A Deoxyribonucleic Acid Phosphatase-Exonuclease from Escherichia coli

I. PURIFICATION OF THE ENZYME AND CHARACTERIZATION OF THE PHOSPHATASE ACTIVITY*

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A major problem regarding deoxyribonucleic acid polymerase action is the nature of the deoxyribonucleic acid template. Alterations of DNA may reduce or enhance its priming capacity, and both kinds of change have complicated assays of polymerase purification. When extensively purified polymerase was chromatographed on hydroxylapatite, over 90% of the protein and all the activity, as measured with the deoxyadenylate-deoxythymidylate copolymer as primer, were found in a single symmetrical peak. However, the amount of enzyme recovered was less than 20% as determined with calf thymus DNA as primer. Eluted earlier in the chromatogram was a minute protein peak (less than 2% of the total), free of polymerase but capable of restoring the main polymerase peak to full activity with the thymus DNA primed assay. This new enzyme has been purified extensively from Escherichia coli extracts and identified as a phosphatase highly specific for a phosphate residue esterified to the 3'-hydroxyl end of a DNA chain. Given a 3'-phosphoryl terminus on DNA, as shown in Scheme 1, the enzyme releases inorganic phosphate and then proceeds as an exonuclease with the stepwise release of 3'-hydroxyl termini of a DNA chain. Such a 3'-phosphoryl terminus is highly specific for a phosphate residue esterified to the 3'-hydroxyl group.

The present report deals with the purification of this DNA phosphatase and the specificity of its action. The preceding paper reveals another aspect of the action of this enzyme, the capacity to cleave 5'-nucleotidyl residues stepwise from the 3'-hydroxyl end of a DNA chain. The enzyme thus hydrolyzes phosphomono-or diester bridges between the phosphate and the 3'-hydroxyl group. Given a 3'-phosphoryl terminus on DNA, as shown in Scheme 1, the enzyme releases inorganic phosphate and then proceeds as an exonuclease with the stepwise release of 5'-mononucleotides.

The ability of this DNA phosphatase activity to remove the 3'-phosphoryl end groups of high molecular weight oligonucleotides provides a useful reagent for studying the effect of such end groups in the DNA-synthesizing system (1).
pH 8.0 (Step 5), and was assayed by a modification of the method of Razzell and Khorana (9). Further purification was achieved by chromatography on phosphocellulose. A column of phosphocellulose (1 cm × 8 cm) was equilibrated with 0.01 M succinate buffer, pH 6.0, containing 0.001 M EDTA. Approximately 10 mg of the ammonium sulfate fraction were dialyzed against the above buffer and applied to the resin. After the resin was washed with 5.0 ml of the same buffer, elution of the enzyme was accomplished by 5.0-ml portions of 0.01 M succinate buffer, pH 6.0, containing the following concentrations of KCl: 0.05 M, 0.10 M, 0.20 M, and 0.50 M. Approximately 70% of the activity applied to the column was recovered in the 0.2 M and 0.5 M eluates. This preparation had a specific activity 4 times greater than the ammonium sulfate fraction and had no measurable activity (less than 0.1% of its phosphodiesterase activity) on \(3'P\)-labeled 5'-dAMP or on \(3'P\)-labeled 3'-deoxynucleotides.

**Nucleic Acids and Nucleotides**—Calf thymus DNA was isolated by the method of Kay, Simmons, and Dunn (10). 32P-labeled DNA (specific activity, 20 μc per μmole of phosphate) was isolated from \(E. coli\) as described by Lehman (11). DNA containing no radioactive label was isolated from \(E. coli\) by the same procedure. \(3'2P\)-Labeled DNA (25 μc per μmole of phosphate) isolated from \(Bacillus subtilis\) (6B 19) was a gift of Dr. Walter Bodmer. 32P-Labeled ribosomal RNA (10 μc per μmole of phosphate) was isolated from \(E. coli\) by a modification of the method of Littauer and Eisenberg (12). DNA was removed from the final preparation (less than 0.1%) by treatment with pancreatic DNase. Soluble RNA was prepared as previously described (13), and the synthetic oligonucleotides, TpTpTp and TpTpTPpTP, prepared as described elsewhere (14, 15), were generously provided by Dr. A. Nussbaum and Dr. A. Duffield. \(E. coli\) alkaline phosphatase released 0.80 and 0.95 mole of inorganic orthophosphate per mole of TpTpTp and TpTpTPpTP, respectively. 32P-Labeled rCTP (6 × 10⁶ c.p.m. per μmole) was prepared as previously described (16); unlabeled deoxyribonucleotide triphosphates were purchased from the California Corporation for Biochemical Research. All concentrations of DNA and RNA are expressed as nucleotide equivalents.

**Other Reagents**—DEAE-cellulose (type 40, Brown Company) and Whatman phosphocellulose (P-70, W. and R. Ralston, Ltd.) were processed according to Peterson and Sober (17). Streptomycin sulfate was kindly donated by Merck Sharp and Dohme Company. Crystallized bovine plasma albumin was obtained from Armour and Company. p-Nitrophenyl phosphate was obtained from the California Corporation for Biochemical Research.

### Methods

#### Preparation of 32P-Labeled Substrates

**Mononucleotides**—32P-Labeled \(E. coli\) DNA (1.25 μmoles) was hydrolyzed to its constituent 3'-mononucleotides by the consecutive action of micrococcal DNase and calf spleen phosphodiesterase as previously described (18). The resulting mixture of mononucleotides was acidified to pH 4.0, adsorbed to Norit, washed twice with 0.001 M HCl, and then eluted by means of three extractions with 1.0 ml of 50% ethanol containing 0.3% ammonium hydroxide. Of the radioactivity initially present in the DNA, 85% was recovered as the 3'-mononucleotides. 32P-Labeled 5'-dAMP and dT3MP were isolated from 32P-labeled \(E. coli\) DNA as described previously (19).

**3' Phosphoryl Terminated DNA**—32P-Labeled \(E. coli\) DNA was partially digested with micrococcal nuclease, producing oligonucleotides terminated at one end by a 3'-phosphoryl group (20–22). The incubation mixture (20 ml) consisted of 1 mmole of glycine buffer, pH 9.2, 0.2 mmole of CaCl₂, 20 μmoles of 32P-labeled DNA, and 74 units of micrococcal nuclease. After incubation for 180 minutes at 37°C, 29% of the radioactivity had become acid-soluble. The reaction mixture was chilled and dialyzed against 100 volumes of 1 M KCl for 12 hours at 4°C to remove the dialyzable oligonucleotides. This was repeated three times (total of 48 hours dialysis), followed by a 12-hour dialysis against 0.02 M KCl. After this extensive dialysis, approximately 97% of the remaining DNA was acid-precipitable. The dialyzed solution represented the 3'-phosphoryl-terminated DNA and was stored at −20°C. No attempt was made to remove the nuclease, as this activity is not detectable in the absence of added calcium ion (4).

A similar preparation of 32P-labeled 3'-phosphoryl-terminated DNA was prepared by partially digesting \(E. coli\) DNA with the calf spleen deoxyribonuclease (3). The reaction mixture (4.7 ml) consisted of 100 μmoles of sodium acetate buffer, pH 5.0, 1.0 μmole of 32P labeled \(E. coli\) DNA, and 1.0 unit of calf spleen DNase. After incubation for 60 minutes at 37°C, 2% of the radioactivity had become acid-soluble. The reaction mixture was then treated in the manner described for the micrococcal nuclease digest.

Denatured 32P-labeled 3'-phosphoryl-terminated DNA was prepared by heating the dialyzed (0.02 M KCl) micrococcal nuclease digest in a boiling water bath for 10 minutes, then quickly cooling it in an ice bath.

**3' Phosphoryl Terminated Acid-soluble DNA Oligonucleotides**—To 1.0 ml of the micrococcal digest described above was added 1.0 ml of cold 10% trichloroacetic acid. After centrifugation to remove insoluble material, the supernatant fluid containing the acid-soluble 32P-labeled oligonucleotides (30% of total DNA)

1 In this paper, the term DNA will denote the long acid-insoluble fragments used as substrates as well as the native DNA from which they were derived.

2 A 0.02-ml aliquot of the reaction mixture was removed and added to 0.28 ml of water. The acid-soluble radioactivity was then determined as described in the assay for micrococcal nuclease.
was collected and extracted four times with ether, neutralized, evaporated to dryness, and dissolved in 2.0 ml of water. 

5'-Phosphoryl-terminated DNA—32P-Labeled E. coli native DNA was partially digested with E. coli endonuclease to produce oligonucleotides terminated at one end by 5'-phosphoryl groups (2). The incubation mixture (1.5 ml) consisted of 100 μmoles of Tris buffer, pH 7.5, 5 μmoles of MgCl₂, 1.5 μmoles of 32P-labeled DNA, and 5 units of endonuclease. After incubation for 180 minutes, 21% of the radioactivity had become acid-soluble. To prevent further action by the enzyme (23), 30 mmoles of soluble RNA were added. The reaction mixture was then treated in the manner described for the micrococcal nuclease digest.

3'-Phosphoryl-terminated RNA—32P-Labeled E. coli ribosomal RNA was partially digested with pancreatic RNAse, an enzyme which produces oligonucleotides terminated at one end by 2',3'-cyclic or by 3'-phosphoryl groups (24, 25). The incubation mixture (3.0 ml) consisted of 100 μmoles of Tris buffer, pH 7.5, 5 μmoles of 32P-labeled ribosomal RNA, and 0.002 μg of crystalline pancreatic RNAse. After 120 minutes at 37°, 24% of the radioactivity had become acid-soluble. The reaction mixture was treated in the manner described for the micrococcal nuclease digest.

A mixed polymer of ribo- and deoxyribonucleotides was synthesized as described by Berg, Fancher, and Chamberlin (26). It was designed to include 32P-rCMP residues interspersed among the four deoxyribonucleotides with a frequency of about 1 per 10 nucleotides. The incubation mixture (10.0 ml) contained 0.04 M Tris buffer, pH 7.8, 0.004 M 2-mercaptoethanol, 0.002 M MnCl₂, 0.4 μmole each of dATP, dGTP, dTTP, and dCTP, 2.0 μmole of 32P-labeled rCTP (6 × 10⁶ c.p.m. per μmole), 5 mg of calf thymus DNA, and 16 μg of 32P DNA polymerase (phosphocellulose fraction). After 2 hours at 37°, the mixture was heated at 70° for 5 minutes to destroy the polymerase; a small precipitate that formed was removed by centrifugation. The mixture was dialyzed against 1 liter of 2 M NaCl for 3 days, with daily changes of the dialysate, and then for 3 hours against 0.02 M NaCl-0.01 M Tris, pH 7.8. As judged by 32P content, 22.1 mmoles of ribo-cytidylate were in a polymeric form precipitable by 3.5% perchloric acid; 0.2 mmoles was acid-soluble. Assuming an equal incorporation of ribo- and deoxyribocytidylate and 1 cytosine nucleotide per 4.6 nucleotides, approximately 200 mmoles of newly synthesized mixed polymer were recovered in the dialyzed product.

Alkaline treatment of 2.0 ml of the product (containing approximately 40 mmoles of newly synthesized mixed polymer) was accomplished by adjustment with 0.2 ml of 4 N NaOH and incubation at 37° for 24 hours. The polymer was precipitated with 3.5% perchloric acid, washed with 0.01 N HCl, and dissolved in 1.0 ml of 0.1 M Tris buffer, pH 7.8; the recovery of labeled polymer was 46%.

RNase treatment of 1.0 ml of the product was performed by incubation with 200 μg of pancreatic RNase (previously heated at 100° for 5 minutes at pH 5) in 0.1 M potassium phosphate buffer, pH 6.5, for 2 hours at 37°. The polymer was precipitated, washed, and dissolved in 0.5 ml of Tris buffer, as with the alkaline-treated product; the recovery of labeled polymer was 58%.

Assay of E. coli DNA Phosphatase Activity

This assay measures the formation of acid-soluble, Norit-nonadsorbable products arising from 32P-labeled 3'-phosphoryl-terminated DNA. The incubation mixture (0.30 ml) contained 20 μmoles of potassium phosphate buffer, pH 7.0, 3 μmoles of MgCl₂, 0.3 μmole of 2-mercaptoethanol, 50 mmoles of 32P-labeled 3'-phosphoryl-terminated DNA (micrococcal nuclease digest), and 0.04 to 0.40 unit of enzyme. The reaction mixture was incubated for 30 minutes at 37°; then 0.20 ml of a cold solution of calf thymus DNA (2.5 mg per ml) and 0.50 ml of cold 10% trichloroacetic acid were added. After 5 minutes at 0°, the resulting precipitate was removed by centrifugation for 5 minutes at 12,000 × g, and 0.50 ml of the supernatant fluid was pipetted into a tube containing 0.70 ml of H₂O, 0.15 ml of Norit-carrier solution (27), 0.05 ml of 1 N HCl, and 0.20 ml of a Norit suspension (20% packed volume), all at 0°. The mixture was shaken for 1 minute; after 5 minutes at 0° the Norit was removed by centrifugation, and the supernatant fluid was quantitatively transferred to a tube containing 0.20 ml of Norit suspension. The mixture was treated as previously, 1.0 ml of the supernatant fluid was pipetted into a planchet, and 1 drop of 3 N KOH was added to the aliquot; the solution was taken to dryness, and the radioactivity was measured.

The supernatant fluids obtained from control incubations, with enzyme omitted, contained 0.02 to 0.06% of the added radioactivity. The two Norit adsorption steps were necessary to remove acid-soluble oligonucleotides present in the substrate (approximately 2 to 3%), and to remove acid-soluble oligonucleotides or mononucleotides produced as the result of nuclease action. Omission of the second Norit adsorption step increased the control values by 50 to 100%.

A unit of DNA phosphatase activity is defined as the amount causing the production of 1.0 mpmole of acid-soluble, Norit-nonadsorbable 32P in 30 minutes. The radioactivity produced was proportional to the enzyme concentration at levels of 0.04 to 0.40 unit of enzyme. Thus, with the addition of 1.7, 2.4, 3.4, 1.2, and 6.6 × 10⁻⁵ μg of protein (phosphocellulose pervaporate), specific activities of between 57,000 and 59,000 were found. The release of inorganic orthophosphate was also proportional to the length of incubation (Fig. 2) over a period of 150 minutes.

The 32P-labeled 3'-phosphoryl-terminated DNA produced by calf spleen DNase, the 5'-phosphoryl-terminated DNA, and the 3'-phosphoryl-terminated RNA were tested as substrates under the same assay conditions as described above. When 32P-labeled mononucleotides or 3'-phosphoryl-terminated acid-soluble oligonucleotides were tested as substrates, the acid precipitation was omitted, and only one Norit treatment was included. Inorganic phosphate produced by enzymatic action on 3'-TPcTPc was determined directly from the reaction mixture after removal of protein by trichloroacetic acid. The hydrolysis of p-nitrophenyl phosphate was measured by the formation of p-nitrophenol according to Razzell and Khorana (9).

Other Methods

Protein was determined, after precipitation with cold trichloroacetic acid, by the method of Lowry et al. (28). Inorganic orthophosphate was determined by the method of Chen, Toribara, and Warner (29), deoxyribose by the method of Dische (30), and ribose by the method of Mejaun (31). 32P was measured with approximately 80% efficiency in a Nuclear-Chicago model D-47 windowless gas flow counter.

Dilutions of enzyme for assay were routinely made in a solution composed of 0.03 M Tris buffer, pH 7.4, bovine plasma albumin, 0.5 mg per ml, and 0.001 M 2-mercaptoethanol.
**RESULTS**

*Purification of E. coli DNA Phosphatase-Exonuclease*

Unless otherwise indicated, all operations were carried out at 0–4°. All centrifugations were performed at 15,000 × g for 10 minutes. The purification procedure and results of a typical preparation are summarized in Table I. Due to variations resulting from the use of different extracts or fractions aged for varying periods, it is advisable to carry out a trial fractionation for the streptomycin and acetone steps.

**Preparation of Extracts**—The growth and preparation of cell-free extracts from *E. coli* strain B was as described for DNA polymerase (3). Four hundred fifty grams of packed cells, wet weight, yielded 2000 ml of extract (Table I).

**Streptomycin Precipitation**—To 2500 ml of extract were added 2500 ml of Tris-HCl buffer, 0.05 M, pH 7.5, containing 0.001 M EDTA, and then with constant stirring 360 ml of 5% streptomycin sulfate were added over a 45-minute period. After 10 minutes, the suspension was centrifuged and the supernatant fluid collected (Fraction II, Table I).

**Acetone Fractionation**—To 5000 ml of streptomycin supernatant, made 0.001 M with glutathione, were added, with stirring, 325 ml of acetone over a 45-minute period. The suspension was stirred for 10 minutes and centrifuged, and the precipitate was discarded. To the supernatant fluid were added, with stirring, an additional 1425 ml of acetone over a 90-minute period. The suspension was stirred for 10 minutes and then centrifuged. The resulting precipitate was dissolved in 1000 ml of potassium phosphate buffer, 0.01 M, pH 7.5, containing 0.001 M 2-mercaptoethanol (Fraction III). All procedures in the acetone fractionation, including centrifugation, were carried out at 0°.

**DEAE-cellulose Fractionation**—A column of DEAE-cellulose (85 cm² × 18 cm) was prepared and washed with approximately 5 liters of potassium phosphate buffer, 0.10 M, pH 7.5. When the pH of the eluate was that of the washing solution, the column was eluted with 10 liters of potassium phosphate buffer, 0.01 M, pH 7.5, containing 0.001 M 2-mercaptoethanol. The acetone fraction (1,000 ml) was applied to the column at the rate of 1,000 ml per hour. The adsorbent was then washed with 800 ml of potassium phosphate buffer, 0.01 M, pH 7.5, containing 0.001 M 2-mercaptoethanol. A linear gradient of elution was applied, with 0.01 M and 0.16 M potassium phosphate at pH 7.5 as limiting concentrations. The total volume of the gradient was 13,000 ml, and 0.001 M 2-mercaptoethanol was present throughout the gradient. The flow rate was 2,000 ml per hour, and 200-ml fractions were collected at 6-minute intervals. Approximately 80% of the activity was eluted in a peak between 4.0 and 5.5 resin bed volumes of effluent. The fractions which contained enzyme of specific activity greater than 1,600 units per mg of protein (approximately 50% of the starting activity) were pooled. This solution (1,800 ml) was dialyzed for 8 hours against 15 volumes of potassium phosphate buffer, 0.02 M, pH 6.5, containing 0.001 M 2-mercaptoethanol (Fraction IV).

**Phosphocellulose Fractionation**—A column of phosphocellulose (25 cm² × 14 cm) was prepared and washed with 10 liters of potassium phosphate buffer, 0.02 M, pH 6.5, containing 0.001 M 2-mercaptoethanol. The dialyzed DEAE-cellulose fraction (1,800 ml) was applied to the column at the rate of 1,500 ml per hour, and the adsorbent was then washed with 1,250 ml of potassium phosphate buffer, 0.02 M, pH 6.5, containing 0.001 M 2-mercaptoethanol. A linear gradient of elution was applied with 0.02 M and 0.28 M potassium phosphate at pH 6.5 as limiting concentrations. The total volume of the gradient was 4,300 ml, and 0.001 M 2-mercaptoethanol was present throughout the gradient. The flow rate was 750 ml per hour, and 25-ml fractions were collected at 6-minute intervals. Of the applied activity, 85% was eluted in a sharp peak between 6.2 and 7.2 resin bed volumes of effluent. The peak fractions containing enzyme of specific activity ranging from 44,000 to 59,000 units per mg of protein (50% of the starting activity) were pooled (Fraction V).

**Phosphocellulose Pervaporation**—The phosphocellulose fraction, 166 ml, was reduced in volume by pervaporation (pressure dialysis) against 4 liters of potassium phosphate buffer, 0.10 M, pH 7.4, containing 0.001 M reduced glutathione. A 30-cm length of Visking size 8 dialysis tubing was filled with the phosphocellulose enzyme solution. With one end open to the atmosphere, by means of a glass tube passed through a rubber stopper, the tubing was placed in a 4-liter suction flask containing buffer. A vacuum was applied to the flask by means of a water suction pump, and after approximately 20 hours the solution was reduced to the desired volume. As the dialysis tubing would only contain 50 ml of solution, even after expansion by pressure, it was necessary to add additional enzyme at intervals as the volume decreased. After pervaporation to a total volume of 13 ml, 87% of the activity in the phosphocellulose fraction was recovered (Fraction VI).

An aliquot of the phosphocellulose pervaporate was dialyzed against Tris buffer, 0.05 M, pH 7.5, containing 0.001 M reduced glutathione and 0.10 M potassium chloride, in order to remove phosphate from the enzyme preparation. An aliquot was stored at 0°; the remainder of the phosphocellulose pervaporate was further dialyzed against potassium phosphate buffer, 0.10 M, pH 7.5, containing 0.001 M reduced glutathione to assure removal of 2-mercaptoethanol, and was then frozen at −10°.

The phosphocellulose pervaporate represented a 1300-fold purification over the starting extract and contained 15% of the activity initially present. In all the experiments to be described, the phosphocellulose pervaporate was used as the enzyme source.

**Properties of Purified Enzyme**

**Stability**—The *E. coli* extract was stored for 2 weeks at 0° without appreciable loss in activity. The streptomycin supernatant fluid (Fraction II) was frozen and stored at −20° for 3 months without loss of activity. The phosphocellulose pervaporate (Fraction VI) was stored at 0° in potassium phosphate

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**Table I**

<table>
<thead>
<tr>
<th>Fraction and step</th>
<th>Total units of DNA phosphatase</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Extract</td>
<td>178</td>
<td>38</td>
</tr>
<tr>
<td>II. Streptomycin</td>
<td>160</td>
<td>54</td>
</tr>
<tr>
<td>III. Acetone</td>
<td>112</td>
<td>103</td>
</tr>
<tr>
<td>IV. DEAE-cellulose</td>
<td>60</td>
<td>2,380</td>
</tr>
<tr>
<td>V. Phosphocellulose</td>
<td>30</td>
<td>48,600</td>
</tr>
<tr>
<td>VI. Phosphocellulose pervaporate</td>
<td>26</td>
<td>48,800</td>
</tr>
</tbody>
</table>

*We acknowledge the valuable assistance of F. Sample in this purification procedure.*
Experiment 2, the effect of several cations in a reaction mixture containing an optimal MgCl₂ level (0.01 M) is shown. The pH of each buffer was determined at room temperature and 0.05
maleate at the same concentration. One hundred percent represents the release of 0.208 mmpmole of Pi. The pH of each buffer was
employed; potassium phosphate buffer was replaced by Tris-
phosphate pervaporate). Conditions of standard assay were
employed except for the replacement of potassium phosphate buffer, pH 7.0.

Other Enzyme Activities—Evidence presented in the succeeding
paper (32) indicates that this enzyme is also an exonuclease.
The presence of a trace of ribonuclease is noted in the next paper in studies on ribosomal RNA as a substrate. DNA polymerase assays showed no activity (less than 0.002 polymerase unit per 360 DNA phosphatase units). The lack of 5'-nucleotidase or conventional phospho-
monoesterase activity in the purified preparation will be docu-
mented below (see Table IV). The presence of a trace of ribo-
nuclease is noted in the next paper in studies on ribosomal RNA as a substrate (32).

Effect of pH on Rate of Reaction—The optimal pH range for the purified enzyme is 6.8 to 7.4 in potassium phosphate buffer, and 6.7 to 7.0 in Tris-maleate buffer (Fig. 1). Maximal activity was obtained at approximately pH 7.0 with either buffer.

Effect of Divalent Cations—The purified enzyme requires added magnesium ion for maximal activity. In the absence of added MgCl₂, only 5% of maximal activity was observed. The presence of EDTA in the reaction mixture (0.01 M) eliminated this residual activity, suggesting that it was the result of traces of contaminating metal ions. Under the conditions of the assay in either potassium phosphate or Tris-maleate buffer, the optimal Mg²⁺ concentration was 1 × 10⁻³ M. At 6.6 × 10⁻³ M and 3.0 × 10⁻³ M, 65% and 80%, respectively, of maximal activity was observed. In Tris-maleate buffer, Mn²⁺ could only partially replace Mg²⁺. Thus, at an optimal Mn²⁺ concentration (1 × 10⁻³ M), only 50% of the maximal activity seen with Mg²⁺ was observed. The addition of Mn²⁺ at 3.3 × 10⁻³ M to the optimal Mg²⁺ concentration resulted in a 44% inhibition. Mg²⁺ could not be replaced by other divalent cations such as Zn²⁺ or Ca²⁺. The addition of either of these produced no stimulation at concentrations ranging from 3.3 × 10⁻⁵ M to 3.3 × 10⁻⁴ M. The presence of Zn²⁺ in the complete reaction mixture was found to produce a striking inhibition. Thus, addition of Zn²⁺ at 3.3 × 10⁻⁵ M resulted in a 90% inhibition even in the presence of optimal Mg²⁺ concentration. Ca²⁺ produced no such inhibition at concentrations up to 3.3 × 10⁻³ M. These results are summarized in Table II.

Sulfhydryl Requirement, Inhibition by p-Chloromercuribenzoate—If 2-mercaptoethanol was omitted from the standard incubation mixture, 90% of the maximal activity was obtained. However, on incubation for longer periods (180 minutes), complete loss of activity was observed, which could be completely pre-
vented by the presence of 0.001 M 2-mercaptoethanol or 0.001 M reduced glutathione during the period of incubation (32) (Fig. 2). Cysteine could only partially replace 2-mercaptoethanol (80% of maximal activity after incubation for 180 minutes). Sulf-
hydryl sensitivity could also be demonstrated by inhibition of the reaction with p-chloromercuribenzoate. With 2-mercapto-
ethanol omitted from the enzyme diluent and reaction mixture,

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Studies on Enzymatic Specificity

Identification of Product as Inorganic Orthophosphate—The acid-soluble, Norit-nonadsorbable radioactivity resulting from the action of DNA phosphatase on 3'-phosphoryl-terminated DNA was chromatographed on a Dowex 1 column, chloride form, in the presence of unlabeled inorganic orthophosphate (Fig. 3). A peak of radioactivity, containing 98% of the initial $^{32}$P, was obtained which chromatographed identically with that of known inorganic orthophosphate.

Quantitative Release of 3'-Phosphoryl-terminal Groups as Inorganic Orthophosphate—Either with the addition of excess enzyme or with prolonged incubation, the release of orthophosphate could be shown to reach a limit. The further addition of enzyme resulted in no additional orthophosphate release. However, when an additional equal amount of substrate was added to the reaction mixture, a further release of orthophosphate occurred, equal to that initially produced (Fig. 4). It could also be demonstrated that the amount of orthophosphate released by the DNA phosphatase was identical with that released by E. coli alkaline phosphatase, acting on the same substrate.

The quantitative removal of the monoester phosphate from a number of 3'-phosphoryl-terminated DNA preparations could be demonstrated (Table III). As indicated above (Table III), 3'-phosphoryl-terminated DNA prepared either by micrococcal nuclease or by calf spleen nuclease treatment was a substrate for the purified enzyme (Table IV). In contrast, 5'-phosphoryl-terminated DNA prepared by E. coli endonuclease treatment was not a substrate under the conditions tested.

Failure of Small Polynucleotides to Serve as Substrates—This is shown by the lack of action on the acid-soluble 3'-phosphoryl-terminated oligonucleotides and the synthetic substrate TpTpTp (Table IV). Further evidence of the requirement for large polynucleotides is the lack of activity on 3'-mononucleotides; 5'-mononucleotides were also ineffective. The addition of any of these compounds to the standard assay produced no inhibition.

Preference for Double Stranded DNA—This is indicated by the diminished rates (30 to 50%) observed with 3'-phosphoryl-terminated DNA denatured by heating and quick cooling (see "Methods"). In addition, the rate of $P_i$ release was proportional to enzyme concentration only up to a release of 0.20 mmole of $P_i$, compared to 0.4 mmole with the unheated substrate. Although quantitative removal of $P_i$ could be accomplished, judged by the total amount of $P_i$ released by an excess of E. coli alkaline phosphatase, approximately 5 times more enzyme was required for the limit to be reached.

Failure of RNA to Serve as Substrate—The requirement for a
polydeoxyribonucleotide is shown by the absence of activity on 
32P-labeled 3'-phosphoryl-terminated E. coli ribosomal RNA 
(prepared by pancreatic RNAse treatment). This preparation 
produced no inhibition when added to the standard assay. 
This RNAse-treated 32P-labeled ribosomal RNA was a substrate for 
the E. coli alkaline phosphomonesterase (Table IV). The 
failure of the enzyme to act as an exonuclease on a double 
stranded copolymer of adenylate and uridylate (32) strongly 
suggests that this inactivity on ribosomal RNA is not due to 
its lack of a double stranded structure.

DNA with Phosphorylribonucleotide Terminus Serves as Sub-
strate—DNA chains to which mixed polymers of 32P-labeled 
ribocytidylate and unlabeled deoxyribonucleotides were added 
were hydrolyzed with alkali or with RNAse (see "Methods") 
and then tested as substrates for the phosphatase. After 
alkaline treatment, every chain should be terminated by a 3'-(or 2')-phosphorylribonucleoside (I in Scheme 2); after RNAse 
treatment, most chains should be terminated by a 3'-phosphoryl-
ribonucleotide. DNA phosphatase action releases P1, and thus 
provides II as a substrate for exonuclease action and subsequent 
release of 32P-5'-ribocytidylate. The data in Table V, combined 
with those in the succeeding report on exonuclease attack on 
the mixed polymer (32), support the reactions shown in Scheme 
2. The data indicate that prior exposure of alkaline-treated 
mixed polymers to the nonspecific E. coli alkaline phosphatase 
increases the release of 32P-5'-ribocytidylate by the DNA phos-
phatase-exonuclease; a similar experiment with the RNase-
treated mixed polymer shows no such effect by the alkaline 
phosphatase. These data suggest that the DNA phosphatase 
acts very slowly (or not at all) on the 2'-phosphoryl-terminated 
chains produced by alkaline hydrolysis.

**DISCUSSION**

The identity of the phosphatase and exonuclease functions 
of this enzyme will be discussed in the succeeding paper. We 
wish to dwell here on the specificity and significance of the 
phosphatase action. The structure of the substrate required 
for its action is a DNA chain terminated by a 3'-phosphoryl 
group; phosphate is not released from DNA chains terminated 
by a 5'-phosphoryl group. Lack of activity on acid-soluble 
oligonucleotides and diminished rates on heated DNA suggest a 
preference for helical structure in the DNA. The enzyme acts 
on a chain in which the terminal nucleotide contains ribose in 
place of 2'-deoxyribose with indications of a preference or speci-
ficity for the 3'-phosphoryl as compared to the 2'-phosphoryl 
ester; ribosomal RNA partially degraded by ribonuclease to 
yield 3'-phosphoryl groups is not a substrate.

An enzyme with similar physical and catalytic properties has 
been identified in Bacillus subtilis as well as in E. coli (34). In 
both organisms the DNA polymerases are strongly inhibited by 
DNA chains with 3'-phosphoryl termini and are therefore de-
pendent on the removal of these residues by the DNA phospha-
tase for initiation of DNA synthesis. This was clearly illustrated 
in a study of replication of B. subtilis DNA by E. coli polymerase. 
Without pretreatment of the DNA with the DNA phosphatase, 
a substantial fraction of the primer failed to react and did not 
form hybrid molecules with the newly synthesized strands.

The presence of the DNA phosphatase in E. coli and B. subtilis

### TABLE III

**Quantitative release of inorganic orthophosphate by DNA phosphatase and alkaline phosphatase**

<table>
<thead>
<tr>
<th>Extent of reaction</th>
<th>Extent of Amount of</th>
<th>DNA</th>
<th>Alkaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cleavage</td>
<td>substrate</td>
<td>phosphatase</td>
</tr>
<tr>
<td>Nuclease</td>
<td>% acid-soluble</td>
<td>mmoles</td>
<td>mmoles</td>
</tr>
<tr>
<td>Micrococcal</td>
<td>5</td>
<td>40</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>80</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>40</td>
<td>1.49</td>
</tr>
<tr>
<td>Spleen</td>
<td>2</td>
<td>40</td>
<td>0.007</td>
</tr>
</tbody>
</table>

![Fig. 4. Limit of P1 released from 3'-phosphoryl-terminated DNA. The incubations were carried out under standard assay conditions (see "Methods") in several tubes, with the addition of 0.40 unit of Fraction VI to each. At 160 minutes, an additional 40 mmoles of substrate were added and the subsequent release of P1 was followed. P1 released by E. coli alkaline phosphatase is shown by the dashed line. The incubation mixture for the alkaline phosphatase consisted of 30 mmoles of Tris buffer, pH 8.0, 3 mmoles of MgCl2, 40 mmoles of 32P-labeled 3'-phosphoryl-terminated DNA, and 0.02 mg of E. coli alkaline phosphatase in a volume of 0.30 ml. The assay for release of inorganic phosphate was identical with that for the DNA phosphatase. Longer incubation or additional enzyme resulted in no further release of phosphate.](image-url)
raises the question as to the natural occurrence and origins of such 3'-phosphoryl-ended DNA molecules. Are such groups present with a characteristic frequency in DNA in vivo? If not, are such groups created by shearing forces employed in the isolation of DNA with some disposition perhaps for the DNA to be cleaved at certain points? These places in the chain may represent the previous interposition of non-nucleotide residues between diester linkages. The presence of the DNA phosphatase not only poses these questions but also provides a reagent which may help in their solution. Combined with the use of mp-labeled DNA of high specific radioactivity, this enzyme will permit the quantitative determination of 3'-phosphoryl termini and a study of the conditions in vivo and in vitro which may help in their solution. Combined with the use of 3*P-alkaline phosphatase is described in Fig. 4, and the assay procedure for release of inorganic phosphate was identical, for each compound tested, with that used for the DNA phosphatase assay on the same compound. The 3'-phosphorylated terminated RNA, the "acid-soluble" 3'-phosphoryl-terminated oligonucleotides, TpTpTp, and the 3'- or 5'-mononucleotides produced no inhibition (less than 10%) in the standard assay.

Other ways in which this new enzyme may be exploited are now be checked by means of the phosphatase. Alkaline treatment or RNase treatment of what appeared by conventional physical criteria to be identical, native DNA preparations have been extremely erratic. The basis for this inhomogeneity would appear to be in large measure due to 3'-phosphoryl termini as judged by DNA phosphatase action. The specificity and controlled use of various exonucleaseic enzymes and identification of the type of phosphodiester bond cleavages by endonucleases can be facilitated by use of DNA phosphatase. Finally, the possibility that ribonucleotides may occur in DNA chains at a very low frequency may now be checked by means of the phosphatase. Alkaline treatment of 32P-labeled DNA should expose 3'-phosphoryl ends susceptible to the action of the enzyme and result in the release of 32P-inorganic phosphate; the inability of the enzyme to act on RNA chains reduces the threat of RNA contamination as a serious nuisance. Although the purification and characterization of the DNA phosphatase have been accomplished by means of a partially degraded DNA substrate, there is evidence that this enzyme also acts on native DNA. Native DNA preparations incubated with the compounds listed. The incubation mixture for the compound tested, with that used for the DNA phosphatase assay on the same compound. The 3'-phosphorylated terminated RNA, the "acid-soluble" 3'-phosphoryl-terminated oligonucleotides, TpTpTp, and the 3'- or 5'-mononucleotides produced no inhibition (less than 10%) in the standard assay.

### Table IV

**Specificity of DNA phosphatase**

Activity was measured as described in "Methods" with replacement of the usual 3'-phosphoryl-terminated DNA substrate by the compounds listed. The incubation mixture for the E. coli alkaline phosphatase is described in Fig. 4, and the assay procedure for release of inorganic phosphate was identical, for each compound tested, with that used for the DNA phosphatase assay on the same compound. The 3'-phosphorylated terminated RNA, the "acid-soluble" 3'-phosphoryl-terminated oligonucleotides, TpTpTp, and the 3'- or 5'-mononucleotides produced no inhibition (less than 10%) in the standard assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
<th>Relative activity (DNA phosphatase)</th>
<th>Total Pi released by E. coli alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-Phosphoryl-terminated DNA</td>
<td>40</td>
<td>100†</td>
<td>0.82</td>
</tr>
<tr>
<td>Micrococcal nuclease</td>
<td>40</td>
<td>91</td>
<td>0.10</td>
</tr>
<tr>
<td>Spleen nuclease</td>
<td>40</td>
<td>48‡</td>
<td>0.79</td>
</tr>
<tr>
<td>Heat-denatured 3'-phosphoryl-</td>
<td>40</td>
<td>0.10</td>
<td>1.80</td>
</tr>
<tr>
<td>terminated DNA</td>
<td>40</td>
<td>&lt;0.10</td>
<td>1.52</td>
</tr>
<tr>
<td>3'-Phosphoryl-terminated RNA</td>
<td>40</td>
<td>&lt;0.30</td>
<td></td>
</tr>
<tr>
<td>5'-Phosphoryl terminated DNA</td>
<td>40</td>
<td>&lt;0.30</td>
<td></td>
</tr>
<tr>
<td>&quot;Acid-soluble&quot; 3'-phosphoryl-</td>
<td>40</td>
<td>1.0</td>
<td>24.0</td>
</tr>
<tr>
<td>terminated oligonucleotides</td>
<td>112</td>
<td>&lt;0.3</td>
<td>30.6</td>
</tr>
<tr>
<td>TpTpTp</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'-dAMP, dTMP, dCMP, dGMP</td>
<td>30</td>
<td>0.2</td>
<td>28.0</td>
</tr>
<tr>
<td>(mixture)</td>
<td>16</td>
<td>0.2</td>
<td>15.9</td>
</tr>
<tr>
<td>5'-dTMP</td>
<td>16</td>
<td>0.3</td>
<td>15.3</td>
</tr>
<tr>
<td>dNTP</td>
<td>400</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>3'-Phosphoryl terminated RNA</td>
<td>40</td>
<td>1.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Micrococcal nuclease</td>
<td>40</td>
<td>91</td>
<td>0.10</td>
</tr>
<tr>
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</tr>
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<td>Heat-denatured 3'-phosphoryl-</td>
<td>40</td>
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<td>1.80</td>
</tr>
<tr>
<td>terminated DNA</td>
<td>40</td>
<td>&lt;0.10</td>
<td>1.52</td>
</tr>
<tr>
<td>3'-Phosphoryl-terminated RNA</td>
<td>40</td>
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<td></td>
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<td>5'-Phosphoryl terminated DNA</td>
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<tr>
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<td>5'-dTMP</td>
<td>16</td>
<td>0.3</td>
<td>15.3</td>
</tr>
<tr>
<td>dNTP</td>
<td>400</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

* Nucleotide equivalents.
† Represents a turnover number of 1800 μmoles of Pi released per minute per mg of the phosphocellulose pervaporate fraction; activity on other compounds is expressed relative to this.
‡ Enzyme proportionality only up to a release of 0.20 μmole of Pi.

### Scheme 2

The sequential action of the DNA phosphatase-exonuclease on a mixed polymer terminated by ribocytidylate and sub-terminal deoxynucleotides By and Bz.

### Table V

**Extent of action of DNA phosphatase-exonuclease on alkali- or RNase treated mixed (ribo deoxynucleotidyl) polymer**

Each incubation mixture contained (in 0.3 ml) 0.05 ml of alkalitreated or RNase-treated 32P-labeled mixed polymer (see "Methods"), 0.07 M Tris buffer, pH 7.8, and 0.01 M MgCl2. Mixtures 2 and 3 contained 20 μg of E. coli alkaline phosphatase. After 1 hour at 37°C, 0.03 ml of 0.01 M meroaptoetanol, 0.02 ml of 1 M potassium phosphate buffer, pH 7.0, and 0.01 ml of 0.5 M HCl were added to each tube. Mixtures 1 and 3 also received 16 μg of DNA phosphatase-exonuclease. Incubation was for 2 hours at 37°C. Release of 32P into acid-soluble form from the polymer was determined as in nuclease assays (see "Methods").

<table>
<thead>
<tr>
<th>Mixture and enzyme addition</th>
<th>Alkali-treated</th>
<th>RNase-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1. DNA phosphatase-exonuclease</td>
<td>65*</td>
<td>69</td>
</tr>
<tr>
<td>2. E. coli alkaline phosphatase</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>3. Both enzymes</td>
<td>93</td>
<td>60</td>
</tr>
</tbody>
</table>
with the DNA phosphatase contain (or develop) 3'-phosphoryl termini which are released as Pi by this enzyme. The values obtained from E. coli DNA and T7 phage DNA preparations were approximately 0.03 and 0.01% of the phosphate residues, respectively. These native samples of DNA, when treated with the enzyme, are more active as primers by a factor of 2 to 10. From this it may be inferred that there are inhibitory 3'-phosphoryl groups whose removal leads to this increased priming activity. The addition of such 3'-phosphoryl-terminated DNA to a DNA polymerase reaction, primed by native DNA, results in a striking inhibition of polymerase action (1).

**SUMMARY**

1. A new and highly specific phosphatase has been purified approximately 1300-fold from extracts of *Escherichia coli*. The enzyme quantitatively releases inorganic orthophosphate from 3'-phosphoryl-terminated deoxyribonucleic acid.
2. The enzyme is less active on heat-denatured 3'-phosphoryl-terminated DNA than on the unheated preparation. The enzyme fails to act as a phosphatase on 5'-phosphoryl-terminated DNA.
3. The enzyme attacks DNA with a phosphorylribonucleotide terminus. Unlike *E. coli* alkaline phosphatase, the DNA phosphatase is unable to act on 3' or 5'-deoxymononucleotides or on deoxyoligonucleotides of short chain length.
4. The DNA phosphatase activity is associated with an exonuclease activity described in the following paper.

**REFERENCES**


* L. Bertuch and A. Kornberg, unpublished results.

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A Deoxyribonucleic Acid Phosphatase-Exonuclease from Escherichia coli: I. PURIFICATION OF THE ENZYME AND CHARACTERIZATION OF THE PHOSPHATASE ACTIVITY

Charles C. Richardson and Arthur Kornberg


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