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

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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 T2 DNA byendonucleolytic cleavages. Duplex T2 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 T2 DNA is ascribed to an endonuclease activity. Form I PM2 DNA is nicked to yield first relaxed circles and then linear DNA which is subsequently hydrolyzed only from the chain termini. The linear duplex DNA intermediates consist of a discrete series of fragments (11 are usually resolved on agarose gels) with initial molecular weights ranging from 6.3 x 10^6 (the intact PM2 DNA size) to approximately 1 x 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 10^5 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(adenosine 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'-monoester-forming endonuclease. 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 columns, as described by Janaki and Oleson (18), except that bovine serum albumin was omitted. We are greatly indebted to Dr. Oleson 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 -20° for over 1 year in solutions containing 5 mM Tris/acetate, pH 8.8, 0.005% Triton X-100 (Sigma), and 50% glycerol.

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 exonuclease, has been used routinely in studies of nucleic acid sequence, structure, and composition for almost 20 years. The enzyme has been known to hydrolyze 5'-monophosphate-terminated oligonucleotides by a consecutive liberation of 5'-mononucleotides from 3' termini (13). Trinucleotides bearing a 3'-mononucleotide are relatively resistant but, with a 10-fold greater enzyme concentration, are degraded by first releasing a 3',5'-mononucleotide diphasphate 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).

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One unit of enzyme liberates acid-soluble material from heat-denatured calf thymus DNA (0.6 mg/ml) at a rate of 4.0 A495 units/min at 37 °C in 0.026 M ammonium acetate (pH 5.0).

DNA – T2 and T2 DNA were obtained by published procedures (20-22). The PM2 DNA was further purified by CsCl-ethidium bromide banding (23) and ethidium bromide was removed by 1-butanol alcohol extraction. Col El DNA was a gift of Dr. L. Liu and was further purified by dye- buoyant equilibrium centrifugation (23, 24). T2 (29)DNA (30), T2, T2 st (0), and λDNA* were prepared as described elsewhere.

**Digestion with Venom Phosphodiesterase** – Unless otherwise noted, the standard 37 °C reaction mixture contained 20 mM Tris, pH 9.1, 1 mM Mg2+, 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 <10 mM. 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 supernatant was measured.

Hydrolysis of T2 (29)DNA was monitored by an acid-soluble assay previously described (30).

**Preparation of Forms I, II, and III PM2 DNA** – Pancreatic DNase I was used to introduce ~1 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 Mg2+, 1 mM EDTA, 0.1 µg/ml of bovine serum albumin (crystallized, Pasteur bovine serum albumin from Miles Laboratories), 30.4 µg/ml of form 1 PM2 DNA, 180 µg/ml of ethidium bromide, and 5 µg/ml of DNase I. 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 8.0, 1 mM EDTA. The ligase reaction mixture was 10 mM Tris, pH 8, 2 mM Mg2+, 0.76 mM EDTA, 50 µg/ml of bovine serum albumin, 5 mM 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 18 mM, phenol extraction, and dialysis against 20 mM Tris, pH 9.1. The ligase reaction mixture at 30 °C yielded approximately 75% closed circular DNA (determined by the fluorescence 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 once (27). The reaction mixture concentrations were 377 pg/ml form I PM2 DNA, 1.8 pg/ml of E. coli ligase. The reaction was carried out at either 30 or 37 °C on separate samples, taking precautions to avoid temperature perturbations upon addition of EDTA.

**Fluorescence Assay** – The fractional amount of form I DNA was determined by a sensitive ethidium bromide fluorescence method (28). The fluorescence was measured at ambient temperatures before and after heat denaturation using an Aminco fluorocolorimeter equipped with a Corning 7-51 excitation filter (365 nm peak) and a Wratten 23A emission filter. The cell compartment was not thermostated and, therefore, precautions were taken to ensure that all samples were temperature-equilibrated and readings were taken immediately after inserting the cuvette.

In this study, the per cent of DNA other than form I, and, therefore, not spontaneously renaturable, was taken to be the per cent decrease in the fluorescence intensity resulting from digestion. This is an approximation which assumes no significant dye fluorescence in the presence of single-stranded DNA and an equal fluorescence in the presence of all forms of duplex DNA. Samples of known composition were made by mixing in different proportions stock solutions consisting of >95% of either form I or form II PM2. Fluorescence assays of these solutions gave per cent of form II DNA within 5% of that expected.

**Protein Electrophoresis** – Venom phosphodiesterase was electrophoresed on 12-cm 5% polyacrylamide gels at pH 4.3 according to published procedures (29). Gels stained with Coomassie blue were scanned at 550 nm using a Gilford model 2400 spectrophotometer equipped with a linear transport.

**Duplex DNA Electrophoresis** – PM2 and T7 DNA were electrophoresed on agarose gels using a buffer of 40 mM Tris/HC1, pH 8.0, 5 mM sodium acetate, and 0.05 mM EDTA. Gels of 14 cm length were made in tubes (6 x 0.5 cm) according to a published procedure (30). A horizontal slab gel system, described elsewhere (31), was also used, in which case the DNA was usually precipitated by ethanol (32) and redissolved in the gel buffer prior to electrophoresis. The PM2 DNA was sometimes electrophoresed with 0.5 µg/ml of ethidium bromide in the gel and buffer but except for slight changes in relative mobilities, the dye did not alter the results.

Molecular weight markers were obtained from the Eco RI digestion of either λ or T5 DNA. On 0.7% (w/v) agarose gels (22 V, 19 h) the mobilities relative to bromphenol blue are linearly related to the logarithm of the molecular weight for RI digest fragments of 4.7, 5.7, 5.3, 5.0, and 2.1 million daltons (30). On these gels form I DNA migrates fastest followed by unit length form III and then form II. To quantitate relative amounts of form I, II, and III during the course of phosphodiesterase digestion of PM2 DNA, reaction products were run on 1.4% agarose gels 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 equal quantities of form I PM2 (containing <10% form II) and form II PM2 (containing <10% form I). The quantitation procedure gave results within 2% 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.

**Single-stranded DNA Electrophoresis** – Two different systems were employed. The first employed 0.6% agarose gels (6 x 14 mm) containing 0.036 M Tris, 0.03 M NaH2PO4, and 1 mM EDTA, pH 7.7. Electrophoresis was for 12 to 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/EDTA/EDTA buffer in the upper reservoir was cooled to 4 °C prior to use.

For both systems alkali-denatured T2 st(0) DNA was used for molecular weight markers. A linear relationship between relative mobilities of ethidium bromide (blue tracking dye) and logarithm of molecular weight 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 T2 DNA** – Phosphodiesterase has an endonuclease activity on single-stranded DNA as evidenced by the initial digestion products of heat-denatured compared to native T2 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 106 daltons in size, the cleavages are internal.

From the results shown in Table I, it is possible to calculate the order of magnitude for the rate of cleavage which produces large fragments from initially intact denatured DNA. The estimated endonuclease rate on single-stranded DNA is 10-11 to 10-12 cleavages per min per unit of enzyme and is shown in Table II relative to the rate on form I PM2 DNA (see below).

The rates of hydrolysis of double- and single-stranded T2 DNA were also compared by measuring the production of acid-soluble material (Fig. I). The single-stranded DNA is rendered acid-soluble more rapidly than the double-stranded DNA.
extrapolation from Table II data yields a value of ~1.5% acid-soluble material at 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 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 15 min.

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Relative Rate</th>
<th>Method</th>
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<tbody>
<tr>
<td>SS T&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Acid-soluble</td>
<td>1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>A&lt;sub&gt;obs&lt;/sub&gt; acid-soluble, Fig. 1</td>
</tr>
<tr>
<td>DS T&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Acid-soluble</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>A&lt;sub&gt;obs&lt;/sub&gt; acid-soluble, Fig. 1</td>
</tr>
<tr>
<td>Form I PM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Form II</td>
<td>2 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Agarose gels</td>
</tr>
<tr>
<td>Form I PM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Form II</td>
<td>1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Fluorescence assay</td>
</tr>
<tr>
<td>SS T&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Large fragments</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;-10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Agarose gels, Table I</td>
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<tr>
<td>Form II PM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Form III</td>
<td>2 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Agarose gels</td>
</tr>
<tr>
<td>Form II PM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Form III</td>
<td>2 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Agarose gels, initial substrate form 1</td>
</tr>
<tr>
<td>Form I PM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Form III</td>
<td>2 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Agarose gels, initial substrate form 2</td>
</tr>
</tbody>
</table>

* 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 x 10<sup>6</sup> 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 PM<sub>2</sub> 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 10<sup>6</sup>. The reaction rates were determined at enzyme concentrations differing by as much as 10<sup>5</sup>, 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<sub>7</sub> DNA, 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 PM<sub>2</sub> 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′ Termini of Duplex T<sub>7</sub> 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.

The terminally directed nature of the hydrolysis of linear duplex DNA was demonstrated by the following experiment. At various times during the digestion of duplex T<sub>7</sub> DNA with phosphodiesterase, samples of the reaction mixture were
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 per cent acid-soluble DNA. The experimental results (filled circles) are compared with the calculated change in molecular weight (solid line) for exclusive terminally directed hydrolysis of the 26 x 10^6 dalton duplex. Allowing for some uncertainty in the molecular weight values 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 T₇ [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 mM in l-serine and 0.1 mM in zinc acetate. Mung bean nuclease (37), a known single strand specific endonuclease (19, 20), was then added to a final concentration of 0.6 unit/ml and the per cent acid-soluble products was determined after incubation at 37° for 10 and 20 min. No increase in acid-soluble material was observed under conditions where, if the DNA were all single-stranded, mung bean nuclease would render it 100% acid-soluble. If phosphodiesterase were acting according to mechanism A, DNA which was 13% acid-soluble would be 26% acid-soluble after hydrolysis of the 5' single-stranded tails by mung bean nuclease. We conclude that phosphodiesterase digests linear duplex DNA simultaneously from the 3' and 5' termini (Fig. 2, mechanism B).

The question mark in mechanism B, Fig. 2, indicates that we do not know how many nucleotides are released from the 3' end before a cleavage occurs on the opposite strand. However, the reaction with mung bean nuclease shows that there is less than 1% of a T₇ DNA molecule or less than 200 nucleotides per strand.

Mechanism of Action on Form II DNA—Phosphodiesterase also exhibits an endonuclease activity on form II PM2 DNA as shown by the reaction time course illustrated in Figs. 4 and 5. 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 II DNA is converted to a series of discrete linear DNA fragments varying in size from approximately 6 x 10⁶ to 1 x 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 x 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 x 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 T₇ DNA.

In a separate experiment (enzyme concentration of 8 x 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 concentration of $4 \times 10^{-4}$ and $8 \times 10^{-4}$ 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 II 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 ~$10^3$ and ~$10^3$ 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 ends to the ultimate mononucleotide products. To explain the appearance of the numerous discrete linear fragments, it is necessary to postulate that the form I DNA sometimes experiences at least two simultaneous single strand cleavages which can occur at a number of unique sites. The same pattern of discrete fragments is observed over a range of variations in temperature, ionic strength, and enzyme concentration, but it is seen only when form I DNA is the initial substrate. In an experiment (not shown) with an initial substrate of form II DNA (produced by limited pancreatic
terase phosphodiesterase activity. Both activities co-migrate with the percent of form I DNA present. The exonuclease activity was assayed with bis-p-nitrophenyl phosphate, a synthetic determined by the fluorescence assay (28) which measures known exonuclease activity. The endonuclease activity was PM2 DNA were investigated and compared to those of the initial products of phosphodiesterase digestion were unit DNase I hydrolysis), gel electrophoresis revealed that the unique reactive sites is also unknown. The terminally directed activity on form III DNA refers to mechanism B in Fig. 2.

Effect of Mg^{2+} Both active with no added Mg^{2+} but both are stimulated by small amounts (<10 mM) and inhibited by larger amounts.

Effect of EDTA Inactivates Inactivates

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 Mg^{2+}, but both are stimulated by small amounts of Mg^{2+} and are inhibited by larger amounts. The dependence on Mg^{2+} 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

**FIG. 6.** Mechanism of action of phosphodiesterase on form I PM2 DNA. Reaction pathways are shown for both the production of unit length and fragmented form III DNA intermediate. For the latter reaction, the form II molecule is illustrated with two nicks in one strand although nothing is known concerning the maximum number of nicks per strand or the strand specificity. The number of possible unique reactive sites is also unknown. The terminally directed activity on form III DNA refers to mechanism B in Fig. 2.

**Fig. 7.** Electrophoretic mobility of endo- and exonuclease activities. Phosphodiesterase (0.26 unit) was electrophoresed on two pH 4.3, 5% polyacrylamide gels at 2 mA/tube for 4 h. One gel was stained with Coomassie blue and scanned for protein. The other was frozen and cut into 1 mm slices which were refrigerated overnight in tubes containing 0.2 ml of 0.1 M Tris, pH 9.1. Exonuclease activity was determined by the standard bis-p-nitrophenyl phosphate assay. Endonuclease activity on form I PM2 DNA was assayed by the fluorescence method.

DNase I hydrolysis), 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.

**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 percent 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

**TABLE III**

<table>
<thead>
<tr>
<th>Property</th>
<th>Endonuclease activity on form I PM2</th>
<th>Exonuclease activity on bis-p-nitrophenyl phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent inactivation by heat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 9, 37°, 100 min</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>pH 9, 65°, 15 min</td>
<td>38</td>
<td>50</td>
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<td>pH 6, 65°, 15 min</td>
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<td>91</td>
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<td>Per cent inactivation by reduc-</td>
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<tr>
<td>0.5 mm l-Dithioerythritol, 5</td>
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<td>87</td>
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<tr>
<td>min</td>
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<td>1 mm L-Cysteine, 5 min</td>
<td>80</td>
<td>98</td>
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<td>pH Optimum</td>
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<td>9.0</td>
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<td>Electrophoresis</td>
<td>Both activities co-migrate with sin-</td>
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<td></td>
<td>gle protein band.</td>
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<tr>
<td>Effect of Mg^{2+}</td>
<td>Both active with no added Mg^{2+}</td>
<td>Both active with no added Mg^{2+} but both are stimu-</td>
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<td></td>
<td>but both are stimulated by small</td>
<td>lated by small amounts (&lt;10 mM) and inhibited by</td>
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<td></td>
<td>amounts of Mg^{2+} and are inhibited</td>
<td>larger amounts.</td>
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<td>by larger amounts.</td>
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The dependence on Mg^{2+} 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.
An Endonuclease Activity of Venom Phosphodiesterase

Phosphodiesterase also possesses an endonuclease activity on form I DNA, but the rate of hydrolysis is ~10^6 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 ~10^6 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, or mononucleotides, they are 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 Tt gene 32 protein (40), and a number of single strand specific nucleases (44-52). The cleavage site of S, nuclease on form I SV40 DNA in high salt is in the same region as the gene 32 protein binding site and this region is most easily denatured at high pHo (46). It is likely that the torsional stress produced by the superhelical turns results in the formation of kinks or bends in the double helix (27). The base pairing would be distorted at such sites, which are probably A + T rich regions of low thermodynamic stability.

It is of interest to compare the phosphodiesterase activity on form I DNA with that of other single strand specific nucleases. The rate of cleavage on form I DNA is reported to be less than that on single-stranded DNA for phosphodiesterase (Table II), mung bean nuclease (27), and S, nuclease (48), but the ratio varies with each enzyme. The rep BC DNase, on the other hand, can cleave circular single-stranded DNA (53), but is completely inactive on form I 54 or Col El DNA (55).

Unlike phosphodiesterase, the single strand specific nucleases from Neurospora crassa (45, 59) and human aneuploid cells (51) are inactive on form II DNA but S, (46, 48, 49) and the Pseudomonas BAL 31 (50) enzymes produce unit length form III from form II DNA by acting opposite the original nick. For these latter examples, so with phosphodiesterase, form I DNA is more reactive than form II. Whether or not the initial nicks are enlarged to gaps by exonuclease action prior to the endonuclease cleavage of the opposite strand is not clear. Form II DNA, made by limited action of pancreatic DNase I on form I DNA, and form II DNA, an intermediate in the phosphodiesterase digestion of form I DNA, are cleaved by phosphodiesterase at the same rate. S, and the Pseudomonas nucleases also show endonuclease activity on DNA with pre-existing nicks (49, 50).

The single strand specific nucleases do not have endonuclease activity on duplex linear DNA at a rate that is significant compared to rates on form I or single-stranded DNA. However, exonuclease-like action, which releases acid-soluble products from the ends of duplex linear DNA, has been reported for S, (56), Pseudomonas nuclease (50), and mung bean nuclease (20). It is possible that the terminally directed activity of these enzymes is due to the relative thermodynamic instability of the helix at the chain ends, but evidence for this conclusion has only been demonstrated for mung bean nuclease (20). Compared to other single strand specific endonucleases, phosphodiesterase seems to have a relatively high ratio of duplex DNA exonuclease activity to single-stranded or form I DNA endonuclease activity, but little quantitative data are available. Table II shows that phosphodiesterase cleaves ~15 phosphodiest er bonds at the termini of duplex T7 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 ~15 to 1 is not constant but depends on reaction conditions. A ratio of ~7 to 1 can be calculated for the action of Pseudomonas nuclease 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 MgSO4 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.

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