Action of Venom Phosphodiesterase on Deoxyribonucleic Acid*

EDWARD J. WILLIAMS,† SHAN-CHING SUNG,‡ AND M. LASKOWSKI, SR.

From the Biochemical Laboratory for Cancer Research, Marquette University School of Medicine, Milwaukee 3, Wisconsin

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The observation of Boman and Kaletta (1, 2) that venom phosphodiesterase is capable of hydrolyzing the so called native thymus deoxyribonucleic acid has been confirmed in several laboratories (3-5). Boman (6) noticed that under appropriate conditions the reaction may be carried almost to completion. All these experiments were performed with comparatively crude preparations of phosphodiesterase.

In the present paper the progress of degradation of deoxyribonucleic acid by highly purified phosphodiesterase has been followed by three independent methods: pH-stat titration, viscosimetry, and spectrophotometry. Similar measurements were made with pancreatic deoxyribonuclease I (a typical endonuclease) and with micrococcal deoxyribonuclease. The results were compared, and they led to the conclusion that phosphodiesterase degrades deoxyribonucleic acid to completion, essentially by exonucleolytic action (7).

EXPERIMENTAL PROCEDURE

Phosphodiesterase—Phosphodiesterase from the venom of Bothrops atrox was prepared by a method similar to that described previously (8), but modified to improve both yield and purity of the preparation.

Acetone Fractionation—Step 1. The procedure of Koerner and Sinsheimer (9) was modified. Venom (2 g) was dissolved in 120 ml of cold water, stirred for 30 minutes at 0°, and filtered on Büchner funnel, with Whatman No. 3 paper. The clear solution was placed in an ice bath and was treated with 80 ml of cold 0.5 M acetate buffer, pH 4.0, and then with 145 ml of acetone (-20°) to attain a concentration of 42%. The mixture was stirred for 30 minutes, and centrifuged in a Servall refrigerated centrifuge at 0° for 15 minutes. The heavy yellow precipitate (Precipitate 1) was discarded. The clear supernatant solution was transferred to a bath at -17°, and 55 ml of -20° acetone were added with stirring. The mixture was stirred for 1 hour and was centrifuged at -17°. The precipitate (Precipitate 2) was dissolved in water (10 ml per g of original venom) and was kept frozen. It was stable for a week. This precipitate contained most of phosphodiesterase and only about 0.001% of the 5'-nucleotidase originally present in venom. Table I illustrates the distribution of the two enzymes in the three precipitates. The introduced modification (cf. Table I in the previous paper (8)) increased the yield of phosphodiesterase about 2-fold (60% recovery instead of 30% by the previous procedure), and decreased the contaminating 5'-nucleotidase.

When larger batches of phosphodiesterase had to be prepared, this step was performed with 10-g quantities of dried venom, proportional increases of other reagents, and the use of the GSA rotor of refrigerated Servall centrifuge. With the large scale experiments the removal of 5'-nucleotidase was less efficient; the final third precipitate contained about 2 to 3 times more 5'-nucleotidase than was normally found in small scale experiments.

Ethanol Fractionation—Step 2. When the amount of Precipitate 3 (Step 1) equivalent to 20 g of venom was accumulated, batches were thawed and combined. Water and 1 M acetate buffer, pH 6.0, were added to attain the final absorbancy at 280 mμ of 10.0 and a final molarity of acetate buffer of 0.1. The inert protein was removed with 0.5 volume (33%) of ethanol (95% counted as 100%) at 0°, and the phosphodiesterase was precipitated with an additional 1.5 volumes of ethanol (33 to 66% precipitation) at -17°.

Step 3. The precipitate from Step 2 was dissolved in 0.1 M Tris buffer, pH 8.9, and the volume was adjusted to an absorbancy at 280 mμ of 10.0. The fractionation between 33 and 66% was repeated as in the previous step. The precipitate was dissolved in a small volume of 0.1 M Tris, pH 8.9.

Continuous Paper Electrophoresis—Step 4. The solution of enzyme from Step 3 (total absorbancy at 280 mμ of about 180) was dialyzed against 0.5 M Tris buffer, pH 8.9, and was introduced on the middle tab of the Beckman-Spinco model CP continuous flow electrophoresis apparatus, previously equilibrated with the same buffer. Protein concentration was 5 to 10 A280 units per ml, charging rate 14 ml per hour, applied potential 700 volts. Under these conditions protein was distributed throughout 12 of the 16 tubes, forming two or three distinct peaks. About 80% of the phosphodiesterase appeared in tubes 6 to 8 (tube 1 was closest to the cathode).

Chromatography on Carboxymethyl Cellulose—Step 5 was carried out as described previously (8). This step was essential since the endogenous DNase present in venom (8, 10), and partly carried through the previous steps, appeared in peak 1, whereas phosphodiesterase appeared in peak 2 (Fig. 1, (8)).

Chromatography on Diethylaminoethyl Cellulose—Step 6 was performed as previously described (8). This step eliminates the final traces of contaminating 5'-nucleotidase. Contrary to the

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previously observed distribution (Fig. 2, (8)), only a small amount of phosphodiesterase appeared in peak 1. The bulk of the phosphodiesterase of high potency appeared in peak 2. The yield in each step and the extent of purification are summarized in Table II. Phosphodiesterase was kept frozen until ready to use. Phosphodiesterase thus obtained also acted on ATP, producing AMP and pyrophosphate.

**Assay**—For the purpose of purification, phosphodiesterase was assayed with calcium di-p-nitrophenyl phosphate as suggested by Sinsheimer and Koerner (11), except that Mg++ was omitted. Beginning with step 4, crystalline bovine serum albumin (Armour and Company) was added to stabilize the enzyme (0.5 mg per 3.0 ml of incubation mixture). Activity was expressed in nM/mg per minute and potency as activity per Azs0 of the enzyme solution. 5'-Nucleotidase was determined as previously described (8) and expressed in the same units. Endogenous DNase was determined viscometrically at pH 5.0.

**Micrococcal DNase**—Micrococcal DNase was prepared according to Cunningham et al. (12) and was the gift of Dr. Cunningham.

DNA was prepared according to Kay et al. (13). It contained 15% moisture, and, on a dry weight basis, 8.4% P, 14.3% N, and 31% of total ash; sulfur was absent. The moisture and P values agree with values found by Cavalleri (14) for a similar preparation of DNA. The N value is in good agreement with the average value reported by Donnec (13), although the percentage of phosphorus is low; e (P) of our preparation was 6300 in 0.2 M NaCl.

The stock solutions of DNA were prepared by dissolving 75 to 95 mg of air-dried DNA in 50 ml of 0.2 M NaCl at 5°. Test solutions were then prepared from these stock solutions, deionized water and salt, HCl, NaOH, and Tris buffer solutions. The chemicals were analytical grade and were used without further purification. In a few experiments stock solutions were prepared from DNA freed from salt by dialysis. The total phosphorus content of each stock solution was determined by the method of Fiske and SubbaRow (15).

**Spectrophotometric Analyses**—Calibrated 1-cm quartz cuvettes and a Beckman model DU spectrophotometer equipped with thermostagers and a photomultiplier attachment were used for spectrophotometric analyses.

**Viscosimetric Measurements**—Viscosimetric measurements were performed with an Ostwald viscosimeter having a flow time of 10.5 seconds and an average velocity gradient $\beta = (8V) / (3 \pi r \eta)$ as 280 sec⁻¹ for deionized water. The value of $\beta$ for the DNA solution employed ranged between 77 and 106 sec⁻¹. No kinetic energy corrections were made.

Paper chromatography was performed according to Bergkvist (16).

**pH-Stat Titrimetry**—The essential features of the pH-stat apparatus were a constant voltage transformer, a Leeds and Northrop line operated pH-meter, a Leeds and Northrop Speedomax, type G recorder, and a cam-driven (by a constant torque, 3/4 horse-power motor) calibrated 1-ml syringe. pH could be maintained constant within 0.014 pH unit. Enzymatic hydrolyses were carried out in a water-jacketed cell under a flow of nitrogen, with magnetic stirring. The concentration of NaOH which was used to maintain the pH ranged between 1.60 and 10.0 X 10⁻³ M, depending on the rate and extent of different hydrolysis reactions. The normality of the NaOH was determined by standard volumetric procedure at the beginning and end of each pH-stat titration and the average value was used in the ensuing calculations. All measurements were made at 25°.

Before use in pH-stat experiments, phosphodiesterase was dialyzed against dilute NaOH (pH 9.0) in the cold. Dialysis was performed in a closed system to prevent absorption of CO₂. The pH was checked at each change of the dialysate and at the conclusion of dialysis.

**RESULTS AND DISCUSSION**

Although the optimal conditions for the action of phosphodiesterase on several synthetic and natural substrates of low molecular weight are now established (17-19), this is not the case with DNA. Boman (6) reported that with 0.1% DNA, the reaction proceeded almost to completion.

On the basis of Boman’s observation, conditions were sought to carry the reaction to completion. With 0.3 unit of phosphodiesterase per ml, 0.007% of DNA, in the presence of 10⁻³ M NaCl and 10⁻³ M CaCl₂, hydrolysis (see Curve A, Fig. 1) was completed in 3 hours. The rate of hydrolysis for the first 5 minutes was of the order of 2 nmoles of nucleotide phosphate liberated per minute per mg of enzyme. The completion of the reaction was calculated from pH-stat data and was confirmed by paper chromatography. Recovery of mononucleotides was almost quantitative; no ultraviolet-absorbing material was left at the origin or detected in any other region outside that of mononucleotides. In our hands, no significant difference be-
Table III shows the effect of an increased NaCl concentration on the extent of hydrolysis of DNA at $10^{-3}$ in the reaction medium when phosphodiesterase acted on DNA. The concentration of bivalent cations required was found to be related to the concentration of monovalent cations present in the reaction medium when phosphodiesterase acted on DNA. The results obtained with phosphodiesterase prompted experiments in which the effects of sodium and magnesium ions on DNase I were studied. It has been known for sometime that DNase I is inhibited by high concentrations of sodium chloride in the reaction medium (20). It has now been observed that the inhibition produced by NaCl may be reversed by an increased concentration of Mg++. An example is shown in Fig. 2. A progress of enzymatic degradation of the DNA molecule has been studied extensively only with DNase I (21-23). Assuming a polydisperse substrate and using light scattering and pH stat data, Thomas (21) has shown that the degradation of DNA with DNase I at 25° is best fitted by a plot of $M(P)/M$ against $2pu3/2h + 1$ for a value of $h = 2$; $M(P)/M$ is the ratio of the weight average molecular weight ($M_w$) of the polymer molecules at a given stage of degradation to the molecular weight of the molecules before the addition of enzyme. The term $2pu3$ is a measure of the number of diester bond cleavages per parent Z-average molecular weight ($M_z$) polymer molecule. The fact that $M(P)/M$ for sodium chloride concentration is considerably lower. The reason for using $10^{-3}$ NaCl was to approach as closely as possible conditions comparable with methods other than the pH stat.

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Time-activity curves of purified venom phosphodiesterase measured by pH-stat titration. Percentage of hydrolysis = \((\text{moles of NaOH} \times 100)/\text{(total moles of diester bonds)}\). Curve A: \(X, 0.001 \text{M} \text{MgCl}_2, 0.01 \text{M} \text{NaCl}, 0.007\% \text{DNA}; \Box, 0.001 \text{M} \text{CaCl}_2, 0.01 \text{M} \text{NaCl}, 0.007\% \text{DNA}; \Diamond, 0.001 \text{M} \text{NaCl}, 0.007\% \text{DNA}.\) Curve B: \(\Delta, 0.001 \text{M} \text{MgCl}_2, 0.007\% \text{dialyzed DNA}; \bigcirc, 0.001 \text{M} \text{MgCl}_2, 0.007\% \text{dialyzed DNA}.\) Curve C: \(\bullet, 0.0005 \text{M} \text{MgCl}_2, 0.007\% \text{dialyzed DNA}.\) Curve D: \(\triangle, 0.0005 \text{M} \text{MgCl}_2, 0.007\% \text{dialyzed DNA}.\) The concentration of enzyme in all experiments was 0.3 unit per ml (6.7 μg per ml).

**Table III**

Effect of increased NaCl concentration on rate of degradation of DNA by phosphodiesterase*

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>$0.01 \text{ M NaCl}$</th>
<th>$0.01 \text{ M MgCl}_2$</th>
<th>$0.11 \text{ M NaCl}$</th>
<th>$0.01 \text{ M MgCl}_2$</th>
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<td>0.09</td>
<td>0.17</td>
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<td>0.10</td>
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<td>0.13</td>
<td>0.56</td>
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<td>0.14</td>
<td>0.63</td>
<td>0.14</td>
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<td>0.15</td>
</tr>
<tr>
<td>90</td>
<td>0.67</td>
<td>0.15</td>
<td>0.67</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Phosphodiesterase, 0.3 unit per ml; 0.05 μM Tris buffer (pH 8.70), DNA concentration, 0.000% (on air dry weight basis). Incubated in a thermostated Beckman model DU spectrophotometer at 30°, and read against blank containing no enzyme.

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** The reversal of NaCl inhibition of DNase I by increased concentration of MgCl$_2$. All tubes contained 0.004% dialyzed DNA, 0.1 μM Tris buffer, pH 7.2, 0.001 μM MgCl$_2, 1$ μg per ml of DNase I. Tubes B and C in addition to the above contained 0.1 μM NaCl. At the time indicated by arrows (11 minutes) tubes A and B received 0.1 ml of 2 μM MgCl$_2$ to adjust its concentration to approximately 0.05 μM. Incubation was carried in a thermostated model DU Beckman spectrophotometer at 30°, and tubes were read against a blank containing 0.004% DNA in 0.1 μM Tris buffer.

![Another series of experiments](http://www.jbc.org/)

In another series of experiments, dialyzed DNA was hydrolyzed by phosphodiesterase in the presence of: (a) $10^{-3}$ μM MgCl$_2$, (b) $10^{-2}$ μM MgCl$_2$, and $10^{-2}$ μM NaCl, (c) $3 \times 10^{-4}$ μM MgCl$_2$. The extent of hydrolysis was lowest in (a), highest in (c).
tor $\sqrt{2h + 1}$ takes into consideration the number of hydrogen bonds which are required to hold two DNA strands together when one or more diester bond cleavages have been produced in each of the strands. As Thomas has shown, the fact that $M(P)/M$ may be replaced by $[\eta]/[\eta]_0$ (ratio of the intrinsic viscosities) indicates that the flexible rod relationship, $[\eta] = KM$, is true for the degradation of DNA with DNase I during the early stages of digestion.

Fig. 3 illustrates our viscosity pH-stat data together with Thomas’ theoretical curve assuming an $M_0$ value of $7.5 \times 10^6$ for our DNA ($M_0 = 5 \times 10^6$) and using the approximation suggested by Thomas that at a DNA concentration of 0.002%, $[\eta] = \left[ \frac{\eta_p}{c} \right]$, where $\eta_p$ is the specific viscosity, $c$ is the concentration of DNA, and $\bar{g}$ is the average velocity gradient of a given solution in the viscometer. Although our DNA concentrations are approximately 4 times as large as those used by Thomas, the fit of our data for the early stages of degradation is surprisingly good. Conway and Butler (24) have published data which suggest that the reduced specific viscosity at DNA concentrations comparable to those used by us need be corrected only for velocity gradient effects.

For an exonuclease, during the initial phase of the reaction, one may postulate that the decrease in viscosity (and molecular weight) is proportional to the number of internucleotide bonds cleaved. A hypothetical straight line was drawn on the assumption that cleaving of 5% of the internucleotide bonds reduced the viscosity by 5%. Fig. 3 shows that the fit of the hypothetical curve and the experimental data is quite good. A comparison with the DNase I curve and data leaves little doubt that the venom diesterase attacks DNA essentially by exonucleolytic action.

The results obtained with micrococcal DNase represent an intermediate type of nucleolytic behavior. Obviously, the predominant action is endonucleolytic, yet the change in viscosity per cleavage is smaller than would be expected on the assumption of random cleavages. A plausible explanation is that some exonucleolytic splits follow the endonucleolytic action. This assumption is corroborated by the recent results of Dekker et al. (25–27).

A sharp distinction between endo- and exonucleolytic types of action, seen in the viscometric studies, prompted the investigation in which absorbancy change was correlated with the number of splits. It has been expected that the magnitude of the optical perturbation caused by a cleavage of an internucleotide bond will be dependent on the location of the bond in the molecule and will thus reflect the endo- or exonucleolytic type of action. This expectation was confirmed.

Fig. 4 illustrates changes in absorbancy per cleavage during the degradation of DNA by the three enzymes. The ordinate represents difference spectra values at 259 mÅ. Direct spectra curves of DNA before and after complete digestion with venom phosphodiesterase showed that the spectral perturbation was both bathochromic and hyperchromic, a result which agrees with the previous observation on dinucleotides (28).

It is now generally accepted that the action of DNase I asymptotically approaches a standstill when approximately 25 to 30% of the internucleotide linkages have been broken ($\Delta_{259} \approx 4500$). Kunitz (29) was the first to notice that the optical effects subside earlier than the liberation of tritatable groups. This observation has been confirmed.

The initial cleavages produced by DNase I result in a hyperchromic effect which is considerably greater than either that for micrococcal DNase or, particularly, that for phosphodiesterase. An apparent lack of optical effect during the second part of the reaction with DNase I is puzzling. A highly speculative explanation is that the optical effect due to cleavages of internucleotide bonds is balanced by an opposite optical effect resulting from reforming of double-stranded structures. Since the longest chains remaining in the DNase I digest are estimated as between 8 and 12 nucleotides, and it is known that almost all combinations of nucleotides are present in the digest, it seems conceivable that double-stranded molecules may be formed even at 37°. Recent findings of Lipsett et al. (30), which show that even dinucleotides may form double-stranded structures, substantiate such an explanation.

The curve representing the digestion with phosphodiesterase (Fig. 4) is S-shaped. One might speculate that near the mid point of the digestion a cleaving of one internucleotide linkage produces a more pronounced rearrangement in the helical structure than a corresponding cleavage either at the end or the beginning of digestion.

With micrococcal DNase the optical effect per cleavage is consistently higher than with phosphodiesterase. This would be expected because of the endonucleolytic character of micrococcal DNase. The unexpected finding is that the final optical effect is equal for both enzymes in spite of the fact that phosphodiesterase cleaves 100% of bonds, and micrococcal DNase 70%. The
comparison of values of specific absorbancy of 5′- (31) and 3′- (32) deoxyribomononucleotides indicates that these values are rather similar. The observed discrepancy cannot, therefore, be ascribed to the difference of breaking C3′-P (phosphodiesterase) versus P-C5 bond (micrococcal DNase), and remains unexplained.

**SUMMARY**

An improved method for preparation of venom phosphodiesterase with a higher potency and in better yield has been described. Purified phosphodiesterase quantitatively degrades intact thymus deoxyribonucleic acid to mononucleotides; no other products have been detected. The rate of the reaction is dependent on the MgCl₂ or CaCl₂ concentration and is inhibited by high NaCl and substrate concentrations. The degradation of deoxyribonucleic acid by phosphodiesterase follows the course expected for exonucleolytic action and differs strikingly from that of deoxyribonuclease I, a typical endonuclease. Micrococcal deoxyribonuclease represents an intermediate type of enzyme in which endonucleolytic action predominates at the early stages of reaction. For each of the three enzymes studied the optical effect per diester bond cleavage varies during the digestion. The three different curves thus obtained reflect endo- or exonucleolytic type of action.

**REFERENCES**

Action of Venom Phosphodiesterase on Deoxyribonucleic Acid
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