The Deoxyribonucleases of Escherichia coli

I. PURIFICATION AND PROPERTIES OF A PHOSPHODIESTERASE

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There are a number of enzymes present in cell-free extracts of Escherichia coli which can degrade deoxyribonucleic acid (DNA) and smaller polynucleotides derived from it (1-3). One of these enzymes, although unable to hydrolyze high molecular-weight DNA at an appreciable rate, will rapidly hydrolyze DNA which has undergone some prior degradation; it is in this respect analogous to the classical phosphodiesterase of snake venom (4). The purpose of this report is to describe in detail the purification and properties of this enzyme which will be referred to as the Escherichia coli phosphodiesterase.

This diesterase has been found to hydrolyze E. coli and calf thymus DNA's to their constituent 5'-mononucleotides once these polymers have undergone some degradation as a result either of heating or limited treatment with pancreatic DNase. The E. coli phosphodiesterase also is capable of degrading appropriately pretreated bacteriophage DNA's bearing glucosylated hydroxymethyl cytosine to their constituent mononucleotides. In this respect, it differs from the venom diesterase, which is unable to catalyze the cleavage of most of the linkages in which glucosylated hydroxymethyl cytosine is involved (5-7).

In further contrast to the venom diesterase, the E. coli phosphodiesterase cannot hydrolyze either free dinucleotides or the 5'-terminal dinucleotide portion of a polydeoxyribonucleotide chain.

EXPERIMENTAL

Materials

Crystalline pancreatic DNase and RNase were products of the Worthington Biochemical Corporation. Purified human semen mononuclease was the generous gift of Dr. L. A. Heppel. 5'-Nucleotidase from bull semen was prepared by the method of Heppel and Hilmoe (8). Venom phosphodiesterase was prepared from Crotalus adamanteus venom by the method of Sinsheimer and Koerner (9).

P32-labeled DNA was isolated from E. coli grown to a limit on P32-orthophosphate. Glycerol-lactate (10) medium, 200 ml, containing 0.6 µmole of orthophosphate per ml (specific activity, 50 µecuries per µmole) was inoculated with 0.2 ml of a 7-hour nutrient broth culture of E. coli strain B. After 18 hours of growth, the cells were harvested and washed twice with 0.9% KCl. The packed cells (0.7 g) were suspended in 4.5 ml of 0.14 M NaCl containing 0.01 M sodium citrate, and then 320 mg of recrystallized sodium dodecyl sulfate were added. The suspension was stirred for 1 hour at room temperature during which time it became clear and extremely viscous. Two volumes of 95% ethanol were added and the heavy fibrous precipitate which formed was transferred to a polyethylene tube and was homogenized for several minutes by means of a glass pestle, with 5 ml of 1.4 M NaCl-0.01 M sodium citrate. The suspension was centrifuged at 12,000 X g for 10 minutes and the slightly opalescent, viscous supernatant fluid was drawn off. This extraction process was repeated twice on the residual precipitate and the supernatant fluids were combined. Upon the addition of 2 volumes of cold 95% ethanol to the supernatant fluids, a fibrous precipitate was formed which was dissolved in 5 ml of 0.14 M NaCl-0.01 M sodium citrate. Sodium dodecyl sulfate, 22.5 mg, was added and the solution was again stirred at room temperature for 1 hour. Solid NaCl was added to a final concentration of 1 M, and after the resulting precipitate was removed by centrifugation for 20 minutes at 12,000 X g the supernatant fluid was again treated with 2 volumes of ethanol and the precipitated DNA was dissolved in 3 ml of 0.14 M NaCl-0.01 M sodium citrate. Pancreatic RNase, 40 µg, was added and the solution was incubated at room temperature for 15 minutes. The solution was mixed with 1.5 ml of a Norit suspension (20% packed volume) and stirred at 0° for 5 minutes; the Norit was removed by centrifugation. This process was repeated and the Norit was washed with 2 ml of 0.14 M NaCl-0.01 M sodium citrate. The supernatant solution obtained after Norit treatment, and the wash were combined and treated with 2 volumes of ethanol. The DNA fibers which formed were dissolved in 4 ml of 0.02 M NaCl and the solution was centrifuged for 30 minutes at 105,000 X g. Two volumes of cold ethanol were added to the supernatant fluid and the resulting DNA fibers were dissolved in 4 ml of 0.02 M NaCl. The DNA prepared in this way had a molar extinction coefficient at 260 mp of 6.9 X 10^3 based on deoxyribose and a reduced viscosity of 52 (g/100 ml)-1. Protein contamination was of the order of 2% or less.

Unlabeled E. coli DNA was isolated in the same way from cells grown in M-9 glucose-salts medium (11).

Calf thymus DNA was isolated by the method of Kay et al. (12).

T2r+ bacteriophage was grown and purified by the method of Herrick and Barlow (13). The DNA was isolated from the phage by osmotic shock (14), followed by removal of the intact bacteriophage and ghosts by centrifugation for 90 minutes at 15,000 X g. The DNA was deproteinized by shaking with an equal volume of 10% octanol in chloroform for 30 minutes, then precipitated with 2 volumes of cold 95% ethanol, and finally dissolved in 0.02 M NaCl.

1 This procedure for the isolation of E. coli DNA which is a modification of the procedure of Zamenhof et al. (31) was developed by S. R. Kornberg, to whom I am grateful for permission to describe the details in this paper.
DNA from bacteriophage χX 174 was the generous gift of Dr. Robert L. Sinsheimer.

Partially degraded P32-labeled DNA was prepared by treating the P32-labeled E. coli DNA with pancreatic DNase. The reaction mixture (5.0 ml) consisted of the following: E. coli DNA, 2 μmoles of P containing 0 μc of P32; pancreatic DNAse, 0.005 μg; bovine plasma albumin, 500 μg; Tris buffer, pH 7.5, 50 μmoles; and MgCl2, 5 μmoles. After incubation at 37° for 1 hour, the mixture was chilled in ice and 0.1 volume of cold 70% (weight per volume) trichloroacetic acid was added. The suspension was kept at 0° for 10 minutes, then centrifuged at 12,000 × g for 5 minutes. The precipitate was dissolved in 4 ml of 0.02 N NaOH, then precipitated by the addition of 4 ml of cold 1 N perchloric acid; the solution and reprecipitation were repeated. The final precipitate was dissolved in 2 ml of 0.02 N NaOH; 0.1 N HCl was added to pH 7 and the volume was adjusted to 8 ml with distilled water. Such a solution contained approximately 0.2 μmole of P per ml.

Calf thymus DNA labeled with C14 deoxyctydylate at its 3'-hydroxyl end was prepared by treating calf thymus DNA with uniformly labeled C14-deoxyctydylidine triphosphate in the presence of purified E. coli polymerase (15). 5'-dCMP32 was isolated from P32-labeled E. coli DNA as described previously (2). Unlabeled deoxynucleoside monophosphates were purchased from the California Foundation for Biochemical Research.

Diethylaminoethyl cellulose (DEAE-cellulose) was purchased from Brown and Company, Berlin, New Hampshire. Protamine sulfate was obtained from Eli Lilly and Company. Crystaline bovine plasma albumin was purchased from Armour and Company.

Methods

Assay of E. coli Phosphodiesterase—This assay measures the conversion of a partially degraded (but acid-insoluble) DNA labeled with P32 to acid-soluble fragments. The incubation mixture (0.30 ml) contained 20 μmoles of glycyine buffer pH 9.2, 2 μmoles of MgCl2, 10 μmoles of partially degraded P32-DNA (2 μc per μmole of P), and 0.05 to 0.25 unit of enzyme. The mixture was incubated at 37° for 30 minutes; 0.2 ml of a solution of calf thymus DNA (2.5 mg per ml) was added as “carrier” and then 0.5 ml of cold 0.5 N perchloric acid was added. After 5 minutes at 0°, the precipitate was removed by centrifugation at 10,000 × g for 3 minutes, and 0.2 ml of the supernatant fluid was pipetted into a planchet. After addition of a drop of 1 N KOH the solution was taken to dryness and the radioactivity determined.

The supernatant fluids obtained from control incubations (enzyme omitted), contained 0.3 to 0.4% of the added radioactivity. A unit of enzyme is defined as the amount causing the production of 10 μmoles of acid-soluble P32 in 30 minutes. The radioactivity made acid-soluble was proportional to the enzyme concentration at levels from 0.05 to 0.25 unit of enzyme. Thus, with the addition of 0.005, 0.01, 0.02, and 0.04 ml of a 1:50 dilution of crude E. coli extract, 300, 367, 379, and 334 units of enzyme per ml of extract, respectively, were obtained.

Deoxyxypentose was measured by the method of Dische (16). Protein was determined according to the method of Lowry et al. (17). Phosphate was determined by the method of Fiske and SubbaRow (18). P32 was measured with a thin window Geiger-Müller tube. C14-containing samples were assayed in a windowless gas flow counter.

Paper electrophoresis was carried out at room temperature by the method of Markham and Smith (19), in 0.02 M ammonium formate buffer pH 3.5, at a potential of 1200 volts. Under these conditions, 2 hours were sufficient to achieve a complete separation of the four deoxynucleoside 5'-phosphates.

Paper chromatographic separations were run for 14 to 24 hours in the solvent systems described by Markham and Smith (19) modified in the following way: Solvent 1, ammonium sulfate saturated in water, 80 parts; 1 M sodium acetate, 18 parts; isopropanol, 2 parts (volume per volume per volume). Solvent 2, n-propanol, 60 parts; concentrated ammonium hydroxide, 30 parts; water, 10 parts (volume per volume per volume). Solvent System 1 was used with Whatman No. 3MM paper and Solvent System 2 with Whatman No.1 paper. Whatman No.3MM paper was used for electrophoresis.

RESULTS

Purification of E. coli Phosphodiesterase

Sonic extracts of E. coli strain B were prepared as described previously (2). All steps in the purification were carried out at 0-4° (Table I).

Protamine Precipitation and Elution—To 800 ml of extract were added, first, 800 ml of glycyglycine buffer (0.05 M, pH 7.0) and then slowly, with stirring, 160 ml of a 2% protamine sulfate solution. After 10 minutes, the suspension was centrifuged at 10,000 × g for 15 minutes. The gummy precipitate was transferred to a Waring Blender and homogenised with 1600 ml of potassium phosphate buffer (0.07 M, pH 8.0) for 5 minutes at low speed. The suspension was centrifuged for 3 hours at 44,000 × g in a Spinco model L centrifuge, and the supernatant fluid was collected.

Concentration of Protamine Eluate—To 1,600 ml of protamine eluate were added 904 g of cold ammonium sulfate. After 10 minutes at 0°, the precipitate was collected by centrifugation for 30 minutes at 10,000 × g and dissolved in 300 ml of potassium phosphate buffer (0.05 M, pH 6.8).

Ammonium Sulfate Fractionation—To 300 ml of concentrated protamine eluate were added 30 g of ammonium sulfate, and after 10 minutes at 0°, the resulting precipitate was collected by centrifugation for 10 minutes at 12,000 × g; it was dissolved in 36 ml of potassium phosphate buffer (0.02 M, pH 7.5).1

DEAE-Cellulose Chromatography—A column of DEAE-cellulose (20) (10 × 2.2 cm) was prepared and equilibrated with potassium phosphate buffer (0.02 M, pH 7.5). Ammonium sulfate fraction, 40 ml, which had been previously dialyzed against potassium phosphate buffer (0.02 M, pH 7.5) was added to the column, and the adsorbent was washed with 10 ml of the same buffer. A linear gradient was applied with 0.1 M and 0.5 M potassium phosphate at pH 6.5 as limiting concentrations; 150 ml of each buffer was used and the flow rate was 70 ml per hour. Fractions were collected at 5-minute intervals. Over 90% of the activity was eluted in a discrete peak between 3.6 and 5.0 resin-bed volumes of effluent. The peak fractions which con

1 A second active fraction is precipitated from solution at higher ammonium sulfate concentrations. Preliminary experiments indicate that this fraction contains another phosphodiesterase differing from the one described here with regard to Mg2+ requirement and pH optimum.
May 1960

I. R. Lehman

Studies on Enzymatic Mechanism

Stepwise Hydrolysis of DNA—When DNA terminally labeled at its 3'-hydroxyl end with C14-deoxyctydylate was treated with the E. coli phosphodiesterase, 70% of the radioactive deoxyribonucleotides of the molecule were made acid-soluble at a time (2 hours) when less than 3% of the unlabeled nucleotides had been released, judged by the appearance of acid-soluble ultraviolet absorbing material (Fig. 1B). This result is similar to that observed with venom diesterase (Fig. 1A), an enzyme which attacks DNA or polynucleotides stepwise from the end bearing a free 3'-hydroxyl group (15, 21, 22). Digestion of the terminally labeled DNA with pancreatic DNase, which attacks DNA in a random manner (23), resulted in the appearance at 2 hours of 77% of the radioactivity and 55% of the ultraviolet absorbing material in an acid-soluble form (Fig. 1C). It therefore appears that the phosphodiesterase from E. coli carries out a stepwise attack on DNA starting from the 3'-hydroxyl end in a manner analogous to venom diesterase.

Effect of State of DNA on Enzymatic Rate—The E. coli phosphodiesterase hydrolyzes intact DNA at about one-hundredth the rate observed with DNA which had been treated with pancreatic DNase. When the DNA was preheated at 100° for 10 minutes, the enzymatic rate was over 300-fold greater than that observed with unheated DNA as substrate. DNA from the bacteriophage φX 174, which has been shown to be single-stranded (24), was hydrolyzed without prior degradation, over 200 times as rapidly as intact E. coli DNA, and at a rate nearly equivalent to that of the heated DNA (Table I).

In a mixture composed of heated and unheated DNA, the E. coli phosphodiesterase is preferentially able to degrade the DNA which has been heated (Table III). When a mixture composed of heated P32-labeled E. coli DNA and unheated, unlabeled E. coli DNA was treated with the phosphodiesterase, all of the P32 was made acid-soluble, together with an amount of ultraviolet absorbing material corresponding to the total amount of heated DNA added to the mixture. In the reverse situation (heated, unlabeled DNA and heated P32-labeled DNA), less than 2% of the radioactive DNA was made acid-soluble at a time when 88% of the added unlabeled DNA was converted to an acid-soluble form as measured by the appearance of acid-soluble ultraviolet absorbing material.

DNA, when heated, undergoes a sharp helix coil transition resulting from the rupture of the hydrogen bonds of the molecule and the collapse of the macromolecular structure. This transition can be easily measured by observing the rise in optical density (the hyperchroismic) of the DNA solution. The temperature at which the transition occurs and the shape of the transition curve are related to the nature of the DNA and the ionic strength of the medium (25).

The helix coil transition as measured optically was observed to...
TABLE II

Effect of state of DNA on activity of E. coli phosphodiesterase

The reaction mixture contained 170 μmoles of DNA-P, 50 μmoles of glycine buffer pH 9.7, 2 μmoles of MgCl₂, and 9 units of concentrated DEAE fraction in a final volume of 0.30 ml. After 30 minutes at 37°, 0.2 ml of a “carrier” calf thymus DNA solution (2.5 mg per ml) and 0.5 ml of cold 1 N perchloric acid were added. After 5 minutes at 0°, the precipitate was removed by centrifugation and the optical density of the supernatant fluid at 260 μm was determined. For each DNA sample a control tube lacking enzyme was run and this value was subtracted from the optical density obtained in the reaction mixture containing enzyme. The optical density was converted to nucleotide equivalents with the use of a molar extinction coefficient of 10,000.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Acid-soluble nucleotide formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated E. coli DNA</td>
<td>0.3*</td>
</tr>
<tr>
<td>Heated E. coli DNA</td>
<td>104</td>
</tr>
<tr>
<td>E. coli DNA pretreated with pancreatic DNase</td>
<td>32</td>
</tr>
<tr>
<td>0 X 174 DNA</td>
<td>68</td>
</tr>
</tbody>
</table>

* This value was obtained by measuring the formation of acid-soluble P₂⁺ from P₂⁺-labeled E. coli DNA at a specific radioactivity of 10 μc per μmole of P.
† The DNA was dissolved in 0.02 M NaCl containing 0.025 M Tris buffer, pH 7.5, and heated for 10 minutes at 100° and then chilled.
‡ 0.5 μmole of E. coli DNA-P was treated with 0.002 μg of pancreatic DNase for 60 minutes in the presence of 10⁻³ M Mg⁺⁺ and 37° Tris buffer, pH 7.5.

TABLE III

Specific hydrolysis of heated DNA in a mixture of heated and unheated DNA

The reaction mixtures (0.30 ml) contained glycine buffer pH 9.7, 20 μmoles; MgCl₂, 2 μmoles; P₂⁺ E. coli DNA, 75 μmoles of P₂⁺; and P₂⁺ E. coli DNA, 15 μmoles of P (1 μc per μmole of P) as indicated, and 20 units of concentrated DEAE fraction. After 30 minutes at 37°, “carrier” thymus DNA and perchloric acid were added and the optical density at 260 μm and radioactivity of the supernatant fluids were determined as for Table II.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Heated DNA</th>
<th>Unheated DNA</th>
<th>% Heated hydrolyzed</th>
<th>% Unheated hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P₂⁺</td>
<td>P₂⁺</td>
<td>Radioactivity</td>
<td>Radioactivity</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Take place at the same temperature (about 90°) as the transition of DNA from an inactive to an active enzymatic substrate. Furthermore, there is a close correspondence in the shape of the two transition curves (Fig. 2).

Characterization of Enzymatic Products—Calf thymus DNA which had been heated at 100° for 10 minutes and then treated with the E. coli diesterase, was converted completely to acid-soluble products. More than 95% of the phosphate of this digest was sensitive to semen monooesterase. 

In attempting to characterize the end products produced by this enzyme from DNA (E. coli, T2 bacteriophage, or calf thymus) which had undergone partial degradation with pancreatic DNase, it was observed that the extent to which these DNA’s were converted to mononucleotides depended strikingly on the degree to which they were pretreated with the pancreatic DNase (Fig. 3). If DNA (in this case, DNA from phage T2) was incubated directly with the purified enzyme, no monooesterase-sensitive phosphate could be detected (Curve 5, Fig. 3). Exposure of the phage DNA to a low concentration of pancreatic DNase (about 0.005 μg of DNase per μmole of DNA-P for 1 hour) followed by incubation with the diesterase resulted in the conversion of 95% of the phosphate of thedigest to a monoesterase-sensitive form (Curve 1, Fig. 3).

When a digest produced as in Curve 1 was chromatographed on a Dowex 1-formate column, according to the method of Privat de Garilhe and Laskowski (26), 94% of the material initially added to the column was recovered in the form of mononucleotides; no nucleosides were observed. The ratio of isolated deoxyadenylate plus thymidylate to deoxyguanylate plus hydroxymethyl deoxyadenylate was roughly equivalent to the mononucleotide phosphate alone, since it has been observed that the monoester phosphate of di- and oligonucleotides is split only with difficulty by the human semen monooesterase.

4 Phosphate sensitive to monooesterase includes the phosphate of mononucleotides and the terminal phosphate of polynucleotides. Under the conditions of the assay, this value is roughly equivalent to the mononucleotide phosphate alone, since it has been observed that the monoester phosphate of di- and oligonucleotides is split only with difficulty by the human semen monooesterase.
deoxycytidylic¢ of 1.82 (Table IV) agrees well with the values reported by Wyatt and Cohen (27) for the constituent bases of T2 phage DNA as determined by formic acid hydrolysis of this DNA.

The deoxyadenylate isolated from the digest upon treatment with the specific 5'-nucleotidase from bull semen was completely dephosphorylated, so that this mononucleotide was 5'-dAMP. It may be inferred that the other mononucleotides of the digest also contained a 5'-phosphoryl group. The same nucleotidase preparation produced no measurable orthophosphate upon incubation with 5'-AMP.

When the E. coli phosphodiesterase was incubated with DNA which had been degraded to a limit with pancreatic DNase (about 10 μg of DNase per pmole of DNA-P for 7 hours), the level of monoesterase-sensitive phosphate increased from 4% (the result of the action of the pancreatic enzyme) to a level of 43%, but did not exceed this limit even upon further addition of enzyme (Curve 2, Fig. 3). Addition of snake venom phosphodiesterase to this digest raised the level of monoesterase-sensitive phosphate to 92% within 2 hours. Venom diesterase alone, added to a pancreatic DNase limit digest of T2 bacteriophage DNA, yields a final limit digest which contains some 70% mononucleotides together with an oligonucleotide fraction (30%) in which is concentrated most of the glucosylated hydroxymethyl cytosine of the DNA (5).

When a limit digest of DNA (in this case, DNA from calf thymus) produced as shown in Curve 2, Fig. 3, was chromatographed on a Dowex 1-formate column, 49% of the digest was recovered in the form of the four deoxynucleosides; the remainder could be completely accounted for as dinucleotides. Partial resolution of the dinucleotide fraction was achieved by chromatography of the digest on DEAE-cellulose. A column of DEAE-cellulose (20 X 1 cm) was equilibrated with 0.01 M ammonium bicarbonate at pH 8.6; the equilibration and chromatography were carried out at 4°. Five milliliters of digest containing a total of 210 optical density units at 260 μm were added to the column. The column was washed with about 100 ml of 0.01 M ammonium bicarbonate buffer and a linear gradient containing a total of 210 optical density units at 260 μm were added to the column. The column was washed with about 100 ml of 0.01 M ammonium bicarbonate buffer and a linear gradient was applied with limiting concentrations of 0.08 M and 0.1 M ammonium bicarbonate, pH 8.6. The elution pattern (Fig. 4) shows four incompletely resolved peaks in addition to the four deoxynucleosides; elution with 0.2 ml of 0.01 M ammonium bicarbonate at pH 8.6; the equilibration and chromatography were carried out at 4°.

The deoxynucleotides were not separated in this chromatopgram. They will be described in a subsequent publication.

To avoid the added complexity introduced by the presence of glucosylated and nonglucosylated hydroxymethyl cytosine nucleotides in T2 DNA, a digest of calf thymus DNA was used in those studies.

These experiments were begun in conjunction with Dr. Leon Heppel and Dr. Herbert Sober at the National Institutes of Health. I am indebted to Drs. Heppel and Sober for the hospitality of their laboratories and for their guidance in resolving and identifying the dinucleotide fractions of the enzymatic digest.

### Table IV

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP</td>
<td>3.00</td>
</tr>
<tr>
<td>dTMP</td>
<td>2.97</td>
</tr>
<tr>
<td>dGMP</td>
<td>1.68</td>
</tr>
<tr>
<td>dHMCMP*</td>
<td>1.62</td>
</tr>
</tbody>
</table>

\[ A + T = 1.82\]

\[ G + HMC = 1.82\]

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*Hydroxymethyl deoxycytidine monophosphate.*

\[ A = dAMP, T = dTMP, G = dGMP, HMC = dHMCMP.\]
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Fig. 4. DEAE-cellulose chromatography of limit digest produced from calf thymus DNA by the E. coli phosphodiesterase.

The dried fractions were then dissolved in 0.2 ml of water and subjected to paper electrophoresis at pH 3.5 for 2 hours. Each of the fractions, with the exception of V, was found to consist of several constituents. They were eluted from the paper (recovery, 82 to 94%) and aliquots were chromatographed on paper with both Solvents 1 and 2. In each case the electrophoretic fractions were found to consist of a single component. An aliquot of each electrophoretic fraction was treated with venom phosphodiesterase; one-half of the reaction mixture was chromatographed in Solvent 1 and the remainder analyzed by paper electrophoresis at pH 3.5. The constituent deoxymononucleotides were identified by their \( R_F \) values in these two systems as compared with known 5'-deoxynucleotide markers. The mononucleotides were then eluted from the chromatograms and the concentration of each determined. With the exception of those fractions which contained only a single type of nucleotide, all of the fractions were found to consist of two nucleotide components present in equimolar proportions. In the case of those fractions which contained only a single type of nucleotide, the total and monoesterase-sensitive phosphate of each was determined and ratio was found to be very nearly 2:1. These results show that the non-mononucleotide fraction of the digest was composed entirely of dinucleotides.

The determination of which of the two possible isomeric pairs predominated in a given dinucleotide was carried out by the method of Sinsheimer (28). Each dinucleoside diphosphate was treated first with purified semen monoesterase to convert it to the corresponding dinucleoside monophosphate, then treated with venom diesterase to yield a nucleoside and a 5'-mononucleotide. The nucleoside component is derived from the end bearing a terminal 5'-phosphoryl group; the nucleotide component is obtained from the end bearing a 3'-hydroxyl group. The split products were analyzed by paper electrophoresis at pH 3.5, a procedure which permits identification of the nucleotide, and by paper chromatography in Solvents 2 and 3, procedures by which both the nucleotide and nucleoside can be identified. In each instance, one of the two isomeric pairs either was present exclusively or was present in great excess over the other (Table V). Sinsheimer (28) and Privat de Garilhe et al. (29) have isolated all of the isomeric dinucleotides in a pancreatic DNase limit digest of calf thymus DNA (about 16% of the total) and have found that the sequence p-Purine-p-Pyrimidine was either absent or very rare, with the exception of d-pGpC in which the reverse sequence predominated. In the dinucleotides of the limit digest produced by the E. coli phosphodiesterase (approximately 50% of the total), precisely the same situation was found to exist (Table V).

To eliminate the possibility that some inhibitor is developed during the degradation of DNA by high concentrations of pancreatic DNase, which in turn prevents the splitting of dinucleotides by the E. coli phosphodiesterase, d-pTpT, d-pCpT, and d-pGpG were isolated from the limit digest and treated with excess enzyme. One-tenth \( \mu \) mole of each dinucleotide was incubated with 30 units of enzyme for a period of 3 hours and the reaction mixture was chromatographed in Solvent 2, together with the untreated dinucleotides. In no case was any splitting observed. Hydrolysis of even 10% of the dinucleotide could have been detected by this procedure. Treatment of the dinucleotides with
venom diesterase resulted in quantitative conversion to their constituent mononucleotides. It was also conceivable that the termination of a dinucleotide with a 5'-phosphoryl group prevented its cleavage by the diesterase. To test this possibility, d-pGpG was treated first with purified semen monoesterase to convert it to the dinucleoside monophosphate (d-GpG), then incubated with the enzyme and chromatographed as before. As in the case of d-pGpG, no hydrolysis was observed.

Properties of the Purified Enzyme

Absence of Nucleotidase—The purified enzyme is free of any detectable acid or alkaline nucleotidase activity. Thus, when 50 mmoles of 5′-dCMPmb (specific activity, 6 μc per μmole) were incubated with 10 units of enzyme for 30 minutes at either pH 5 or 9, less than 0.01 mpmole of the P32 was converted to a detectable acid or alkaline nucleotidase activity. Thus, when low concentrations of RNA resulted in only 12% of the total P to an acid-soluble form. Amino acid acceptor RNA (32), kindly provided by Dr. Paul Berg.

Activity on RNA—The purified enzyme preparation hydrolyzes RNA very slowly. Incubation of 9 units of enzyme with 0.1 μmole of purified E. coli RNA-P for 2 hours resulted in the conversion of only 12% of the total P to an acid-soluble form. A similar result was obtained with the use of RNA preheated at 100° for 10 minutes. The same quantity of partially degraded DNA from E. coli was converted completely to acid-soluble products under the same conditions. RNA did not prevent the hydrolysis of the DNA by this enzyme preparation, since incubation of partially degraded DNA labeled DNA with a 20-fold excess of RNA (unlabeled) resulted in a complete conversion of the DNA to acid-soluble products and again only about 10% hydrolysis of the RNA. In view of the large amounts of enzyme which must be added to observe any degradation of RNA, it is not clear whether the RNAase activity of the purified enzyme preparation is a function of the diesterase or may be attributed to a contaminating enzyme.

Magnesium Requirement—Under the conditions of the assay, no significant difference in activity could be detected at Mg++ concentrations ranging from 1.5 X 10⁻⁵ to 3 X 10⁻⁴ M. In the absence of added Mg++ 1/3 of maximal activity was observed. This residual activity was completely eliminated by the addition to the reaction mixture of Versene (0.02 M), suggesting that the enzyme has an absolute requirement for some cation and that the residual activity is the result of traces of the metal ion present in the enzyme preparation. Mn++ could not be effectively replaced by a number of other divalent cations. Thus Mn++ and Ca++ each, at concentrations of 3 X 10⁻⁴ or 6 X 10⁻⁴ M, completely abolished enzymatic activity. Zn++ gave 84% of maximal activity at a concentration of 3 X 10⁻⁴ M, and less than 10% at 6 X 10⁻⁵ M.

pH Optimum—The pH optimum for the purified enzyme is between pH 9.2 and 9.8 (0.07 M glycine buffer). At pH 10.7 (0.07 M glycine) about 30% of optimal activity and at pH 7.5 (0.07 M Tris buffer) about 20% of optimal activity was observed. Enzymatic activity is enhanced by phosphate ion. Thus at pH 8.5 (0.07 M glycine), where the activity was 60% of optimal, the addition of 0.07 M K2HPO4 (pH 8.5) increased the activity to the optimal level.

Stability—The concentrated DEAE fraction showed no significant loss of activity when stored at -20° for 2 months. A loss of about 60% in activity was observed after storage at -20° for 6 months.
It is apparent from the data presented here that the substrate attacked most readily by the *E. coli* phosphodiesterase is a DNA which has undergone collapse of its secondary structure as a result of heating. The rapid cleavage of DNA from the bacteriophage ΦX 174 further suggests that single-stranded DNA is the optimal substrate for this enzyme. The extremely low order of enzymatic activity observed with intact, double-stranded DNA as substrate can, therefore, most probably be attributed to the inability of the enzyme to attack an extended, hydrogen-bonded structure, and not to the low molar concentration of chain ends present in a solution of intact DNA. The latter alternative might have been anticipated on the basis of the exonucleytic mechanism of enzymatic cleavage. In view of the striking difference in hydrolytic activity on heated and ΦX 174 DNA as compared with double-stranded DNA, a classification of enzymes attacking polydeoxynucleotides might be proposed which defines phosphodiesterases as those enzymes which can only attack single-stranded polydeoxynucleotide chains and deoxyribonucleases as enzymes capable of attacking both the single- and double-stranded forms.

An interesting property of the *E. coli* phosphodiesterase is its apparent inability to cleave dinucleotides. One might postulate that the enzyme begins at the 3'-hydroxyl end of a polydeoxynucleotide chain and hydrolyzes the chain in a stepwise fashion, producing 5'-deoxymononucleotides until the terminal dinucleotide is reached, whereupon cleavage ceases. This picture of the enzymatic mechanism is supported by three lines of evidence:

1. The enzyme appears to be an exonuclease, initiating hydrolysis at the 3'-hydroxyl end of the polydeoxynucleotide chain.
2. The *E. coli* phosphodiesterase cannot cleave dinucleotides (d-pGpG, d-pTpT and d-pCpT) when these are incubated directly with the enzyme.
3. A limit pancreatic DNase digest of DNA is converted completely to a mixture of mono- and dinucleotides by the *E. coli* phosphodiesterase.

A limit pancreatic DNase digest of calf thymus DNA consists of about 1% mononucleotides, 16% dinucleotides, and the remainder of oligonucleotides ranging in size from tri- to octanucleotides; the products are on the average of the magnitude of tetranucleotides (30). Treatment of this digest with the *E. coli* diesterase results in the formation of a final limit digest consisting of one-half mononucleotides and one-half dinucleotides. On the other hand, incubation of heated DNA or DNA subjected to minute amounts of pancreatic DNase with the diesterase yields a final digest consisting, as far as can be detected, entirely of mononucleotides. This result is to be anticipated on the basis of the proposed mechanism. In the latter case, the terminal dinucleotide of a DNA chain some 1,000 to 10,000 nucleotides long would represent only 0.2 to 0.02% of the total nucleotide equivalents and would not be detected by the techniques used. In the former case, the average polynucleotide chain length is 4, so that terminal dinucleotide represents some 60% of the total.

It is also of interest to note that the sequence of purines and pyrimidines in the isomeric dinucleotides found in an *E. coli* diesterase digest produced from a pancreatic DNase limit digest is precisely that observed in the dinucleotides of the pancreatic DNase digest. That is, the sequence p-Pyrimidine-p-Purine is always the predominant one, with the exception of d-pGpC. This result can be explained by assuming that the pancreatic enzyme may cleave the DNA chain so as to produce a polynucleotide terminating at its 5'-end in the sequence p-Pyrimidine-p-Purine; this sequence being split only rarely. Occasionally, depending upon further specificity considerations, the pancreatic enzyme may cleave this terminal dinucleotide from the polynucleotide chain to produce a free dinucleotide. When this second cleavage does not occur, an oligonucleotide results. Upon exposure of the latter to the diesterase, the same dinucleotide would be produced; its frequency would, however, be increased, since it would result from any chain terminated by that particular dinucleotide. Therefore, the dinucleotides produced by the *E. coli* diesterase under these conditions may be considered to be a reflection of the specificity of the pancreatic DNase.

The inability of the *E. coli* enzyme to cleave the terminal 5'-dinucleotide of a polydeoxynucleotide chain may provide a means of identifying the nucleotides situated at the end of such a chain; it may therefore be a useful reagent in polydeoxynucleotide end group analysis.

An important difference between the *E. coli* phosphodiesterase and the diesterase from snake venom is in the ability of the bacterial enzyme to degrade appropriately pretreated bacteriophage DNA's bearing glucosylated hydroxymethyl cytosine completely to their constituent mononucleotides. The venom diesterase is unable to hydrolyze most linkages in which glucosylated hydroxymethyl cytosine is involved. The availability of an enzyme which can degrade the bacteriophage DNA's to their constituent mononucleotides (with the exception of the terminal dinucleotide of the chain) has permitted a detailed analysis of all the glucosylated hydroxymethyl cytosine nucleotides present in these DNA's.

The partially degraded P32-labeled DNA used in the detection of the *E. coli* phosphodiesterase and its subsequent purification provides an extremely sensitive substrate for surveying tissue extracts for their various DNases and diesterase levels. The sensitivity of this technique is limited only by the specific radioactivity of the DNA. Furthermore, one may observe phosphodiesterases which would otherwise go undetected were one to use the conventional diesterase substrate, Ca[sp(3-nitrophenyl)-phosphate]. The latter is not split by a diesterase such as is described here.

**SUMMARY**

A phosphodiesterase has been purified about 140-fold from extracts of *Escherichia coli*, which has the following properties.

1. It shows only slight activity with intact, double-stranded deoxyribonucleic acid (DNA), but rapidly hydrolyzes heated, double-stranded DNA or DNA from bacteriophage ΦX 174 to 5'-mononucleotides. Hydrolysis proceeds in a stepwise manner, beginning at the 3'-hydroxyl end of the chain.

2. The enzyme is unable to cleave free dinucleotides or the 5'-terminal dinucleotide of a polydeoxynucleotide chain. As a result, its action on a pancreatic DNase limit digest of calf thymus DNA consists of about 1% mononucleotides, 16% dinucleotides, and the remainder of oligonucleotides ranging in size from tri- to octanucleotides; the products are on the average of the magnitude of tetranucleotides (30).

3. A limit pancreatic DNase digest of DNA is converted completely to a mixture of mono- and dinucleotides by the *E. coli* phosphodiesterase.

4. The enzyme appears to be an exonuclease, initiating hydrolysis at the 3'-hydroxyl end of the polydeoxynucleotide chain.

5. The *E. coli* phosphodiesterase cannot cleave dinucleotides (d-pGpG, d-pTpT and d-pCpT) when these are incubated directly with the enzyme.

6. A limit pancreatic DNase digest of calf thymus DNA consists of about 1% mononucleotides, 16% dinucleotides, and the remainder of oligonucleotides ranging in size from tri- to octanucleotides; the products are on the average of the magnitude of tetranucleotides (30).

7. Treatment of this digest with the *E. coli* diesterase results in the formation of a final limit digest consisting of one-half mononucleotides and one-half dinucleotides.

8. On the other hand, incubation of heated DNA or DNA subjected to minute amounts of pancreatic DNase with the diesterase yields a final digest consisting, as far as can be detected, entirely of mononucleotides. This result is to be anticipated on the basis of the proposed mechanism. In the latter case, the terminal dinucleotide of a DNA chain some 1,000 to 10,000 nucleotides long would represent only 0.2 to 0.02% of the total nucleotide equivalents and would not be detected by the techniques used.

9. In the former case, the average polynucleotide chain length is 4, so that terminal dinucleotide represents some 60% of the total.

10. It is also of interest to note that the sequence of purines and pyrimidines in the isomeric dinucleotides found in an *E. coli* diesterase digest produced from a pancreatic DNase limit digest is precisely that observed in the dinucleotides of the pancreatic DNase digest. That is, the sequence p-Pyrimidine-p-Purine is always the predominant one, with the exception of d-pGpC. This result can be explained by assuming that the pancreatic enzyme may cleave the DNA chain so as to produce a polynucleotide terminating at its 5'-end in the sequence p-Pyrimidine-p-Purine; this sequence being split only rarely. Occasionally, depending upon further specificity considerations, the pancreatic enzyme may cleave this terminal dinucleotide from the polynucleotide chain to produce a free dinucleotide. When this second cleavage does not occur, an oligonucleotide results. Upon exposure of the latter to the diesterase, the same dinucleotide would be produced; its frequency would, however, be increased, since it would result from any chain terminated by that particular dinucleotide. Therefore, the dinucleotides produced by the *E. coli* diesterase under these conditions may be considered to be a reflection of the specificity of the pancreatic DNase.

11. The enzyme is unable to cleave free dinucleotides or the 5'-terminal dinucleotide of a polydeoxynucleotide chain. As a result, its action on a pancreatic DNase limit digest of calf thymus DNA consists of about 1% mononucleotides, 16% dinucleotides, and the remainder of oligonucleotides ranging in size from tri- to octanucleotides; the products are on the average of the magnitude of tetranucleotides (30).
thymus DNA in which the average chain length is 4, produces a digest consisting of one-half mononucleotides and one-half dinucleotides. In the case of a DNA chain several thousand nucleotides long, the 5'-terminal dinucleotide represents only a very small proportion of the total, hence more than 95% of the chain can be hydrolyzed to mononucleotides.

3. The E. coli phosphodiesterase, in contrast to venom phosphodiesterase, is able to degrade bacteriophage DNA's bearing glucosylated hydroxymethyl cytosine, to their constituent mononucleotides.

4. The purified enzyme is most active at an alkaline pH (9.2 to 9.8) and requires Mg++ for optimal activity.

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REFERENCES