 mM EDTA, 2 mM MgCl₂, and 10% glycerol. The column was washed with 2 liters of the equilibration buffer and the enzyme eluted with 0.32 M potassium phosphate, pH 7.4, 5 mM GSH, 0.5 mM EDTA, 9 mM MgCl₂, and 10% glycerol. Fractions containing polymerase activity were pooled, and pulvizerized (NH₄)₂SO₄ was added over 2 hours with stirring to a final concentration of 0.39 g/ml. After an additional 30 min of stirring, the precipitate was collected by centrifugation at 16,000 × g for 20 min. The precipitate could be stored at −20° for 1 week without significant loss of activity. If (NH₄)₂SO₄ was added more quickly, a loss of activity sometimes resulted.

Step 4: Gel Filtration—The ammonium sulfate precipitate from Step 3 was dissolved in 20 ml of 0.3 M potassium phosphate, pH 7.4, 0.5 mM EDTA, 2 mM MgCl₂, 25 mM 2-mercaptoethanol, and 10% glycerol (Fraction III) and passed through a Sephadex G-200 column (11.7 cm × 41 cm) equilibrated with the same buffer. DNA polymerase III elutes ahead of DNA polymerase II but there is considerable overlap. DNA polymerase III activity eluted at about 75% of the total eluted polymerase III activity were pooled, and dialyzed against 0.15 M potassium phosphate, pH 7.4, 5 mM GSH, 0.5 mM EDTA, and 10% glycerol. The nucleus substrate was sonicated, heat-denatured Escherichia coli [SH]DNA. DNA-cellulose and the activity eluted with 0.15 M buffer. Peak fractions containing about 80% of the total activity were pooled and concentrated by dialysis against 30% polyethylene glycol, 0.1 M potassium phosphate, pH 6.8, 5 mM GSH, 0.5 mM EDTA, and 20% glycerol for 5 hours, followed by dialysis for 4 hours into 0.15 M potassium phosphate, pH 6.8, 5 mM GSH, 0.5 mM EDTA, and 50% glycerol (Fraction VI).

Step 7: DNA-cellulose Chromatography—DNase I-treated DNA-cellulose was resuspended overnight in 10 mM Tris-HCl, pH 7.5/0.5 mM EDTA. A column (5.4 cm² × 3.7 cm) was then prepared and washed successively with 80 ml of 0.025 M potassium phosphate, pH 6.8, 5 mM GSH, 0.5 mM EDTA, 2.5 mM MgCl₂, and 10% glycerol (Buffer B); 60 ml of Buffer B plus 0.3 mg/ml of albumin; and 60 ml of Buffer B alone. About one-half of Fraction VI was applied to the column by diluting aliquots 1:3 with 5 mM GSH, pH 7.0. The column flow rate was maintained at about 30 ml/hour. The column was washed successively with 20 ml of Buffer B, 30 ml of Buffer B plus 0.1 M KCl, and 40 ml of Buffer B plus 0.14 M KCl. Activity was eluted by Buffer B plus 0.34 M KCl (Fig. 1). The pooled fractions containing polymerase were dialyzed 5 hours into 0.15 M potassium phosphate, pH 6.8, 7 mM GSH, 0.5 mM EDTA, and 50% glycerol (Fraction VII) and stored at −20°. Failure to prewash the column with albumin resulted in nearly a total loss of polymerase activity; presumably albumin saturates nonspecific adsorption sites. No albumin was found in the final product, Fraction VII, as indicated by the absence of a 67,000 dalton band on sodium dodecyl sulfate-polyacrylamide gels.

The DNA-cellulose column could be used repeatedly after first washing with Buffer B alone; rewashing with albumin was not necessary.

Step 7: Hydroxylapatite—As an alternative to DNA-cellulose, Fraction VI may be purified on hydroxylapatite (Fraction VII). Fifty units of Fraction VI were diluted 3-fold with 5 mM GSH, pH 7.0, and applied to a column of hydroxylapatite (0.7 cm² × 2.4 cm) equilibrated with 0.05 M potassium phosphate, pH 6.8, 5 mM GSH, and 10% glycerol. The column was washed with 2.5 ml of 0.1 M potassium phosphate, pH 6.8, 5 mM GSH, 10% glycerol, and then developed by a 13-ml linear gradient containing 0.1 M to 0.4 M potassium phosphate, pH 6.8, gradient containing 5 mM GSH and 10% glycerol. Polymerase activity eluted at about 0.15 M buffer. Fractions containing activity were made to 50% glycerol and stored at −20°. Although

FIG. 1. Co-purification of the polymerase and exonuclease activities of DNA polymerase III on DNA-cellulose. About 700 units of Fraction VI were applied to a column (5.4 cm² × 3.7 cm) of DNA-cellulose and the activity eluted with 0.025 M potassium phosphate, pH 6.8, 5 mM GSH, 0.5 mM EDTA, 2.5 mM MgCl₂, 0.34 M KCl, and 10% glycerol. The nucleic substrate was sonicated, heat-denatured Escherichia coli [SH]DNA.
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The Fraction VII polymerase III had about 4500 times the specific activity of the high speed supernatant material and the enzyme appears to be nearly homogeneous. When 4 µg of a concentrate of Fraction VII were analyzed by polyacrylamide gel electrophoresis at pH 8.0, one major protein band was observed (Fig. 2A) which had the same mobility as DNA polymerase activity (Fig. 2B), both correspond to an Rf of 0.46. Thirteen micrograms of Fraction VII had also been applied to a third gel which was not stained. The protein was eluted from the section of this gel corresponding to the position of the polymerase, Rf 0.43 to 0.49, and run on a polyacrylamide gel containing 0.2% sodium dodecyl sulfate. A single, sharp 167,000 dalton band was observed with no evidence of a low molecular weight subunit (Fig. 2C). The molecular weight was calculated by comparison with a parallel gel of a series of molecular weight standards (Fig. 2D); DNA polymerase III migrates a little slower than the β' subunit of Escherichia coli RNA polymerase. Equal sections of the gel shown in Fig. 3A above (Rf 0.37 to 0.43) and below (Rf 0.49 to 0.56) the protein band were similarly eluted and the eluates run on sodium dodecyl sulfate gels. From the 0.37 to 0.43 Rf section, a single, very faint, about 165,000 dalton band was observed, representing a slight tailing of the polymerase, and there was no observable protein from the 0.49 to 0.56 Rf section. Five micrograms of the Fraction VII concentrate were also run directly on a sodium dodecyl sulfate gel; the major band, representing about 60% of the total protein, had a molecular weight of 165,000. The average value for the polymerase III protein chain is therefore 166,000.

The molecular weight of native Bacillus subtilis polymerase III was determined from the sedimentation coefficient and Stokes radius by the method of Siegel and Monty (34), assuming that the partial specific volume of the enzyme equals 0.725 cm³/g (40). The determinations were done at two ionic strengths. The results of sedimentation through glycerol gradients of polymerase and protein standards are shown in Fig. 3A. In 0.2 M phosphate, the polymerase activity sedimented with the same velocity as lactate dehydrogenase. The calculated sedimentation coefficient, s20,w, for the polymerase is 7.4 S; in 0.05 M phosphate, the coefficient is 7.8. The distribution coefficient, Kd, of the polymerase on Sephadex G-200 is clearly salt-sensitive, with the calculated Stokes radii being 56 A and 48 A in 0.2 M and 0.05 M phosphate, respectively (Fig. 3B). The enzyme eluted ahead of catalase in 0.2 M phosphate but behind it in 0.05 M phosphate. In Fig. 4, the G-200 filtration pattern of polymerase III in 0.2 M phosphate is displayed and the sedimentation pattern has been reported elsewhere (11). There was no evidence of significant aggregation in the gel filtration or sedimentation results. The calculated molecular weights (rounded to two significant figures) of 170,000 and 150,000 in 0.2 and 0.05 M phosphate, respectively, average to 160,000. Since this value is in good agreement with the value of 166,000 obtained from sodium dodecyl sulfate gels, we conclude that polymerase III consists of a single polypeptide of very high molecular weight.

Absence of Endonuclease Activity

A circular, covalently closed DNA substrate was used to test for contaminating endonucleolytic activity since a single scission per molecule can be detected by alkaline sedimentation. Both native and irreversibly denatured minicircular DNA from...
E. coli 15 were used; the latter substrate provided a measure of single strand specific endonucleolytic activity. After a 60-min 30°C incubation with 2 μM [3H]DNA and 0.25 unit of polymerase, essentially no rupture of either substrate was observed. Fig. 5 shows the result with the native DNA substrate. Since less than 5 × 10⁻⁴ nmol of phosphodiester linkages were hydrolyzed, the polymerase to endonuclease ratio is at least 10⁶. In a control reaction, 0.01 unit of pancreatic DNase I quantitatively converted each of the substrates to material sedimenting near the top of the gradient (data not shown).

**Requirements of Intrinsic Exonuclease Activity**

The intrinsic exonuclease activity has a stricter requirement for MgCl₂ than the polymerase activity. Essentially no exonuclease activity (<2%) is seen in the absence of MgCl₂, either in the presence or absence of 0.5 mM EDTA, whereas addition of the chelator is necessary to abolish totally polymerase activity. The MgCl₂ concentration optimum is quite broad; exonuclease activity was maximal from about 4 to 18 mM and fell to 50 and 68% maximal activity at 1.3 and 26 mM MgCl₂, respectively. Unlike the polymerase activity, the nuclease activity is strictly dependent on Mg⁺ ions. Fig. 7 (right) shows the result with the circular single-stranded homopolymer DNA substrate, which is 10 times more active than the E. coli DNA substrate.

**Fig. 3.** Sedimentation coefficient and Stokes radius of DNA polymerase III. The polymerase was sedimented through 35 to 50% glycerol gradients (A) and filtered through Sephadex G-200 (B) as described under "Materials and Methods." The buffers contained 0.05 M potassium phosphate, pH 7.0 (-), or 0.2 M potassium phosphate, pH 7.0 (---). The Sephadex columns were calibrated with N4 [3H]-phage and [3H]thymidine. The standards were: catalase, LDH, bacterial alkaline phosphatase, BAP, hemoglobin, bovine serum albumin, BSA, alcohol dehydrogenase, ADH, and β-galactosidase.

**Fig. 4.** Filtration of DNA polymerase III through Sephadex G-200. The column buffer contained 0.2 M potassium phosphate, pH 7.0, and the procedure is described under "Materials and Methods." The column was calibrated with N4 [3H]phage and [3H]thymidine, and the symbols for the standards are given in Fig. 3 legend. ADH, alcohol dehydrogenase; BSA, bovine serum albumin.

**Fig. 5 (left).** Absence of endonuclease activity in polymerase III. Minicircular [3H]DNA from Escherichia coli 15 was incubated either in the presence (●) or absence (O) of 0.25 unit of Fraction VII polymerase III and the reaction products analyzed by sedimentation through alkaline sucrose gradients. Each 3.8-ml gradient was collected from the bottom and the radioactivity profiles are shown. The larger [3H] peak represents covalently closed duplex circles and the smaller peak represents linear and single-stranded circular forms.

**Fig. 6 (center).** Salt inhibition of the exonuclease. The standard exonuclease reaction mixture contained 25 μM sonicated, heat-denatured Escherichia coli [3H]DNA. 0.05 unit of Fraction VII polymerase III, and the indicated concentrations of KCl. Maximal activity was observed at 10 mM KCl.

**Fig. 7 (right).** Inhibition of the exonuclease by circular, single-stranded DNA. The standard reaction mixture (50 μl) contained either 6.4 μM denatured Escherichia coli [3H]DNA (●) or chain length 600 or [3H]d(T)₃₆₀ (O), the indicated concentrations of fl DNA, and 0.005 unit of Fraction VII. The incubation was for 10 min at 30°C. The ratio of the reaction velocity with (v) and without (v₀) circular DNA is plotted; v₀ for the homopolymer substrate was 10 times that for the E. coli DNA.
activity appears quite low in the presence of MnCl₂. In the range from 0.5 to 20 mM MnCl₂, with no MgCl₂ present, essentially no nucleolytic activity was seen (<3%). The synthetic activity in the presence of 0.6 mM MnCl₂, the sharp optimal concentration, was 60% of that with 6.5 mM MgCl₂.

The polymerase and nuclease activities also respond differently to spermidine. In the absence of MgCl₂ and in the presence of 0.15 mM EDTA and 3 mM spermidine, about 80% of the optimal polymerase activity with MgCl₂ was seen. However, in the absence of MgCl₂ and either in the presence or absence of 1 mM EDTA, 1 or 3 mM spermidine did not promote any exonuclease activity. In fact, in the presence of 6 mM MgCl₂, 3 mM spermidine caused a 72% inhibition of nuclease activity.

The pH optimum of the exonuclease activity, pH 7.4, is the same as that of the polymerase activity (2). For the exonuclease, at pH 6.4 and pH 8.4 there was 60 and 46%, respectively, of the activity seen at pH 7.4. The exonuclease is quite sensitive to ionic strength. In the presence of 30 mM Tris-HCl, pH 7.4, optimal exonuclease activity occurred at about 10 mM KCl, and at 90 mM KCl there was 50% of maximal activity (Fig. 6). This ionic strength sensitivity is very similar to that seen with polymerase activity (2). Again like polymerase activity, the exonuclease is quite sensitive to sulfhydryl reagents. Maximal activity requires the presence of sulfhydryl compounds, such as 1 mM dithiothreitol, 2 mM GSH, or 3 mM 2-mercaptoethanol. N-Ethylmaleimide at 7 mM and p-chloromercuri phenyl sulfonic acid at 0.8 mM caused about 90% inhibition.

While some nucleases are greatly affected by nucleotides (41), there was no effect of moderate concentrations (≤ 50 μM) of either deoxy- or ribonucleoside triphosphates on the polymerase III exonuclease. Very high concentrations of nucleoside triphosphates are inhibitory. A mixture of each of the four common deoxyribonucleoside triphosphates at 200 μM resulted in a 40% inhibition; this concentration is over 2 orders of magnitude greater than their apparent Kₘ values (see below). B. subtilis polymerase III does not appear to have a "nick translating" activity like the 5' → 3' exonuclease of E. coli polymerase I (42). There is minimal degradation of nicked, minicircular DNA (Fig. 1) and this activity, as well as that on DNase I-treated E. coli DNA, is not stimulated by deoxyribonucleoside triphosphates.

**Exonuclease Activity on Various Templates**

The relative activity of the exonuclease on several substrates is shown in Table II. The exonuclease is considerably more active on single-stranded than double-stranded DNA. Native, duplex E. coli DNA which had been separated from single-stranded DNA fragments by benzozylated, naphthoylated DEAE-cellulose chromatography was at least 100 times less active than the same substrate which had been briefly heat-denatured for 3 min at 100°C. Similarly, poly(dA) essentially abolished the vigorous activity seen on poly(dT). As analyzed in detail in the subsequent section, the exonuclease prefers short single-stranded DNA, such as sonicated, heat-denatured DNA and the nonanucleotide, to long single-stranded DNA. Some physical property of the long substrate must retard hydrolysis. As also indicated in Table II, terminal 3'-phosphoryl groups on single-stranded DNA blocked hydrolysis. The low activity with an equal mixture of 3'-hydroxyl- and 3'-phosphoryl-terminated DNA indicated that 3'-phosphoryl DNA is also an inhibitor of the nuclease activity. The low level of RNase activity indicated by the slow hydrolysis of [3H]-poly(rU) (Table II) is intrinsic to polymerase III since it is OPH(NH)₂-Ura-sensitive (11). Moreover, the addition of 43 μM unlabeled poly(rU) inhibited the digestion of 20 μM single-stranded E. coli [3H]DNA by 84% (12).

**Dependence of Rate of Hydrolysis on Substrate Size**

We have shown that the DNA polymerase III exonuclease hydrolyzes short DNA chains much faster than long DNA chains even at saturating substrate concentrations (Table II and Ref. 12). This large effect of chain length on reaction rate, observed also with some other exonucleases (43, 44), suggests that regions of the DNA substrate distant from the site of catalysis influence activity. A plausible explanation for this general phenomenon pointed out by Huang and Lehman (43) is that the interior nucleotides provide nonproductive binding sites and thus activity falls off as the ratio of interior to 3'-hydroxyl terminal residues increases with increasing chain length. To test directly this mechanism of intrinsic competitive inhibition for the B. subtilis polymerase III exonuclease, the ratio of interior to terminal substrate residues was controlled by adding increasing amounts of circular, single-stranded, fl DNA to a fixed amount of labeled substrate (Fig. 1). According to prediction, the circular DNA is a good inhibitor of the hydrolysis of E. coli DNA; the rate was half-maximal at a concentration of circular DNA about four times that of the linear DNA. The apparently smaller effect on poly(dT) hydrolysis is in large part due to the direct proportionality of the slope of these plots to the measured Kₘ/Vₘₐₓ which, as demonstrated below, is much smaller for the homopolymer substrate. While the linearity of the plots shown in Fig. 7 indicates a simple inhibition scheme, at higher circular DNA concentrations (not shown in the figure) inhibition was less than predicted by the extrapolated line, showing secondary effects.

If intrinsic competitive inhibition occurring at interior nucleotides is the sole effect of chain length on hydrolytic activity, then the kinetics of the reaction should obey the following rate equation:
\[
v = \frac{k_{\text{cat}} E_o S}{S + K_m \left( 1 + \frac{1}{K_i} \right)}
\]

where \( k_{\text{cat}} \) is the catalytic rate constant; \( E_o \), the total enzyme concentration; \( S \), the substrate concentration in terms of 3'-hydroxyl termini; \( K_m \), the dissociation constant for the enzyme-inhibitor complex; and \( i \), the concentration of internal nucleotide inhibitor. Since here \( i \) is defined by \( S \) and \( n \), the chain length of the substrate in nucleotides, then if only a fraction, \( c \), of the internal nucleotides are active competitive inhibitors, this equation can be transformed into:

\[
v = \frac{K_i k_{\text{cat}} E_o S}{K_i + K_m \alpha (n - 1)}\]

Thus the measured, or apparent, \( K_m \) and \( V_{\text{max}} \) (\( K_{\text{app}} \) and \( V_{\text{app}}^{\max} \)) are equal to \( K_m \frac{K_i}{K_i + K_m \alpha (n - 1)} \) and \( K_{\text{cat}} E_o /[K_i + K_m \alpha (n - 1)] \), respectively. This simple competitive case predicts that both \( K_{\text{app}} \) and \( V_{\text{app}}^{\max} \) will vary inversely with \( n \) while the ratio of these two parameters will be constant. These relationships for intrinsic competitive inhibition contrast with competitive inhibition by an added compound where the inhibitor causes an increase in \( K_{\text{app}} \) and does not alter \( V_{\text{app}}^{\max} \).

To test the prediction of the rate equation, \( K_{\text{app}} \) and \( V_{\text{app}}^{\max} \) were measured for the DNA and poly(dT) substrates listed in Table III; the data for the poly(dI) and DNA substrates will be treated separately since these homopolymers are much better substrates and are free of secondary structure. The chain length of the substrates was measured by end group labeling with polynucleotide kinase and/or alkaline velocity sedimentation. Lineweaver-Burk plots for some of the substrates are shown in Fig. 8 and the calculated kinetic constants are listed in Table III. While all the double reciprocal plots were linear, the slopes and intercepts differed markedly from substrate to substrate. For the natural DNA and poly(dT) substrates, \( K_{\text{app}}^{\max} \) varies inversely with \( n \) in the predicted fashion over at least a 3-order of magnitude range as shown by the good agreement between the measured \( K_{\text{app}}^{\max} \) and the \( K_{\text{app}} \) calculated from the value of \( n \) and the rate equation (Table III). In contrast, the magnitude of \( V_{\text{app}}^{\max} \) and \( V_{\text{app}}^{\max}/K_{\text{app}}^{\max} \) does not vary as expected. While \( V_{\text{app}}^{\max} \) does decrease with chain length for the natural DNA substrates, it does not vary according to the predicted hyperbolic function, and the effect of \( n \) on \( V_{\text{app}}^{\max} \) is considerably smaller than on \( K_{\text{app}}^{\max} \). For the poly(dT) substrates, the very high \( V_{\text{app}}^{\max} \) is essentially independent of \( n \) over the range of tested chain lengths. The \( V_{\text{app}}^{\max}/K_{\text{app}}^{\max} \) instead of being constant increases with \( n \) for both DNA and poly(dT) substrates.

Besides a more usual, predominant 3' → 5' exonucleolytic activity, an additional unique 5' → 3' activity has recently been found associated with E. coli DNA polymerase III (45). A 5' → 3' exonuclease may be involved in the processing of Okazaki fragments and in the repair of DNA. Three observations had

**TABLE III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( n )</th>
<th>( V_{\text{app}}^{\max} )</th>
<th>( K_{\text{app}}^{\max} )</th>
<th>( V_{\text{app}}^{\max}/K_{\text{app}}^{\max} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured N4 DNA</td>
<td>46,000</td>
<td>1,000</td>
<td>1.7 × 10⁻⁴</td>
<td>2.1 × 10⁻⁴</td>
</tr>
<tr>
<td>Sheared, denatured Escherichia coli DNA</td>
<td>600</td>
<td>3,000</td>
<td>0.017</td>
<td>0.017</td>
</tr>
<tr>
<td>Sheared, denatured Escherichia coli DNA</td>
<td>90</td>
<td>10,000</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>pG-C-T-T-C-C-C-A</td>
<td>9</td>
<td>≥ 18,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(dT)</td>
<td>600</td>
<td>45,000</td>
<td>0.017</td>
<td>0.019</td>
</tr>
<tr>
<td>Poly(dT)</td>
<td>330</td>
<td>47,000</td>
<td>0.028</td>
<td>0.029</td>
</tr>
<tr>
<td>Poly(dT)</td>
<td>60</td>
<td>55,000</td>
<td>0.08</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Calculated from the value of \( n \) and the rate equation in the text.

* Measured value.

---

**FIG. 8.** The effect of the substrate chain length on the exonuclease activity. Lineweaver-Burk plots for three separate single-stranded DNA templates are shown. The standard exonuclease assay contained 0.05 unit of Fraction VII polymerase III and the indicated 3'-termini concentrations of degraded, heat-denatured Escherichia coli [PH]DNA of chain length 90 (O) or 600 (●) in A and intact denatured N4 [PH]DNA (Δ) in B.
suggested that the *B. subtilis* polymerase III exonuclease degrades in a 3' → 5' direction. First, hydrolysis is inhibited by 3'-phosphoryl-terminated DNA (Table II); second, polymerase III does not promote nick translation; third, bacteriophage λ DNA and *E. coli* DNA partially digested by exonuclease III, both of which contain 5'-terminal single-stranded tails, did not cause any inhibition at a concentration 20 times greater than the labeled, single-stranded DNA substrate (12).

The mode of attack by the exonuclease was explored with a series of doubly labeled substrates. The kinetics of hydrolysis of [5'-32P,3H]poly(dT) indicate a 3' → 5' attack (Fig. 9A). Release of tritium proceeded linearly with time while no significant release of 32P occurred until greater than 90% of the tritium had been hydrolyzed. The time course of degradation of [5'-32P]d(C)6-d[3H]d(T)60 (Table IV, Substrate 1) similarly indicated general 3' → 5' hydrolysis (Fig. 9B). Quite surprisingly, even after 95% of the poly(dT) portion had been degraded, no significant amount of poly(dC) had been hydrolyzed. Hybridization of the poly(dT) portion, the 3' end, with poly(dA) (Substrate 2) effectively blocked this 3' → 5' degradation but did not unmask any digestion of the poly(dC). The result with the reverse substrate, [3H]d(T)110e-[5'-32P]d(C)105 (Substrate 3), explains the unexpected result with Substrates 1 and 2. Very little of either label was released, even after extensive incubations. Poly(dC) is therefore a very poor substrate; poly(dC) can contain unusual secondary structure (46) and this may account for its poor activity. The small amount of tritium which was released (2%) from Substrate 3 was not due to a low level of 5' → 3' activity, since hybridization of this copolymer to poly(dG) (Substrate 4) inhibited the release of both labels, indicating that the tritium released depended upon prior 3' → 5' degradation of the poly(dC) on the 3' end. A control experiment showed that the addition of an equal amount of unlabeled poly(dG) did not inhibit the degradation of labeled poly(dT) (data not shown). Duplexes containing nicks and gaps were also free of attack from the 5' end (Substrates 5 and 7).

The most sensitive test for a 5' → 3' nuclease employed [5'-32P,3H]d(T)110 extended with unlabeled poly(dC) (Substrate 8). The 3' end has been blocked and only a single nucleolytic cleavage at the 5' end removes all of the 32P. After long incubations with polymerase III, the release of label was measured by chromatographing reaction products in Solvent System I. Only 0.6% of the tritium was released while no significant amount of 32P was released (Fig. 10A). Therefore, all the exonucleolytic activity (>99.8%) degrades the substrate in a strictly 3' → 5' direction, and not only showed a 3' → 5' direction of attack but suggested a nonprocessive, or distributive, mode of degradation, since the 32P was released only quite late. Calculations based upon this experiment suggest that the enzyme removes less than 10 nucleotides/encounter. To determine more precisely the number of nucleotides removed per encounter, the hydrolysis of [5'-32P]pG-C-T-T-C-C-C-G-A was followed by homochromatography; the concentration of substrate was about 2000 times that of the enzyme (Fig. 11). A nonprocessive mode is clearly seen. There was quick hydrolysis of the substrate to a mixture of the octa-, hepta-, and hexanucleotides. After about 2.5 min, nearly all the nonanucleotide starting material had been converted to partially degraded chains, while only at later times was the limit digest, [5'-32P]pG-C, finally formed. The regular appearance of each of the degradative intermediates with time implies that the exonuclease removes one, or at most two, nucleotides with each encounter with the substrate; there is no evidence for translocation of the enzyme along its substrate. A parallel experiment with [5'-32P]d(T)110 showed the same distributive pattern of hydrolysis and thus the high rate of digestion of d(T)110 is not due to transformation to a processive mode of attack.

**Limit Digest: a Dinucleotide**

The product of the exonuclease reaction had been shown previously to be 5'-mononucleotides on the basis of chromatography and enzymatic hydrolysis (12). Homochromatography of

\[ \text{Table IV} \]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% total</th>
<th>H released</th>
<th>32P released</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[5'-32P]d(C)6-d[3H]d(T)60</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>[5'-32P]d(C)6-d[3H]d(T)110-poly(dA)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>[5'-32P]d(T)110e-[5'-32P]d(C)105</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>[32P]d(T)110e-[5'-32P]d(C)105-poly(dG)</td>
<td>1.2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>[5'-32P]d(T)110e-[5'-32P]d(C)105-poly(dG)</td>
<td>0.2</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>[5'-32P]d(T)110e-[5'-32P]d(C)105-poly(dA)</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>[5'-32P]d(T)110e-[5'-32P]d(C)105-poly(dA)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>8</td>
<td>[5'-32P]d(T)110e-[5'-32P]d(C)105-poly(dC)</td>
<td>0.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\[ \text{Using the same substrate, 5' → 3' exonuclease was also not detected with Fraction VI polymerase III.} \]
DNA Polymerase III from Bacillus subtilis

FIG. 10. The exonuclease does not degrade in a 5' → 3' direction. A standard exonuclease reaction mixture (0.25 ml) containing 11 μM [5'-32P,3H]d(T)100-poly(dC) was incubated with 0.4 unit of Fraction VII Bacillus subtilis polymerase III (A) or the same amount of Escherichia coli polymerase III (E). After a 45-min 30°C incubation, a 50-μl aliquot was removed and the [3H] and [32P] degradative products examined by paper chromatography (Solvent I). The position of an internal dTMP marker is indicated. The counts at the origin are off the scale and were for [3H] and [32P], respectively, 4334 and 573 in A and 1074 and 172 in B.

FIG. 11. The intrinsic exonuclease degrades in a strictly nonprocessive manner. The homochromatographic display of the exonucleolytic degradation products of [5'-*T]*dG-C-T-T-C-C-C-G-A at the indicated reaction times is shown. The details are under "Materials and Methods." The cellulose thin layer plate was developed until the bromphenol (BP) dye marker approached the top of the plate.

extensively degraded [5'-32P]*dG-C-T-T-C-C-C-G-A implied that the limit digest was a dinucleotide, [5'-32P]*dG-C (Fig. 11). To confirm this identification, the digest was partially degraded with snake venom phosphodiesterase and subjected to electrophoresis at pH 5.5 (Fig. 12A). Before diesterase treatment, the nonanucleotide limit digest migrated as a single peak in a position expected for a dinucleotide, while after diesterase treatment there were two peaks, the undegraded dinucleotide and 5'-dGMP. The limit digest of [5'-32P]*[3H]d(T)100 similarly appears to be a dinucleotide. Chromatography in Solvent System I of extensively degraded [5'-32P]*[3H]d(T)1100 revealed two [32P] peaks (Fig. 12B). About 30% of the [32P] migrated with an internal reference of d(T)3, the limit digest, while the remainder migrated as d(T)3 (47), a partial digest. No [32P]*dTMP was seen.

Polymerase III Can Synthesize in 5' → 3' Direction

Having shown that the exonuclease degrades exclusively from the 3' terminus, a simple but novel experiment was performed to determine the direction of polymerization. The template-primer was [5'-32P]*dG-C-T-T-C-C-C-G-A at the indicated reaction times is shown. The details are under "Materials and Methods." The cellulose thin layer plate was developed until the bromphenol (BP) dye marker approached the top of the plate.

Polymerase III Can Synthesize in 5' → 3' Direction

Having shown that the exonuclease degrades exclusively from the 3' terminus, a simple but novel experiment was performed to determine the direction of polymerization. The template-primer was [5'-32P]*dG-C-T-T-C-C-C-G-A and 0.01 unit of Fraction VII polymerase III was incubated 45 min at 30°C. One-half of this digest was next incubated either with (A) or without (Δ) 100 ng of snake venom phosphodiesterase (5 min, 30°C) and then analyzed using high voltage paper electrophoresis. The position of an internal dGMP marker is shown. B, the standard reaction mixture (0.17 ml) contained 43 μM [5'-32P,*H]d(T)1100 and 0.15 unit of Fraction VII. After a 45-min 30°C incubation, a 20-μl aliquot was removed and chromatographed on paper in Solvent I with internal dTMP and d(T)3 markers.

FIG. 12. The limit digest is a dinucleotide. A, the standard exonuclease reaction mixture (10 μl) containing 8 μM [5'-32P]*G-C-T-T-C-C-C-G-A and 0.01 unit of Fraction VII polymerase III was incubated 45 min at 30°C. One-half of this digest was next incubated either with (A) or without (Δ) 100 ng of snake venom phosphodiesterase (5 min, 30°C) and then analyzed using high voltage paper electrophoresis. The position of an internal dGMP marker is shown. B, the standard reaction mixture (0.17 ml) contained 43 μM [5'-32P,*H]d(T)1100 and 0.15 unit of Fraction VII. After a 45-min 30°C incubation, a 20-μl aliquot was removed and chromatographed on paper in Solvent I with internal dTMP and d(T)3 markers.
polymerase III synthesizes DNA in a 5'→3' direction. A further test of any 3'→5' synthesis could utilize a triphosphate-terminated primer (47, 48).

**Apparent \( K_m \) Values for Deoxyribonucleoside Triphosphates**

We have shown for \( B. \ subtilis \) polymerase III that the measured \( K_m \) for dGTP is 0.6 \( \mu \)M (7). Since the \( K_m \) for a mixture of the four common deoxyribonucleoside triphosphates is at least 1 order of magnitude higher for \( E. \ coli \) polymerase III (49, 50), the \( K_m \) was determined for dCTP, dATP, and dTTP for the \( B. \ subtilis \) enzyme. The results were 0.9 \( \mu \)M for dATP, 1.5 \( \mu \)M for dTTP, and 0.3 \( \mu \)M for dCTP, and thus the difference between the two DNA polymerases remains.

**Incorporation of Ribonucleoside Triphosphates**

Because of the utility of ribonucleoside incorporation by DNA polymerases in sequencing DNA (51), the ability of polymerase III to incorporate rGTP, rCTP, rUTP, and rATP was tested. Either in the presence of a full deoxy- or ribonucleotide complement, or in the presence of a single ribonucleotide, the polymerase was able to utilize only rGTP appreciably. The rGTP was incorporated about 1/2 as well as dGTP in the presence of 0.5 \( \text{mM} \) MnCl\(_2\) and negligibly in the presence of 6 \( \text{mM} \) MgCl\(_2\).

**Lack of Intrinsic Exonuclease in Bacillus subtilis DNA Polym-erase II**

We reported previously that \( B. \ subtilis \) DNA polymerase II had little or no nuclease activity on \( E. \ coli \) DNA (2). In view of the striking dependence of the \( B. \ subtilis \) DNA polymerase III exonuclease on the chain length, secondary structure, and base composition of the substrate, the possible presence of nuclease activity in polymerase II was reinvestigated. The enzyme elution profile from DNA-cellulose, the final purification step, shows no significant nuclease activity under the sharp polymerase peak (Fig. 13). The peak fractions of polymerase were pooled, concentrated, and tested for nuclease using long incubation times. There was still no hydrolysis of native and denatured \( E. \ coli \) DNA and d(T)\(_{250}\), an activity \( >90\% \) of the polymerase activity could have been readily detected.

**DISCUSSION**

In this paper we describe the 4500-fold purification of \( B. \ subtilis \) DNA polymerase III from a mutant strain lacking DNA polymerase I. DNA polymerase II was quantitatively removed during the first five steps. The yield of polymerase III was about 3%. The alternative procedure which substituted hydroxylapatite for DNA-cellulose in the final step gave a three times higher yield of enzyme which was similarly free of contaminating nucleases but had a lower specific activity (Table I). The enzyme from the penultimate phosphocellulose step had a nuclease contamination about equal to the exonuclease activity of the polymerase. The isolation of a relatively large amount of purified enzyme has permitted a more careful characterization of the enzyme than reported previously (2, 3, 12, 14), particularly in regard to its exonuclease activity and physical properties.

The enzyme is nearly homogeneous, yielding a single major band on a nondenaturing gel which is coincident with enzymatic activity (Fig. 2, A and B). The sodium dodecyl sulfate-gel electrophoresis of Fraction VII enzyme showed that the intact polymerase was about 60% of the total protein. The only polypeptide seen on sodium dodecyl sulfate gels of material eluted from the nondenaturating gel has a molecular weight of 166,000. The lack of minor bands on the gel scan shown in Fig. 2C along with the good agreement between the native and denatured molecular weights indicate that the enzyme consists of a single, giant polypeptide chain. Although the mobility of the protein standards on the denaturing gel was linear with the logarithm of molecular weight (Fig. 2D), the calculated value of 166,000 for polymerase III must be viewed in light of the small number of precise standards in this high molecular weight region. Assuming that Fraction VII is 60% pure and that there are \( 2 \times 10^{14} \) bacteria/g of wet, packed cells, then one can calculate from the purification data that there are about 100 polymerase III molecules/cell during logarithmic growth.

The marked effect of phosphate concentration on the calcu-
lateral value of $s_{2n}$ and Stokes radius (Fig. 3) suggests that the shape of the enzyme may be sensitive to ionic strength. The data at higher ionic strength led to a calculated native molecular weight closer to the value obtained from sodium dodecyl sulfate gels, but the difference between the two calculated values was not large since at 0.2 M phosphate the lower $s_{2n}$ was partially compensated by an increased $K_c$. The determinations were done at two ionic strengths to help control for any possible aggregation phenomena, but it was not a significant factor. Using the higher salt data, the calculated relative frictional coefficient, $f/f_n$, is 1.54. This high value is consistent with, but not proof of, a markedly asymmetric molecule (52). Assuming that the enzyme is a prolate ellipsoid with a "compromise" degree of solvation of 0.2 (52), then its axes would be 520 x 52 A, or large enough to extend over 150 base pairs of the DNA helix.

The intrinsic nature of the exonuclease activity in *B. subtilis* DNA polymerase III is well established. The exonuclease activity co-purified with polymerase activity on DNA-cellulose (Fig. 1) and hydroxylapatite (11), and the polymerase to nuclease ratio remained constant upon sedimentation of Fraction VII enzyme (data not shown). Both activities were similarly inhibited by sulfhydryl reagents, high ionic strength, and the specific polymerase III antagonist, OHPb(NH)$_2$Ura.

The exonuclease activity was co-excluded with polymerase activity in the agarose gel monitored, OHPb(NH)$_2$Ura-promoted ternary complex (12). At elevated temperatures, the polymerase and exonuclease activities decayed at the same rate (13). Enzyme purified from a strain which is temperature sensitive because of a mutation in the structural gene for DNA polymerase III showed the same increased thermolability for both activities (13).

The distinction between processive and distributive behavior is an important property of enzymes which synthesize or degrade macromolecules. The processiveness of exonucleases has been generally determined by comparing the relative rates of removal of the terminal and internal nucleotides; degradative intermediates were not followed directly. Hence, some reported nonprocessive exonucleases may actually remove a few hundred nucleotides processively with each enzyme-substrate encounter. Within these limits, polymerase phosphorylase (53), *T. pyovulans* DNA polymerase (54), SP1 DNA polymerase (55), and *Escherichia coli* RNase II (56) and *E. coli* exonuclease I (57) act processively while *Escherichia coli* exonuclease I (57). T4 DNA polymerase’s 3’ → 5’ exonuclease (58), and snake venom phosphodiesterase (55) degrade nonprocessively. Among DNA polymerases, the synthesis catalyzed by calf thymus polymerases $\alpha$ and $\beta$ and *E. coli* polymerases I and II appear to be strictly distributive (59-61), while the T4 enzyme is only partially so (62). Processiveness of an exonuclease has the advantage of preventing the accumulation of degradative intermediates which could interfere with other cellular processes. However, in order to avoid excessive degradation, once the desired structure has been hydrolyzed, a processive nuclease requires a stop and release signal which may be the presence of a neighboring chain, a specific base sequence, or a protein factor. In addition, a processive enzyme must be able to translocate along the DNA chain, which is a very different enzymological event than binding and catalysis. Translocation of the messenger RNA chain on the ribosome, for example, requires specific protein factors and energy in the form of GTP (63). Specific proteins involved in DNA synthesis have been shown recently to make the action of T4 DNA polymerase and *E. coli* DNA polymerase II much more processive (61, 62).

The *B. subtilis* polymerase III exonucleolytic degradation of pG-C-T-T-C-C-G-A and d(T)$_{10}$ demonstrates a strictly nonprocessive mode of degradation in *vitro*. It appears that only one or at most two nucleotides are removed per encounter of enzyme with oligonucleotide. While the experiments were less definitive, the degradation of polynucleotides and the synthesis of DNA also seem distributive. This nonprocessiveness implies the dissociation rate of the enzyme substrate complex is comparable to $k_{on}$ and that translocation along the DNA chain is difficult.

It is sometimes stated (e.g., Ref. 64) that all prokaryotic DNA polymerases have an intrinsic exonuclease that may function to edit mistakes in replication (41), in contrast with eukaryotic DNA polymerases where intrinsic nuclease activity has not been demonstrated (65). Certainly the nuclease activity of several prokaryotic polymerases, including *B. subtilis* DNA polymerase III, has the expected properties of an editor (41, 66). However, we saw no nuclease activity in *B. subtilis* polymerase II at a detection level over 3 orders of magnitude less than the activity of *B. subtilis* polymerase III and there is no evidence for such an activity in the published reports on *B. subtilis* polymerase I (1, 2). The suggestion that the intrinsic nuclease in a *B. subtilis* polymerase may have been lost during purification (41) is germane. The 5’ → 3’ exonuclease of *E. coli* polymerase I can be cleaved off by proteolytic enzymes during purification or by a *B. subtilis* protease (41), and an improved purification of the *Micrococcus luteus* enzyme revealed substantially more 5’ → 3’ exonuclease than found previously (67). However, the *B. subtilis* cells used in this report were harvested in exponential phase and washed twice with a high salt buffer to minimize extracellular protease contamination; no enzymatic, chemical, or genetic manipulation of the intensively studied *E. coli* polymerase I and T4 DNA polymerase has produced a polymerase devoid of 5’ → 3’ exonuclease activity (41, 68, 69); and an intrinsic exonuclease is not needed for faithful synthesis as shown by the results with some eukaryotic polymerases (65). Perhaps in *B. subtilis* an editor need only be wedded to the principal replicative polymerase, polymerase III. With the caution required in interpreting negative results, it seems that *B. subtilis* polymerase II is like the known eukaryotic DNA polymerases in failing to have an intrinsic nuclease activity.

The kinetic analysis indicates that the chain length of the exonuclease substrate influences the rate of the reaction by two opposing mechanisms. One determinant is the increase in the number of interior nonproductive binding sites per 3’-hydroxyl terminus as the chain length increases. This mechanism has been invoked previously (43, 44), but this report provides quantitative proof of this possibility. First, nonproductive binding to interior nucleotides was shown clearly by the potent inhibition of the *B. subtilis* polymerase III-associated exonuclease by single-stranded circular DNA (Fig. 7). Second, the strict inverse relationship over 3 orders of magnitude between $K_{w}$ and chain length (Table III) is also a reflection of this intrinsic competitive inhibition. The ratio $K_{w}/a$ calculated both from the variation in $K_{w}$ with $n$ and from the inhibition by circular DNA are in excellent agreement and are, respectively, 8 and 10 $\mu$M for the natural DNA substrates and 10 and 9 $\mu$M for the poly(dT) substrates. The data do not permit a precise evaluation of the true $K_{w}$ but indicate that it is of the
same order of magnitude as $K/a$. This suggests that the binding of DNA polymerase is about as strong to interior nucleotides as to 3'-terminal nucleotides and therefore the exonucleolytic specificity of the enzyme is dictated at some step subsequent to binding. This avid nonproductive binding must also contribute both to the inhibition of E. coli and B. subtilis polymerase III synthetic activity by single-stranded DNA and to their lowered activity with substrates containing large gaps (41).

The kinetic data also show clearly that intrinsic competitive inhibition is not the sole effect of chain length. $V_{\text{max}}^{\text{max}}$ did not vary with $n$ according to the predicted hyperbolic function and $V_{\text{max}}^{\text{max}}/K_{\text{app}}$ was not independent of $n$. Another mechanism contributing to the chain length effect could be an excluded volume effect whereby the terminus becomes sterically buried as $n$ increases (44). Alternatively, the probability of adventitious folding back of the 3' terminus should increase with $n$ and thus hairpin structures cannot not provide a very poor substrate for a single strand specific exonuclease but also may bind the enzyme tightly since it mimics the DNA conformation necessary for synthesis. The poly(dT) substrates were fabricated specifically to test this latter mechanism since they are devoid of secondary structure. While $V_{\text{max}}^{\text{max}}$ appeared independent of $n$ for these homopolymers, $K_{\text{app}}$ did decrease with $n$ and thus hairpin structures cannot be the sole effect of increasing $n$. Moreover, neither the secondary structure nor the excluded volume mechanism explains the data even in conjunction with intrinsic competitive inhibition, since $V_{\text{max}}^{\text{max}}/K_{\text{app}}$ were larger than predicted by the rate equation for competitive inhibition, an unexpected result.

The simplest resolution is that the accessibility of the 3' terminus to enzymatic attack increases with $n$. This increase exposure is, in fact, implied by the increase of $V_{\text{max}}^{\text{max}}/K_{\text{app}}$ with $n$, since the rate of an enzymatic reaction is proportional to this ratio at low substrate concentrations and thus this ratio is a measure of the intrinsic reactivity of the substrate. A possible physical interpretation is suggested by the observed increase of $V_{\text{max}}^{\text{max}}/K_{\text{app}}$ with approximately the $1/2$ power of $n$. If the substrate is a random coil, then its volume in solution is proportional to $n^{1/2}$ (52) and the local nucleotide density to $n^{-1/2}$. Thus the structure of the polymer becomes more open as $n$ increases and perhaps makes the terminus more accessible to the enzyme. This may also contribute to the higher $V_{\text{max}}^{\text{max}}/K_{\text{app}}$ for poly(dT) than for DNA as the lack of intramolecular hydrogen bonding may permit an even more open structure. It may not be fortuitous that the extrapolated value of $V_{\text{max}}^{\text{max}}$ for $n = 1$ for natural DNA is about equal to the observed $V_{\text{max}}^{\text{max}}$ for poly(dT). While further experiments are needed to substantiate these speculations, the main conclusions are that the chain length markedly influences the rate of the reaction by the opposing mechanisms of intrinsic competitive inhibition and exposure of the terminus to attack. These conclusions about how an enzyme, in effect, measures the size of its giant substrate may extend to other enzymes with macromolecular substrates.

Besides the effect of chain length, the rate of exonuclease activity also depends upon secondary structure and possibly base composition. The exonuclease had very little activity on duplex DNA, such as native E. coli DNA, poly(dA):d(T), and nicked DNA. The minimal activity seen may be real since the analogous T4 DNA polymerase can catalyze the turnover of termini (70) or it may result from single-stranded regions in the DNA preparations. The low activity on poly(dC) may reflect an unusual secondary structure of the homopolymer (46), since the cytidylic acid triplet in pG-C-T-C-C-C-G-A was hydrolyzed essentially as well as the other nucleotides. The best substrate, poly(dT), is always single-stranded.

Although the gram-negative E. coli and the gram-positive B. subtilis are evolutionarily divergent (71, 72), a number of similarities appear among their DNA-related enzymes. Each bacterium has three distinct polymerases (41, 66). Previously, we pointed out that DNA polymerase III from the two sources are quite similar in function, template specificity, reaction requirements, and sensitivity to sulfhydryl reagents, single-stranded DNA, temperature, high ionic strength, and 1-β-D-arabinofuranosylcytosine triphosphate (2). The results in this study permit a more extensive comparison. Both enzymes purified similarly on DEAE-cellulose, phosphocellulose, DNA-cellulose, and hydroxyapatite (14, 73). The native molecular weights are essentially the same and both enzymes synthesize in the 5' → 3' direction (73). The two enzymes catalyze the additional reactions of pyrophosphorolysis of DNA, the exchange of PP, with deoxynucleoside triphosphates, and the hydrolysis of single-stranded DNA from the 3' terminus releasing 5'-mononucleotides until the limit dinucleotide is reached (11, 45, 79).

Despite these similarities, B. subtilis DNA polymerase III is significantly different from the E. coli enzyme. First, only the B. subtilis enzyme is inhibited by arylhydrazinopyrimidines. Second, while the B. subtilis enzyme clearly consists of a single polypeptide chain of 166,000 daltons, the E. coli enzyme probably has two subunits of 140,000 and 40,000 daltons (73). Because the stoichiometry of these subunits is not certain (73), the physical structure of B. subtilis polymerase III is the best known for a host replicative polymerase. Third, while the purified B. subtilis polymerase III clearly has only a 3' → 5' exonuclease, the E. coli enzyme has both 3' → 5' and 5' → 3' exonucleolytic activities (49). Perhaps the E. coli 5' → 3' exonuclease resides in the 40,000 subunit. The exonucleolytic activity of the single subunit T4 DNA polymerase has virtually the same properties as the B. subtilis enzyme (41). Fourth, the apparent $K_m$ values for the deoxynucleoside triphosphates are about 1 order of magnitude lower for the B. subtilis polymerase III than for the E. coli enzyme.

The preferred substrate for B. subtilis and E. coli polymerase III is DNA containing small gaps (41). Replication of long single-stranded templates by the E. coli enzyme requires small molecule and protein cofactors (74, 75) whose precise nature is controversial (reviewed in Refs. 41 and 66). To determine the effect of these cofactors on B. subtilis polymerase III, we exchanged samples of purified B. subtilis polymerase III with Dr. J. Hurwitz for purified E. coli polymerase III and elongation Factors I and II, and the synthesis directed by an RNA-primed φX174 single-stranded DNA template was measured in both laboratories. The results agree, and some of the more extensive data of Vicuna and Hurwitz are shown in Table VI. Several conclusions can be drawn. First, B. subtilis polymerase III, like the E. coli enzyme, can elongate an RNA primer, the possible in vivo substrate (77). Second, the activity of the purified B. subtilis polymerase III is stimulated several-fold by Factors I and II to about one-half the synthetic rate with E. coli polymerase III. Both Factors I and II are required and the activity cannot be due to contaminating E. coli polymerase since the activity is sensitive to OIPb(NH)₃Ura. Third, this factor-dependent synthesis, remarkable considering the wide evolutionary divergence of these bacteria.
strengthens the conclusion of Hurwitz et al. (75) that the protein cofactors are not specific for E. coli polymerase III but instead permit a number of prokaryotic polymerases to replicate a long single-stranded template.

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REFERENCES

DNA Polymerase III from Bacillus subtilis
Purification and characterization of DNA polymerase III from Bacillus subtilis.
R L Low, S A Rashbaum and N R Cozzarelli


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