A Deoxyribonucleic Acid Phosphatase-Exonuclease from Escherichia coli

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

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(Received for publication, July 18, 1963)

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. The enzyme thus hydrolyzes 3'-phosphoryl terminus of a DNA chain. Such a 3'-phosphoryl termi-

Experimental Procedure

Materials

Enzymes—Crystalline pancreatic DNase and RNase were purchased from the Worthington Biochemical Corporation. Endonuclease from E. coli was the concentrated carboxymethyl cellulose fraction, and had a specific activity of 6300 units per mg of protein (2). The 5'-nucleotidase from Crotalus adamanteus venom, specific for 5'-mononucleotides, was purified and assayed as previously described (2). DNA polymerase from E. coli was the phosphocellulose fraction purified and assayed as described in the previous communication (3).

Micrococcal nuclease (4), purified from Micrococcus pyogenes, was a gift of Dr. C. A. Dekker. The enzyme was assayed by incubating 10 μmoles of glycine buffer, pH 9.2, 2 μmoles of CaCl₂, 30 μmoles of ³²P-labeled native E. coli DNA, and enzyme in a volume of 0.30 ml. After incubation for 30 minutes at 37°, the reaction was stopped by chilling and by the addition of 0.20 ml of calf thymus DNA (2.5 mg per ml) as carrier and of 0.50 ml of 7% cold perchloric acid. After 5 minutes at 0°, the precipitate was removed by centrifugation and the radioactivity in the supernatant fluid was determined. A unit of enzyme activity was the amount causing the production of 10 μmoles of acid-soluble DNA nucleotides in 30 minutes. Under these conditions, the micrococcal nuclease preparation had a specific activity of 300,000 units per mg of protein.

The spleen deoxyribonuclease described by Koerner and Sinheimer (5) was a gift of Dr. Walter Bodmer, and had been purified by the procedure of Fredericq and Oth (6). The enzyme was assayed by incubating 10 μmoles of glycine buffer, pH 5.0, 30 μmoles of ³²P-labeled native E. coli DNA, and enzyme in a volume of 0.30 ml. After incubation for 30 minutes at 37°, the 3'-hydroxyl end of a DNA chain. The enzyme thus hydrolyzes phosphomonoo- 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).

* This work was supported in part by Grants GM 07581 and 511 GM 196 from the National Institutes of Health, United States Public Health Service.
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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$^2$ x 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 wash 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 32P-labeled 5'-dAMP or on 32P-labeled 3'-deoxynucleotides.

**Nucleic Acids and Nucleotides**—Calf thymus DNA was isolated by the method of Kay, Simmons, and Dounce (10). 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. 32P-Labeled DNA (25 μc per μmole of phosphate) isolated from Bacillus subtilis (6D 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 0.10% by treatment with pancreatic DNAse. Soluble RNA was prepared as previously described (13), and the synthetic oligonucleotides, TpTpT and TpTpT, 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 TpTpT and TpTpT, respectively. 32P-Labeled rCTP (6 x 10$^6$ c.p.m. per μmole) was prepared as previously described (16); unlabeled deoxyribonucleoside 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 d13M' 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.

32P-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) was

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.

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.

3' 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, 8 μmoles of MgCl₂, 1.5 μmoles of ³²P-labeled DNA, and 5 units of urokinase. After incubation for 180 minutes, 21% of the radioactivity had become acid-soluble. To prevent further action by the enzyme (23), 30 μmoles 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 degraded 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 deoxy nucleotides with a frequency of about 1 per 10 nucleotides. The incubation mixture (10.0 ml) contained 0.04 μm Tris buffer, pH 7.8, 0.004 μm 2-mercaptoethanol, 0.002 μm MnCl₂, 0.4 μmoles each of dATP, dGTP, dTTP, and dCTP, 2.0 μmoles of ³²P-labeled rCTP (6 X 10⁶ c.p.m. per μmole), 5 μg of calf thymus DNA, and 7 μg of 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 μmoles of ribocytidylate were in a polymeric form precipitable by 3.5% perchloric acid; 0.2 μmole was acid-soluble. Assuming an equal incorporation of ribo- and deoxyribocytidylate and 1 cytosine nucleotide per 4.6 nucleotides, approximately 200 μmoles of newly synthesized mixed polymer were recovered in the dialyzed product.

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

R Nase 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 non-adsorbable 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 μmoles 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 x 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 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 μmole of acid-soluble, Norit-non-adsorbable 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, and 6.6 X 10⁻³ μg of protein (protein phosphocellulose per vaporetto), 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 Tr/T₄/T₅ 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 Meijbaum (31). ³²P was measured with approximately 80% efficiency in a Nuclear-Chicago model D-47 windowless gas flow counter.

1 Dilutions of enzyme for assay were routinely made in a solution composed of 0.05 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 °C. All centrifugations were performed at 15,000 x 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 °C.

**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 equilibrated 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 (50% of the starting activity) were pooled (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 2-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 °C; 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 °C.

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 °C without appreciable loss in activity. The streptomycin supernatant fluid (Fraction II) was frozen and stored at -20 °C for 3 months without loss of activity. The phosphocellulose pervaporate (Fraction VI) was stored at 0 °C 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>176</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>
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<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. Allowing maximal activity of the enzyme is recorded; in Experiment 1, the level of cation concentrations were tested. In Experiment 1, the 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 ion. Under the conditions of the assay in either potassium phosphate or Tris-maleate buffer, the optimal Mg ++ concentration was 1 x 10⁻² M. At 6.6 x 10⁻² M and 3.0 x 10⁻³ M, 63% and 80%, respectively, of maximal activity was observed. In Tris-maleate buffer, Mn ++ could only partially replace Mg ++. Thus, at an optimal Mn ++ concentration (1 x 10⁻² M), only 59% of the maximal activity seen with Mg ++ was observed. The addition of Mn ++ at 3.3 x 10⁻³ M to the optimal Mg ++ concentration resulted in a 44% inhibition. Mg ++ could not be replaced by other divalent cations such as Zn ++ or Ca ++. The addition of either of these produced no stimulation at concentrations ranging from 3.3 x 10⁻⁵ M to 3.3 x 10⁻² M. The presence of Zn ++ in the complete reaction mixture was found to produce a striking inhibition. Thus, addition of Zn ++ at 3.3 x 10⁻⁵ M resulted in a 90% inhibition in the presence of optimal Mg ++ concentration. Ca ++ produced no such inhibition at concentrations up to 3.3 x 10⁻³ M. These results are summarized in Table II.

**Table II**

Effect of divalent cations on DNA phosphatase activity

<table>
<thead>
<tr>
<th>Metal addition</th>
<th>Activity (m x 10⁻¹ mmole)</th>
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<tr>
<td>None*</td>
<td>&lt;0.01</td>
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<tr>
<td>None⁺</td>
<td>0.02</td>
</tr>
<tr>
<td>MgCl₂⁺</td>
<td>0.32</td>
</tr>
<tr>
<td>MnCl₂⁺</td>
<td>0.18</td>
</tr>
<tr>
<td>CaCl₂⁺</td>
<td>0.02</td>
</tr>
<tr>
<td>ZnCl₂⁺</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MgCl₂⁺ + CaCl₂</td>
<td>0.32</td>
</tr>
<tr>
<td>MgCl₂⁺ + MnCl₂</td>
<td>0.14</td>
</tr>
<tr>
<td>MgCl₂⁺ + ZnCl₂</td>
<td>0.0033</td>
</tr>
</tbody>
</table>

* EDTA added, 0.01 M.
† Identical results were obtained in potassium phosphate buffer, pH 7.0.

**Fig. 1** pH-activity curve of E. coli DNA phosphatase (phosphocellulose pervaporate). Conditions of standard assay were employed; potassium phosphate buffer was replaced by Tris-maleate at the same concentration. One hundred percent represents the release of 0.208 mmole of P₃. The pH of each buffer was determined at room temperature and 0.05 M.

**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.

**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 (120 minutes), complete loss of activity was observed, which could be completely prevented by the presence of 0.001 M 2-mercaptoethanol or 0.001 M reduced glutathione during the period of incubation (4). Cysteine could only partially replace 2-mercaptoethanol (80% of maximal activity after incubation for 120 minutes). Sulfhydryl sensitivity could also be demonstrated by inhibition of the reaction with p-chloromercuribenzoate. With 2-mercaptoethanol omitted from the enzyme diluent and reaction mixture, importance of maintaining high levels of a sulfhydryl reagent during extended incubation of the purified enzyme will be considered below.

**Other Enzyme Activities**—Evidence presented in the succeeding paper (32) indicates that this enzyme is also an exonuclease. The other E. coli nucleases are not detectable. Absence of activity (less than 10 units per mg of protein) on pTTPTPTPTP (32) eliminates exonuclease 1 (phosphodiesterase (11)) and exonuclease II (33); endonuclease (2) is judged to be absent by lack of inhibition by RNA or the specific antiserum with native DNA as 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 phosphomonoesterase activity in the purified preparation will be documented below (see Table IV). The presence of a trace of ribonuclease is noted in the next paper in studies on ribosomal RNA as a substrate (32).

Recent studies using a preparation of Fraction VI stored at -10°C for 3 months demonstrated a requirement for 0.01 M 2-mercaptoethanol in order to obtain sustained activity under more alkaline incubation conditions such as those optimal for assay of the exonuclease activity (32).
3 × 10⁻² M and 1 × 10⁻⁴ M p-chloromercuribenzoate resulted in a 20% and 50% inhibition, respectively.

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 ³²P, 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 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 Pᵢ release was proportional to enzyme concentration only up to a release of 0.20 mmole of Pᵢ, compared to 0.4 mmole with the unheated substrate. Although quantitative removal of Pᵢ could be accomplished, judged by the total amount of Pᵢ 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
Displacement of Pi by 3'-phosphoryl termini of DNA chains.

**Fig. 4.** Limit of Pi 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 μmole of substrate was added and the subsequent release of Pi was followed. Pi released by E. coli alkaline phosphatase is shown by the dashed line. The incubation mixture for the alkaline phosphatase consisted of 30 μmoles of Tris buffer, pH 8.0, 3 μmoles of MgCl₂, 40 μmoles 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.

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 phosphomonoesterase (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 Terminator Serves as Substrate—DNA chains to which mixed polymers of 32P-labeled ribocytidylate and unlabeled deoxycytidylate 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'-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 specificity 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 dependent on the removal of these residues by the DNA phosphatase 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

Extent of reaction was measured as in the standard assay with an excess of enzyme present (2.0 to 5.0 units). Additional enzyme was added after 20 minutes of incubation in parallel reaction mixtures, and an additional 30-minute incubation was carried out and assayed for further orthophosphate release. This assured that a limit of phosphate released was measured. A similar assay utilizing the release of phosphate by E. coli alkaline phosphatase was carried out (see legend to Fig. 4). The 32P-labeled substrates were prepared by hydrolysis of 32P-labeled E. coli DNA to different extents with either micrococcal or spleen nuclease as described in "Methods." The extent of hydrolysis (acid-soluble radioactivity) and the amount per reaction mixture are recorded.

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<td></td>
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<td>40</td>
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<tr>
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<td>80</td>
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<td>1.58</td>
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<td>40</td>
<td>40</td>
<td>0.08</td>
<td>1.04</td>
<td></td>
</tr>
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<td>Spleen</td>
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<td>40</td>
<td>0.007</td>
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</tbody>
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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 sp3-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 create them.

Other ways in which this new enzyme may be exploited in

**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'-phosphoryl-terminated RNA, the "acid-soluble" 3'-phosphoryl-terminated oligo-nucleotides, TpTpTp, and the 3' or 5'-mononucleotides produced no inhibition (less than 10%) in the standard assay.

<table>
<thead>
<tr>
<th>Compound Description</th>
<th>Amount</th>
<th>Relative Activity (DNA phosphatase)</th>
<th>Total Pi released by <em>E. coli</em> alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-Phosphoryl-terminated DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrococcal nuclease</td>
<td>40</td>
<td>100†</td>
<td>0.82</td>
</tr>
<tr>
<td>Spleen nuclease</td>
<td>40</td>
<td>91</td>
<td>0.10</td>
</tr>
<tr>
<td>Heat-denatured 3'-phosphoryl-terminated DNA</td>
<td>40</td>
<td>&lt;0.10</td>
<td>0.79</td>
</tr>
<tr>
<td>3'-Phosphoryl-terminated RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-Phosphoryl terminated DNA</td>
<td>40</td>
<td>&lt;0.30</td>
<td>1.52</td>
</tr>
<tr>
<td>&quot;Acid-soluble&quot; 3'-phosphoryl-terminated oligonucleotides</td>
<td>40</td>
<td>&lt;1.0</td>
<td>24.0</td>
</tr>
<tr>
<td>TpTpTp</td>
<td>112</td>
<td>&lt;0.3</td>
<td>30.6</td>
</tr>
<tr>
<td>3'-dAMP, dTMP, dCMP, dGMP (mixture)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>&lt;0.1</td>
<td>28.0</td>
</tr>
<tr>
<td>5'-dAMP</td>
<td>16</td>
<td>&lt;0.2</td>
<td>15.9</td>
</tr>
<tr>
<td>5'-dTMP</td>
<td>16</td>
<td>&lt;0.3</td>
<td>15.3</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>400</td>
<td>0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Nucleotide equivalents.
† Represents a turnover number of 1800 mumoles of P1 released per minute per mg of the phosphocellulose per vaporex fraction; activity on other compounds is expressed relative to this.
‡ Enzyme proportionality only up to a release of 0.20 mumole of P1.

**Table V**

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

Each incubation mixture contained (in 0.3 ml) 0.05 ml of alkali-treated 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°, 0.03 ml of 0.01 M 2 mercaptoethanol, 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°. 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. <em>E. coli</em> 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>

* This value is a limit as judged by incubation for periods up to 5 hours. More than 96% of the 32P released was in a nucleotide form as judged by adsorbability to Norit; upon treatment with snake venom 5'-nucleotidase, 65% of the total 32P, i.e. 100% of the acid-soluble 32P, was released as P1. In two other experiments, 48 and 66% of 32P in the polymer was rendered acid-soluble by the DNA phosphatase-exonuclease; in each case all of the 32P in the acid-soluble fraction was then released as P1 by 5'-nucleotidase.

elucidation of DNA structure and its enzymatic replication breakdown are immediately apparent. The priming capacities 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 exonuclease 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 sp3-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

**Scheme 2.** The sequential action of the DNA phosphatase-exonuclease on a mixed polymer terminated by ribocytidylic and sub-terminal deoxynucleotides By and Bz.

**Scheme 2**
with the DNA phosphatase contain (or develop) 3'-phosphoryl termini which are released as P_i 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 or on 3'-phosphoryl-terminated ribonucleic acid.

3. The enzyme attacks DNA with a phosphorylribonucleotide terminus.

4. Unlike E. coli alkaline phosphatase, the DNA phosphatase is unable to act on 3' or 5'-deoxymononucleotides or on deoxynucleotides of short chain length.

5. The DNA phosphatase activity is associated with an exonuclease activity described in the following paper.

REFERENCES


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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