An Endonuclease Activity of Venom Phosphodiesterase Specific for Single-stranded and Superhelical DNA*

(Received for publication, June 29, 1977)

ARTHUR E. PRITCHARD, DAVID KOWALSKI, AND M. LASKOWSKI, SR.

From the Laboratory of Enzymology, Roswell Park Memorial Institute, Buffalo, New York 14263

A homogeneous preparation of venom phosphodiesterase from *Crotalus adamanteus* possesses an intrinsic endonuclease activity, specific for superhelical (form I) and single-stranded DNA. The phosphodiesterase degrades single-stranded T, DNA by endonuclease cleavages. Duplex T, DNA is hydrolyzed by the liberation of acid-soluble products simultaneously from the 3' and 5' termini but without demonstrable internal scissions in duplex regions. Since venom phosphodiesterase is known to hydrolyze oligonucleotides stepwise from the 3' termini, the cleavage at the 5' end of duplex T, DNA is ascribed to an endonuclease activity. Form I PM2 DNA is nicked to yield first relaxed intermediates consisting of a discrete series of fragments (11 are usually resolved on agarose gels) with initial molecular weights ranging from 6.3 × 10^6 (the intact PM2 DNA size) to approximately 1 × 10^6. The cleavage of the form I molecule must, therefore, occur at a limited number of unique sites. The enzyme also cleaves nonsuperhelical, covalently closed circular PM2 DNA but at a 10^4 times slower rate. Both the endonuclease activity on form I DNA and the known exonuclease activity co-migrate on polyacrylamide gels, are optimally active at pH 9, are stimulated by small concentrations of Mg^{2+}, and are similarly inactivated by heat, reducing agents, and EDTA.

Many previous studies on phosphodiesterase have indicated the presence of a relatively minor endonuclease activity (3, 6-12). The ratio of endo- to exonuclease activity has always been reported to be variable and small except in the case of poly(adenoine diphosphate ribose) which is degraded endonucleolytically (12).

Recently, a purification procedure has been developed (13, 14) which yields an enzyme that is homogeneous on polyacrylamide gels and appears to be free from 5'-nucleotidase and 3'-monooester-forming endonucleases. With this preparation, we have investigated the enzyme's intrinsic mechanism of action on form I DNA and single- and double-stranded linear DNA. The substrate specificity of phosphodiesterase is similar to that of known single strand specific endonucleases (15), but its mechanism of action on form I PM2 DNA has some unique features.

**EXPERIMENTAL PROCEDURES**

Enzymes - Phosphodiesterase was prepared from the venom of *Crotalus adamanteus* (Miami Serpentarium Laboratories) by a modification (14) of the method of Dolapchiev et al. (13). Instead of the preliminary inactivation of 5'-nucleotidase, venom was first fractionated with acetone according to Williams et al. (16). This was followed by acid-heat inactivation (17) of the phosphodiesterase-rich fraction to reduce the remaining 5'-nucleotidase. The next two steps: chromatography on concanavalin A-Sepharose and molecular sieving on Bio-Gel 200 remained unchanged (13). The final step of chromatography on quaternary amino ethyl (QAE)-Sephadex was replaced by the affinity chromatography on NADP-agarose column, as described by Janaki and Olesen (18), except that bovine serum albumin was omitted. We are greatly indebted to Dr. Olesen for allowing us to read his manuscript prior to publication. The advantage of NADP column is an additional step removing traces of endonuclease. The preparation was homogeneous on both analytical and sodium dodecyl sulfate gels as previously published (13). Samples were stable at −20º for over 1 year in solutions containing 5 mM Tris/acetate, pH 8.8, 0.005% Triton X-100 (Sigma), and 50% glycerol. Enzyme activity on biotinylated phosphate (Sigma) was determined according to Dolapchiev et al. (13). Enzyme concentration is expressed in units/ml, where 1 unit releases 1 μmol of p-nitrophenol/min.

*Escherichia coli* DNA ligase was purchased from P-L Biochemicals, pancreatic DNase I (DPFF) from Worthington Biochemical Corp., and *Hpa* II enzyme from Miles Laboratories.

Mung bean nuclease was prepared according to Kowalski et al. (19).

Venom phosphodiesterase, often referred to as venom enzyme, has been used routinely in studies of nucleic acid sequence, structure, and composition for almost 20 years. The enzyme has been shown to hydrolyze 5'-monophosphate-terminated oligonucleotides by a consecutive liberation of 5' mononucleotides from 3' termini (1-3). Trinucleotides bearing a 3'-monophosphate are relatively resistant but, with a 10-fold greater enzyme concentration, are degraded by first releasing a 3',5'-mononucleoside diphosphate from the 3' end (4). The nuclease is active on a variety of nucleic acids including native and denatured DNA, RNA, and derivatives containing arabinose (5).

*This research was supported by Contract E11-13225 from ERDA and Grants CA17788 and HL18892 from the National Institutes of Health, and BMS53-06560 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

---

* The abbreviations used are: form I, superhelical covalently closed circular DNA; form II, nonsuperhelical covalently closed circular duplex DNA; form III, linear duplex DNA. One A_{260} unit of DNA is the amount that when dissolved in 1 ml of H_2O gives an absorbance of 1 at 260 nm. A nick refers to a single phosphodiester interruption in one strand of a duplex DNA molecule.
(9). One unit of enzyme liberates acid-soluble material from heat-denatured calf thymus DNA (0.6 mg/ml) at a rate of 4.0 A260 units/min at 37°C in 0.026 M ammonium acetate (pH 5.0).

DNA – T5 and PM2 DNA were obtained by published procedures (20–22). The PM2 DNA was further purified by CsCl-ethidium bromide fractionation and ethidium bromide was removed by 1-butanol alcohol extraction. Col E1 DNA was a gift of Dr. L. Liu and was further purified by dye-4butanol equilibrium centrifugation (23, 24). T5 [3P]DNA (20), T5 T6, T6 st (6), and λ DNA were prepared as described elsewhere (20).

Digestion with Venom Phosphodiesterase – Unless otherwise noted, the standard reaction mixture contained 20 mM Tris, pH 9.1, 1 mM MgCl2, 0.027 mM EDTA, 1.4 mM NaCl, 0.005% Triton X-100, 15 to 24 μg/ml of DNA, and the specified amount of enzyme. Reactions were terminated by the addition of EDTA to a final concentration of 0.1 M. Release of acid-soluble products was determined by adding an equal volume of 0.02 M La(NO3)3 in 0.2 N HCl to the quenched reaction mixture. After centrifugation for 10 min at 7500 rpm, 4°C, the A260 of the supernatant was measured. Hydrolysis of T5 [3P]DNA was monitored by an acid-soluble assay previously described (20).

Preparation of Form I, II, and III PM2 DNA – Pancreatic Nucleases I was used to introduce – nick DNA/molecule in the presence of a saturating amount of ethidium bromide (25, 26). The reaction mixture contained 10 mM Tris, pH 8.0, 4.65 mM MgCl2, 1 mM EDTA, 0.1 mM bovine serum albumin (crystallized Pentex bovine serum albumin from Miles Laboratories), 0.5 μg/ml of form I PM2 DNA, 180 μg/ml of ethidium bromide, and 5 μg/ml of DNAase. Incubation was for 15 min at 20°C followed by the addition of EDTA to 18 mM, phenol extraction, and exhaustive dialysis against 10 mM Tris, pH 9.1, 1 mM EDTA. The ligase reaction mixture was 10 mM Tris, pH 8, 2 mM MgCl2, 0.76 mM EDTA, 50 μg/ml of bovine serum albumin, 5 μM NAD, 30.4 μg/ml of DNase I-nicked PM2 DNA, and 1.8 μg/ml of E. coli ligase. The reaction was carried out at either 30°C or 37°C on separate samples, taking precautions to avoid temperature perturbations upon addition of ligase. The reaction was terminated by the addition of EDTA to 10 mM, phenol extraction, and dialysis against 20 mM Tris, pH 9.1. The ligase reaction at 30°C yielded approximately 75% closed circular DNA (determined by the fluorocentrifugal assay, see below) which was used without further purification. The 37°C reaction yield was approximately 50% and the closed circular DNA was further purified by banding in a CsCl-ethidium bromide gradient (23), followed by 1-butanol extraction and dialysis against 20 mM Tris, pH 9.1.

Unit length form III PM2 DNA was prepared by reacting form I DNA with the restriction nuclease Hpa II which cuts the DNA only at sites free of methylation. The 37°C reaction yield was approximately 50% and the closed circular DNA was further purified by banding in a CsCl-ethidium bromide gradient (23). The fluorescence was measured at ambient temperatures before and after heat denaturation using an Aminco fluorocentrugometer equipped with a Corning 7-51 excitation filter (365 nm peak) and a Wratten 23A emission filter. The cell compartment was not thermostated at 4°C. The other system utilized 0.7% agarose gels electrophoresed at 98 volts for 4 h. The advantage of this system is that form III DNA migrates faster than either forms I or II.

Gels were stained and photographed through a Wratten 23A filter, as described elsewhere (30). Film negatives were scanned at 590 nm using the equipment described above. For quantitation, peaks from the scans were cut and weighed. Differences in band intensities due to limited ethidium binding by form I DNA were found to be insignificant, presumably because the DNA was converted to the nicked form during staining and photography. This was determined by electrophoresing a sample that was made by mixing equimolar quantities of form I PM2 (containing <10% form II) and form II PM2 (containing <10% form I). The quantitation procedure gave results within 3% of that expected.

Duplex DNA molecular weights for one experiment were determined by staining the gels with 0.005% "Stains-All" in 50% aqueous formamide as previously described (33). After destaining, the gels were scanned directly using the equipment described above.

Purification of DNA Electrophoresis – Two different systems were employed. The first employed 0.6% agarose gels (6 x 14 mm) containing 0.036 mM Tris, 0.035 mM NaH2PO4, and 1 mM EDTA, pH 7.7. Electrophoresis was for 12 h at 15 h at 25 V in a water-jacketed apparatus thermostated at 4°C. The other system utilized 0.7% agarose gels electrophoresed at 85 volts for 3 h at room temperature, but the Tris/phosphate/EDTA buffer in the upper reservoir was cooled to 4°C prior to use.

For both systems alkali-denatured T5 st(0) DNA was used for molecular weight markers. A linear relationship between relative molecular weights of the gels and the molecular weight of T5 st(0) DNA was found for major fragments of 17.2, 14.5, 3.8, and 1.6 million daltons (34).

RESULTS

Digestion of Single-stranded Compared to Double-stranded T5 DNA – Phosphodiesterase has an endonuclease activity on single-stranded DNA as evidenced by the initial digestion products of heat-denatured compared to native T5 DNA. The early time course for each of these reactions is summarized in Table I. Under these conditions (Table I), the molecular weight of single-stranded DNA undergoes a large reduction while that of duplex DNA remains unchanged. Since <6.5% of the single-stranded DNA was rendered acid soluble when the products were <1 x 108 daltons in size, the cleavages are internal.

The rates of hydrolysis of double- and single-stranded T5 DNA were also compared by measuring the production of acid-soluble material (Fig. 1). The single-stranded DNA was rendered acid-soluble more rapidly than the double-stranded...
An Endonuclease Activity of Venom Phosphodiesterase

Comparison of phosphodiesterase digestion of single- and double-stranded T, DNA

| Reaction | Single-stranded DNA | Duplex DNA |
|----------|---------------------|------------|
| min      | M₀ × 10⁻⁶           | M₀ × 10⁻⁶  |
| 0        | 18                  | 26         |
| 1        | <1-13               | 26         |
| 4        | <1-8                | 26         |
| 15       | <1                  | 26         |

- Denatured T, DNA (17 µg/ml), prepared by heating at 100° for 40 s and quick cooling on ice, was digested with phosphodiesterase at 0.002 unit/ml under standard conditions. For each time point, 0.47 µg of DNA was electrophoresed on 0.7% agarose with the upper reservoir buffer precooled to 4° as described under "Experimental Procedures." Molecular weights much below 1.5 × 10⁶ may not be resolved on these gels and, therefore, no attempt was made to assign molecular weights in this range. From the data in Table II, extrapolation to an enzyme concentration of 0.002 unit/ml shows that less than 6.5% of the DNA would be acid soluble at 15 min.

- Native T, DNA was digested under conditions identical with the single-stranded DNA. For each time point, 0.14 µg of DNA was electrophoresed at 20 volts for 17 h on 0.7% agarose gels. There was no detectable change in the molecular weight of T, DNA after 15 min of digestion, but the experimental uncertainty was on the order of a million in this molecular weight range. Extrapolation from Table II data yields a value of <1.5% acid-soluble material at 16 min.

| Relative reaction rates on various substrates |
|-------------------------------------------|
| SS T, | Acid-soluble | 1 × 10⁶ | A₁₀₀ acid-soluble, Fig. 1 |
| DS T, | Acid-soluble | 2 × 10⁶ | A₁₀₀ acid-soluble, Fig. 1 |
| Form I PM2 | Form II | 2 × 10⁴ | Agarose gels |
| Form II PM2 | Form II | 1 × 10⁴ | Fluorescence assay |
| Form II PM2 | Form III | 10³-10⁴ | Agarose gels, Table I |
| Form II PM2 | Form III | 2 × 10⁶ | Agarose gels, Initial substrate form I |
| Form II PM2 | Form III | 2 × 10⁷ | Agarose gels, Initial substrate form II |

- SS and DS are single-stranded and double-stranded DNA, respectively.
- Initial reaction rates were calculated as described in the text and are expressed here relative to a rate of 6 × 10⁶ cleavages per min per unit of enzyme on form I' DNA.
- The products are presumably mononucleotides but the fluorescence assay measures only the rate of disappearance of form I' PM2 DNA. No form II or form III DNA intermediates were detected by this assay or gel electrophoresis.

DNA. In contrast to the latter reaction, the rate of the former reaction increases with the extent of hydrolysis. It is possible that single-stranded DNA is hydrolyzed both exo- and endonucleolytically, but no conclusion can be reached from these results.

From the data in Fig. 1, the initial normalized rate of cleavages producing acid-soluble material from single- and double-stranded DNA was calculated for Table II. It was assumed that one cleavage produced one acid-soluble mononucleotide. This is probably correct for the cleavage of duplex DNA, but since oligonucleotides of approximately less than 20 residues are acid-soluble (35), the number of cleavages of single-stranded DNA may be overestimated by as much as 20 times (Table II). However, for the purpose of comparison with the rate on circular duplex DNA (see below), we do not require very precise calculations since the rates shown in Table II encompass a range of 10⁶. The reaction rates were determined at enzyme concentrations differing by as much as 10⁴, but direct comparison is probably valid since the rate of at least one type of cleavage, that producing acid-soluble products from double-stranded T, termini, has been determined to be linearly dependent on the enzyme concentration over the entire range of interest. The enzyme concentration dependence of the cleavage rate on form I PM2 DNA (see below) has also been determined to be linear over a narrower range. Experimental limitations prevent measurement of this rate over a thousand-fold variation in enzyme concentration.

There is also a dependence of reaction rates on the total reaction volume and DNA concentration but these effects are relatively slight and most of the reactions were done in similar volumes with approximately 20 µg/ml of DNA. Using an enzyme concentration of 0.3 unit/ml, the cleavage rate on duplex DNA at 15 µg/ml, determined by the acid-soluble assay, was found to be 50% of that at 125 µg/ml.

Acid-soluble Products Simultaneously from the 3' and 5' Terminals of Duplex T, DNA—Two possible mechanisms for phosphodiesterase digestion of linear duplex DNA are shown in Fig. 2. Mechanism A can be inferred from previous studies which demonstrated that mononucleotides are released starting from the 3' end of an oligonucleotide, proceeding stepwise to the 5' terminus (3, 4, 8). The presence of a single strand specific endonuclease activity, however, logically requires degradation of the 5' single strand tails resulting from 3' to 5'-exonuclease hydrolysis of the opposite strand. This process is described by mechanism B (Fig. 2). In both mechanisms, the hydrolysis is terminally directed and there are no internal single or double strand cleavages.
analyzed for acid-soluble material and the molecular weight of the DNA (native and after alkali denaturation) was determined by agarose gel electrophoresis. Fig. 3 is a graph of the molecular weight of the alkali-denatured DNA versus the percent acid-soluble DNA. The experimental results (filled circles) are compared with the calculated change in molecular weight due to conformational and/or charge effects on single-stranded DNA during agarose gel electrophoresis (36), the agreement is good. In addition, for each experimental point, the recovery of the DNA on the gels was high and no lower molecular weight products were seen. Thus, nicks or double strand cleavages in internal regions (>1000 nucleotides from an end) of the duplex are rare or nonexistent under these conditions.

For reaction times at which the DNA was >50% acid-soluble, alkali treatment of the acid-precipitable DNA was required to obtain single-stranded molecules. Without prior denaturation, agarose gel electrophoresis of the products revealed duplex DNA of approximately 2 times the molecular weight of the single-stranded molecules (data not shown). These results are in agreement with mechanism B but not with mechanism A. According to mechanism A, if >50% of a duplex molecule is rendered acid-soluble, the acid-precipitable material is single-stranded (Fig. 2).

Another experiment capable of distinguishing between the two mechanisms was performed. Duplex T₃ [³²P]DNA was digested with phosphodiesterase under the conditions described in Fig. 3 until 13% of the DNA was acid soluble. The reaction was stopped by lowering the pH to 5 and the solution was made 1 M in l-serine and 0.1 M in zinc acetate. Mung bean nuclease (37), a known single strand specific endonuclease, was added to a final concentration of 0.6 unit/ml and the per cent acid-soluble products was determined by agarose gel electrophoresis (19, 20), was then added to a final concentration of 0.6 unit/ml of phosphodiesterase in 0.05 M Tris/HCl (pH 8.7), 0.005% Triton X-100. After addition of mung bean, aliquots were removed at 0, 17, 34, 72, and 102 min. For the determination of acid-soluble products, 5-µl aliquots were added to 25 µl of a calf thymus DNA solution (1.5 µg/µl) at 37°C and further treated as previously described (20). For agarose gel electrophoresis, aliquots were cooled on ice and combined in a ratio of 10/0.62 with a solution containing 0.8 M acetic acid, 0.02 M EDTA to stop the reaction (final pH ~6). A solution of 1 M NaOH was added to attain a final concentration of 0.1 M. The amount of DNA applied to the gels varied between 1.5 and 3.5 µg, depending on the per cent acid-soluble. In the electrophoresis system employed (see "Experimental Procedures"), the complementary strands of T₃ DNA migrate as two discrete bands at ≤31% acid soluble, while at ≥63% acid soluble, the individual bands were more diffuse and appeared as one broad band. The average mobility was used to calculate the molecular weight and T₃ DNA fragments were used as molecular weight markers.

Undigested T₃ DNA shows two bands of Mₑ = 11 × 10⁶ and 9.6 × 10⁶.

ever, the experiment with mung bean nuclease shows that it is less than 1% of a T₃ DNA molecule or less than 200 nucleotides per strand.

Mechanism of Action on Form I DNA – Phosphodiesterase also exhibits an endonuclease activity on form I PM2 DNA as shown by the reaction time course illustrated in Figs. 4 and 6. Starting with a substrate that is greater than 90% form I, the enzyme first produces form II and form III molecules. Within minutes, all of the form I DNA is converted to a series of discrete linear DNA fragments varying in size from approximately 6 × 10⁶ to 1 × 10⁶ daltons. A total of approximately 11 unique species is produced, with ~50% of the linear DNA less than unit length. The molecular weight of each of these linear intermediates is subsequently reduced by ∼0.5 × 10⁶ after 300 min of digestion. Analysis of gels (not shown) on which alkali-denatured (after phosphodiesterase digestion) samples were electrophoresed revealed a decrease in molecular weight of 0.4 × 10⁶ for the largest linear product in the same time period (Fig. 5). Thus, the linear substrate is degraded only from the termini as was the case, described above, with T₃ DNA.

In a separate experiment (enzyme concentration of 8 × 10⁻⁴ units/ml under standard reaction conditions), the relative amounts of forms I, II, and III PM2 DNA were monitored by agarose gel (1.4%) electrophoresis as a function of phosphodiesterase digestion. In the stages of the reaction covering ~0 to ~70% form III DNA, the data (not shown) indicate that the amount of form II DNA goes through a maximum (see also Fig. 4). There is a linear increase in the amount of form III DNA, but at a slower rate than form I DNA disappears. This indicates that form II DNA is an intermediate in the formation
of form III DNA (see also the following paragraph). The initial rates of the I → II and II → III reactions were calculated from these data and are presented in Table II relative to the rate on form I' DNA (see below). For the purpose of calculation, it was assumed that each reaction involves only one cleavage per molecule, although ~50% of each reaction proceeds by at least two cleavages per molecule to give ultimately linear DNA fragments of less than unit length. The cleavage rate on form II DNA was taken to be the rate of appearance of form III. The cleavage rate on form I DNA under standard reaction conditions at enzyme concentrations of 4 x 10^-4 and 8 x 10^-4 units/ml was also determined by the fluorescence assay (data not shown). This rate (Table II) is in good agreement with that determined by the electrophoresis method.

Using agarose gel electrophoresis, the rate was also determined for the reaction of phosphodiesterase on form II DNA with ~one single strand scission per molecule produced by the limited action of DNase I on form I DNA. This rate, also shown in Table II, is in agreement with that determined as described in the preceding paragraph by assuming that form I DNA was an intermediate in the formation of form III DNA.

Also shown in Table II is the cleavage rate on form I' DNA (nonsuperhelical, covalently closed circles) measured by the fluorescence assay under standard reaction conditions minus NaCl. Reaction rates determined at 0.4 and 0.58 unit/ml were in good agreement after normalization by enzyme concentration. The rate was 4 times less at 30°C using form I' DNA that was prepared by the action of DNA ligase on form II DNA at the same temperature (data not shown). The fluorescence assays used in these experiments showed only a decrease in double-stranded DNA concentration indicating no accumulation of form II or III intermediates. Agarose gels (not shown) also showed no intermediates. These results are consistent with an initial endonucleolytic cleavage of form I' which converts the molecule to a more reactive form. The II → III and III → acid-soluble reactions are ~10^4 and ~10^5 times faster, respectively, than the rate of disappearance of I'.

All of these results are consistent with the mechanism depicted in Fig. 6. The endonuclease activity requires a form I or II substrate. Form III DNA, produced via a form II intermediate, is then hydrolyzed only from the molecular weight markers were those described in Fig. 4. Gels were stained with "Stains-All" and scanned. This procedure revealed the same PM2 digestion products visualized in Fig. 4 plus a smaller fragment that was not seen by staining with ethidium. The molecular weight for the largest linear species is inaccurate probably because for these gels the log of molecular weight is not a linear function of mobility above ~5 x 10^6. This species is initially unit length since it co-migrates with form III PM2 DNA (gels not shown) produced from native PM2 DNA by the cleavage with nuclease Hpa II at a unique site.
The fluorescence assay was used to measure the endonuclease activity on form I PM2 DNA while the exonuclease activity was assayed by the hydrolysis of bis-p-nitrophenyl phosphate. All inactivations and assay reactions for the two activities were performed under essentially identical conditions. Assay reaction mixtures at 37°C contained 1 mM Mg²⁺, 0.07 to 0.1 M Tris (pH 9.1, except for pH dependence assays), 0.005% Triton X-100, and all exonuclease assays were 5 mM in substrate. The specific conditions are as follows. Heat inactivation: the enzyme at 2.6 units/ml was heat-inactivated in 0.1 M Tris/acetate followed by the exonuclease assay at 0.013 unit/ml for 5 min, and the endonuclease assay at 0.0081 unit/ml for 4 min with 1.8 μg of PM2 DNA. Inactivation by reducing agents: the enzyme, at 0.01 unit/ml and 7.8 × 10⁻⁴ units/ml for exo- and endonuclease assays respectively, was inactivated for 5 min at 37°C with 1.2 times the concentration of reducing agent shown in the table. Substrate (0.20 volume) was then added and incubation continued for 5 min. The endonuclease assay contained 3.24 μg of PM2 DNA at 32.4 μg/ml, pH dependence: pH dependence of exonuclease activity was determined by incubation for 3 min in the pH range of 5.5 to 11.5 at an enzyme concentration of 0.040 unit/ml; endonuclease activity was determined in the same pH range using per assay 1.56 μg of PM2 DNA (38 μg/ml) and 8 × 10⁻⁴ units/ml of enzyme for 4 min.

![Fig. 6. Mechanism of action of phosphodiesterase on form I PM2 DNA.](image)

**TABLE III**

| Property | Endonuclease activity on form I PM2 | Exonuclease activity on bis-p-nitrophenyl phosphate |
|----------|------------------------------------|-----------------------------------------------|
| Per cent inactivation by heat | | |
| pH 9, 37°C, 100 min | 7 | 0 |
| pH 9, 65°C, 15 min | 38 | 50 |
| pH 6, 65°C, 15 min | 96 | 91 |

| Per cent inactivation by reducing agents | | |
| 0.5 mM Dithioerythritol, 5 min | | |
| 1 mM L-Cysteine, 5 min | | |
| pH Optimum | 9.0 | 9.0 |

**Effect of Mg²⁺**

Both active with no added Mg²⁺ but both are stimulated by small amounts (<10 mM) and inhibited by larger amounts.

**Effect of EDTA**

Inactivates Inactivates

DNase I hydrolysates, gel electrophoresis revealed that the initial products of phosphodiesterase digestion were unit length form III DNA (more than 50%) and a smear of lower molecular weight fragments. These are the expected products for a form II substrate containing, respectively, one single strand scission and randomly placed multiple single strand cleavages.

**Form I Col E1 DNA was also tested as a substrate** Under conditions which produced the pattern shown in Fig. 4 with PM2 DNA, the Col E1 DNA was converted to four discrete linear fragments, but greater than 90% of the linear molecules were of unit length.

**Endonuclease Activity Is Intrinsic to Venom Phosphodiesterase** – The properties of the endonuclease activity on form I PM2 DNA were investigated and compared to those of the known exonuclease activity. The endonuclease activity was determined by the fluorescence assay (28) which measures the per cent of form I DNA present. The exonuclease activity was assayed with bis-p-nitrophenyl phosphate, a synthetic substrate that has traditionally been used as a measure of phosphodiesterase activity. Both activities co-migrate with the single protein band on polyacrylamide gels (Fig. 7), have similar pH dependence profiles, and are similarly inactivated by heat and reducing agents (Table III). EDTA rapidly inactivates both irreversibly. Neither activity has an absolute requirement for added Mg²⁺, but both are stimulated by small amounts of Mg²⁺ and are inhibited by larger amounts. The dependence on Mg²⁺ concentration is similar but not identical for the two activities. This was not investigated in detail but could be explained by differential effects of ionic strength at the substrate level.

**DISCUSSION**

We have shown that phosphodiesterase has an intrinsic endonuclease activity specific for form I and single-stranded DNA. Double-stranded linear DNA is degraded only from the termini and the rate of this type of cleavage is ~10 times
faster than the endonuclease scission rate for form I DNA. Phosphodiesterase also possesses an endonuclease activity on form I DNA, but the rate of hydrolysis is $10^4$ times slower than the rate of release of acid-soluble products from single-stranded DNA. It is, therefore, reasonable to describe the endonuclease action as single strand specific (see also discussion below on superhelical DNA). It is possible that the low form I DNA endonuclease activity is also intrinsic to phosphodiesterase. The single strand specific nuclease from mung bean is known to have intrinsic endonuclease activity on double-stranded linear DNA (20) and the ratio of single to double strand endonuclease rate is $10^4$ under optimal conditions.

Although phosphodiesterase has a $3'$ to $5'$ polarity of action on single-stranded oligonucleotides (3, 4), we have shown that it hydrolyzes intact linear double-stranded DNA by liberation of acid-soluble products simultaneously from the $3'$ and $5'$ termini (mechanism B, Fig. 2). It can be inferred, therefore, that the action on the $5'$ termini is that of a single strand specific endonuclease, which recognizes a single-stranded $5'$ tail resulting from the release of mononucleotides from the $3'$ end. Some of the details of this mechanism are not yet clear. In particular, it would be useful to know the initial size of products released from the $5'$ end. Although the ultimate products of digestion by phosphodiesterase have always been reported to be $5'$-mononucleotides, oligonucleotides, rather than mononucleotides, may be initially cleaved from the $5'$ ends after limited digestion of the $3'$ termini. This result is expected from a consideration of phosphodiesterase hydrolysis of single-stranded di- and trinucleotides bearing a terminal $3'$-monophosphate. Compared to digestion of $3'$-hydroxyl-terminated substrates, these chains are very resistant and this resistance increases with decreasing chain length (4, 38). Similarly, the rate of attack on single-stranded cyclic oligonucleotides (up to 4 residues) is extremely low compared to that on the linear analogue (6). Apparently, there is a minimum size required for endonuclease activity.

It can be inferred that the endonuclease activity of phosphodiesterase on PM2 DNA is due to single strand like regions in superhelical DNA, but the conclusion lacks ultimate proof. A number of chemical and enzymatic probes have detected such regions in form I DNA. These include formaldehyde (39), methyl mercury hydroxide (40), water-soluble carbodiimide (41, 42), the T4 gene 32 protein (43), and a number of single strand specific nucleases (44-52). The cleavage site of S, nucleases also show endonuclease action on DNA with pre-existing nicks (49, 50).

We have found (data not shown) that phosphodiesterase cleaves $\sim 15$ phosphodiester bonds at the termini of duplex T, DNA in the same amount of time that it takes to introduce a single nick in form I PM2 DNA. However, other experiments (not shown) indicate that this ratio of $\sim 15$ to 1 is not constant but depends on reaction conditions. A ratio of $\sim 7$ to 1 can be calculated for the action of Pseudomonas nucleases on linear and form I PM2 DNA from the data of Gray et al. (50).

Phosphodiesterase cleaves a large fraction of the form I DNA molecules at a minimum of two of a few specific sites resulting in the production of linear molecules of less than unit length. These multiple cleavages must be simultaneous since one nick removes the topological constraint required for superhelicity and only superhelical DNA substrates give the discrete, multiple fragment pattern.

Studies with S, (46, 48, 49) and N. crassa (52) nucleases have shown that superhelical SV40 and polyoma DNA are cleaved at one of a few specific sites, but dX-174 is cleaved at one of many widely distributed sites. Pseudomonas (50) and mung bean (27) nucleases cleave PM2 form I at one of several possible sites, which for mung bean nuclease are not randomly distributed. Thus, the ability to cleave a superhelical DNA at two or more specific sites per molecule appears to distinguish phosphodiesterase from other single strand specific nucleases. However, it is not certain whether this is a unique property of phosphodiesterase or the PM2 DNA. Other single strand specific nucleases may also produce discrete multiple fragments with PM2 DNA under favorable reaction conditions. We have found (data not shown) that phosphodiesterase produces only unit length linear PM2 DNA when the Mg$^{2+}$ concentration is lowered to 0.1 mM.

It is possible that the nuclease-sensitive regions in PM2 DNA correspond to the approximately eight specific $A + T$ rich regions that are the early melting areas in form I DNA (57).
In order to account for the 11 discrete fragments we commonly observe, there must be at least four separate sites of attack, but the exact number has not been determined.

REFERENCES

1. Singer, M. F., Hilmoe, R. J., and Khorana, H. G. (1958) Fed. Proc. 17, 312

22. Wang, J. C. (1974) J. Mol. Biol. 18, 797-816

27. Borzilai, R. (1973) J. Mol. Biol. 74, 739-742

28. Morgan, A. R., and Pulleyblank, D. E. (1974) Biochim. Biophys. Res. Commun. 61, 396-403

29. Brewer, J. M., Poole, A. J., and Ashworth, R. B. (1974) in Experimental Techniques in Biochemistry, Appendix 12, p. 351, Prentice-Hall, Inc., Englewood, New Jersey

30. Helling, E. B., Goodman, H. M., and Boyer, H. W. (1974) J. Virol. 14, 1235-1244

31. McDonell, M. W., Simon, M. N., and Studier, F. W. (1977) J. Mol. Biol. 110, 119-146

32. Manialis, T., Jeffrey, A., and van de Sande, H. (1975) Biochemistry 14, 3787-3794

33. Dahlberg, A. E., Dingman, C. W., and Peaceck, A. C. (1969) J. Mol. Biol. 41, 139-147

34. Hayard, G. S., and Smith, M. G. (1972) J. Mol. Biol. 63, 383-395

35. Cleaver, J. E., and Boyer, H. W. (1972) Biochim. Biophys. Acta 262, 116-124

36. Hayard, G. S. (1972) Virology 49, 342-344

37. Sung, S.-C., and Laskowski, M., Sr. (1963) J. Biol. Chem. 237, 506-511

38. Frivald Garillie, M., and Laskowski, M., Sr. (1965) Biochim. Biophys. Acta 18, 370-378

39. Dean, W., and Lebowitz, J. (1971) Science 175, 5-8

40. Beer, T., and Lebowitz, J. (1971) J. Mol. Biol. 79, 451-470

41. Lebowitz, J., Garon, C. G., Chen, M. C. Y., and Salzman, N. P. (1970) J. Virol. 18, 205-210

42. Chen, M., Lebowitz, J., and Salzman, N. P. (1976) J. Virol. 18, 211-217

43. Delius, H., Mantell, N. J., and Alberts, B. (1972) J. Mol. Biol. 67, 341-350

44. Godson, G. N. (1973) Biochim. Biophys. Acta 305, 59-67

45. Kato, A. C., Bartok, K., Fraser, M. J., and Denhardt, D. T. (1973) Biochim. Biophys. Acta 305, 59-67

46. Beard, P., Morrow, J. F., and Berg, P. (1973) J. Virol. 12, 1303-1313

47. Hornbeak, H. R., and Moss, B. (1974) J. Biol. Chem. 249, 3292-3296

48. Germond, J., Vogt, V. M., and Hirt, B. (1974) Eur. J. Biochem. 43, 591-596

49. Wingard, R. C., Godson, G. N., and Radding, C. M. (1975) J. Biol. Chem. 250, 8848-8850

50. Gray, H. B., Jr., Ostrander, D. A., Hodnett, J. L., Legeriski, R. J., and Robberson, D. L. (1975) Nucleic Acids Res. 2, 1459-1491

51. Pedrini, A. M., Ranzani, G., Pedrali Noy, G., Spadari, S., and Falaschi, A. (1976) Eur. J. Biochem. 70, 275-283

52. Bartok, K., and Denhardt, D. T. (1976) J. Biol. Chem. 241, 590-595

53. Goldmark, P. J., and Linn, S. (1970) Proc. Natl. Acad. Sci. U. S. A. 67, 434-441

54. Goldmark, P. J., and Linn, S. (1972) J. Biol. Chem. 251, 1849-1860

55. Modrich, P., and Zabel, D. (1976) J. Biol. Chem. 251, 5666-5674

56. Shenk, T. E., Rhodes, C., Rigby, P. W. J., and Berg, P. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 989-995

57. Brack, C., Bickle, T. A., and Yuan, R. (1975) J. Mol. Biol. 96, 693-702
An endonuclease activity of venom phosphodiesterase specific for single-stranded and superhelical DNA.
A E Pritchard, D Kowalski and M Laskowski, Sr

J. Biol. Chem. 1977, 252:8652-8659.

Access the most updated version of this article at http://www.jbc.org/content/252/23/8652

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/23/8652.full.html#ref-list-1