Purification of an Endonuclease from the Venom of *Bothrops atrox**

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Since phosphodiesterase is widely used for establishing nucleotide sequence, the properties of its possible contaminants deserve study. A nuclease of the venom of *Bothrops atrox* has been purified approximately 1000-fold from the 42% acetone precipitate, which is a by-product of the preparation of venom phosphodiesterase. The preparation of enzyme thus obtained can split both ribo- and deoxyribonucleic acids at a similar rate. The enzyme has an optimal activity at *p*H 5.0 and requires no magnesium. It acts on DNA as an endonuclease, and produces predominantly tri- or higher oligonucleotides all of which terminate in 3′-monoesterified phosphate. At the early stages of digestion de-Gp-Gp[1] is the most susceptible bond. As the digestion progresses the specificity in respect to the adjacent bases decreases, and the length of the substrate chain becomes more significant. After an exhaustive digestion fragments are obtained in which all four bases in terminal positions occur in an almost random distribution.

In all existing methods of purification of phosphodiesterase[2] from venom, the major effort (Björk, 1961; Williams *et al.*, 1961) has been directed toward freeing phosphodiesterase from 5′-nucleotidase, a specific phosphomonoesterase present in large amounts in venom. Little attention has been paid to other possible contaminants, even though Delezenne and Morel (1919) and Taborda *et al.* (1952) described both ribonuclease and deoxyribonuclease activities in venom. However, in view of our present knowledge, the observed activities could have been caused, at least partly, by venom phosphodiesterase itself. Haessler and Cunningham (1957) described in venom a DNase that had a *p*H optimum at 5.0 and required no Mg^{++}. Laskowski *et al.* (1957) concluded that, in addition to an exonuclease (venom phosphodiesterase) with an optimum at *p*H 8.9, venom contains an endonuclease with an optimum at *p*H 5.0. Among the products of digestion of DNA by the crude venom the latter authors identified small quantities of thymidine 3′,5′-diphosphate, and ascribed its presence to a combined action of a 3′-monoester forming endonuclease and 5′-monoester forming exonuclease. Finally, it was found (Williams *et al.*, 1961) that a DNase of the endonuclease type was present in crude preparations of phosphodiesterase and that the two enzymes are separable by chromatography on columns of CM-cellulose.

The widespread use of venom phosphodiesterase in the study of nucleotide sequences focuses attention on possible contaminants and their properties. The present paper describes the purification of an endonuclease from venom, and some of its properties, including specificity.

EXPERIMENTAL

Materials.—DNA was prepared according to the method of Kay *et al.* (1952), S-RNA was isolated from baker's yeast as described by Holley *et al.* (1961), while the RNA core and the yeast RNA, prepared according to Crestfield *et al.* (1955), were a gift of Worthington Biochemical Corporation, Freehold, N. J. The Poly AU (A:U = 10:1) was a gift from Dr. L. A. Heppel. Venom phosphodiesterase was prepared by Dr. S. C. Sung in this laboratory by a method recently described (Williams *et al.*, 1961). Calcium phosphate gel was prepared according to Keilin and Hartree (1938).

Column Chromatography.—Two types of ion exchangers were used in the purification procedure. Amberlite IRC-50, XE-64, 200–400 mesh, was purchased from Rohm and Haas Company, Philadelphia. DEAE-cellulose, type 20, 0.82 mEq/g, was purchased from Carl Schleicher & Shuell Co., Keene, N. H.

Analysis of Products.—Chromatography on Dowex 1-X2 columns, 200–400 mesh (Volkin and Cohn, 1953; Sinsheimer, 1954) and two-dimensional paper chromatography (Felix *et al.*, 1960) were used to identify the products.

Enzyme Assay.—The diphenylamine method (Dische, 1955) was employed for the first two steps of purification and Kunitz's spectrophotometric

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[1] The abbreviations of the derivatives of nucleic acids are those used by the *Journal of Biological Chemistry*, with the exception that the letters "de," rather than "d," are used to distinguish polydeoxyribonucleotides from polyribonucleotides.

[2] The three different meanings of the term phosphodiesterase have been discussed recently (Schmidt and Laskowski, 1961). For the purpose of this paper the term venom phosphodiesterase will be used to describe a single enzyme belonging to the group of nucleophosphodiesterases which, according to the proposed scheme of classification (Schmidt and Laskowski, 1961; Laskowski, 1961; Sung and Laskowski, 1962), has the following properties: (1) it is nonspecific with respect to sugar, (2) it is an exonuclease, (3) it forms 5′-monoesters, (4) it is an enzyme preferentially cleaving mononucleotides from the terminal of the chain possessing the free 3′-hydroxyl group, and (5) it is capable of hydrolyzing all dinucleotides regardless of their composition.

method (Kunitz, 1950) for the remaining steps. In the first case, 0.5 cc of the enzyme solution, containing 0.8 to 2.5 A_{280} units of protein were added to 1 ml of DNA solution (2 mg DNA per ml, 0.3 M sodium acetate buffer, *p*H 5.0). The mixture was left at 37° for 3 hours, 1.5 ml 15% trichloroacetic solution was added, and the deoxyribose was determined in the supernatant solution with the diphenylamine reagent (Dische, 1955). A unit of nuclease activity is defined as the amount of enzyme that causes an increase in absorbancy at 595 mμ of 0.001 under the conditions of the experiment. Beginning with step 3, Kunitz's spectrophotometric assay was used (Kunitz, 1950), except that the DNA was dissolved in 0.2 M sodium acetate buffer with no magnesium added. Kunitz's units were also used to express activity. Amount of enzyme recommended for assay is 0.005–0.025 of Kunitz's units. Potency in all cases is defined as Kunitz's units/A_{280} of enzyme solution. The activities in steps 1 and 2 were recalculated in Kunitz's units (Table I), on the basis of 1 Kunitz unit = 30,000 A_{595} diphenylamine units, as found for the enzyme preparation of step 3.

The use of the two methods was necessitated by the presence of an interfering agent in the crude venom of *Bothrops atrox*. Upon mixture of venom and DNA solutions at *p*H 5.0 DNA precipitated, and the viscosimetric and spectrophotometric methods were thus rendered useless and the diphenylamine method highly questionable. The latter method was nevertheless employed during the first two steps of purification, since it was the only method possible. The agent that causes the precipitation of nucleic acid could not be removed by dialysis, boiling, or 5×10^{-3} M EDTA; it could be precipitated with 38% acetone at *p*H 7.0 in the cold. The prolonged incubation during the diphenylamine assay (3 hours) was necessitated by the formation of the DNA-containing precipitate, caused by the agent present in the crude venom.

Phosphodiesterase and 5′-nucleotidase were determined by methods previously described (Sinsheimer and Koerner, 1952; Koerner and Sinsheimer, 1957).

Results

Purification of Venom Endonuclease.—The 42% acetone precipitate obtained as a by-product during the preparation of venom phosphodiesterase according to the modified (Williams *et al.*, 1961) method of Koerner and Sinsheimer (1957) served as a starting material.[3] Immediately after centrifugation the precipitate was suspended in a small volume of water and lyophilized. The dry powder was stored in a cold room for several months. The potency of endonuclease (0.002) in this fraction was only slightly higher than in the original venom, but this material was available in large quantities and was already poor in phosphodiesterase.

[3] We are indebted to Dr. K. H. Slotta, 5740 W. 52nd Terrace, Miami 52, Fla., who facilitated contacts with suppliers of this venom in Brazil.

Step 1. Heating to 60° for 30 Minutes.—Four and one half grams of the 42% acetone precipitate of crude venom were suspended in 300 ml of cold water, and the suspension was stirred in the cold room for approximately 12 hours. The non-dissolved material was centrifuged off and discarded. The supernatant was heated in a 60° water bath for 30 minutes, then transferred to an ice-bath. When the solution had cooled to room temperature (24°), the precipitate was removed by centrifugation and discarded. During this step 90% of the contaminating 5′-nucleotidase and about 40% of the remaining phosphodiesterase were removed. An increase of 20–50% in the total activity of the endonuclease, which could have been due to a partial removal of the DNA-precipitating agent, was also noted after this step.

Step 2. Adsorption on 0.03 M *$Ca_3(PO_4)_2$ Gel.*—To the supernatant solution of step 1, enough 0.2 M calcium gel was added to attain a concentration of 0.03 M with respect to calcium phosphate. The suspension was stirred for 30 minutes at room temperature and the calcium phosphate was removed by centrifugation. The supernatant was dialyzed against 20 volumes of water at 4° for approximately 16 hours.[4]

The calcium gel removed most of the remaining 5′-nucleotidase and approximately half of the remaining phosphodiesterase activities. Most important, however, this step removed the agent that causes precipitation of DNA (see above).

Step 3. Ammonium Sulfate Fractionation.—The dialyzed solution from step 2 was brought to 50% saturation with solid ammonium sulfate[5] at 0°, and was centrifuged at 0°. The precipitate was discarded. To the supernatant, solid ammonium sulfate was added to attain 90% saturation. The precipitate was collected and dissolved in approximately one twentieth the original volume of 0.1 M Tris-HCl buffer, *p*H 8.9. The solution was dialyzed against 4 liters of 0.05 M Tris-HCl buffer, *p*H 7.25.

Step 4. First Chromatography on DEAE.—From this point on all manipulations were performed at 4°. The dialyzed solution from the previous step was applied to a 20 × 1 cm DEAE column previously equilibrated with the buffer used for dialysis. The same buffer was used as a starting eluent. The elution pattern is illustrated in Figure 1. The first peak contained the remaining phosphodiesterase and 5′-nucleotidase activities, while the third contained the endonuclease. The endonuclease activity was eluted on the ascending side of the third peak, so that it is not advisable to allow the second peak to trail. The trailing may be avoided by switching to the higher molarity

[4] It is imperative that one proceed with step 2 immediately after step 1. If the unbuffered system is allowed to stand for a prolonged period the endonuclease is also adsorbed on the gel. Should this occur, the enzyme can be eluted by 0.2 M Tris-HCl buffer, *p*H 8.9, with the subsequent steps remaining the same.

[5] Ammonium sulfate for a given saturation is calculated from the formula of Noda and Kuby (1957).

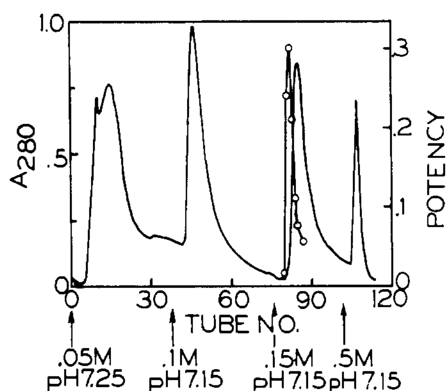

FIG. 1.—Step 4 of the purification procedure. Elution pattern of the first chromatography of endonuclease on DEAE-cellulose. Column 1 × 20 cm, flow rate 3 ml per tube, 4–6 tubes per hour, at 4°. Elution with Tris-HCl buffer of the indicated molarity and *p*H. ——Protein concentration expressed as absorbancy at 280 mμ; O——O potency of venom endonuclease. The contents of tubes 81–90 were pooled and used for further purification.

buffer immediately after the steep portion of the descending part of peak 2 has been eluted. The contents of the tubes with improved endonuclease potency were combined, lyophilized to approximately one third the original volume, and dialyzed against 6 liters of water for 10–12 hours. The lyophilization and the dialysis were repeated once more to insure reduction in volume and removal of salt. At this point the enzyme was frozen and kept at −20° until three or more preparations were accumulated.

Step 5. Chromatography on Amberlite IRC-50.—The combined preparations were dialyzed against 4 liters of 0.05 M sodium acetate buffer, *p*H 5.75, for 12 hours and applied on a 20 × 1 cm Amberlite IRC-50 column, previously equilibrated with the same buffer. The elution was continued with the same buffer till no more protein emerged from the column (Fig. 2). The enzyme emerged with 0.4

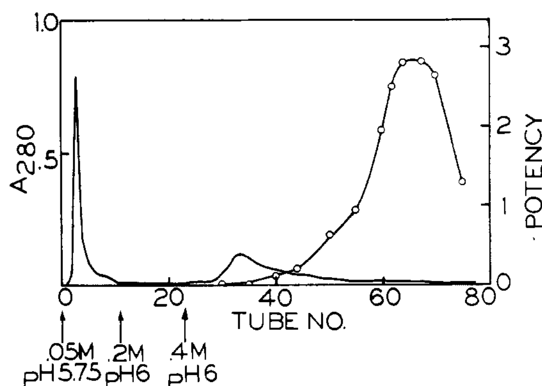

FIG. 2.—Step 5 of the purification procedure. Elution pattern of the chromatography of venom endonuclease on Amberlite IRC-50. Column 1 × 20 cm, flow rate 3 ml per tube, 4–6 tubes per hour at 4°. Elution with sodium acetate buffer of the indicated molarity and *p*H. ——Protein concentration expressed as absorbancy at 280 mμ; O——O potency of venom endonuclease. The contents of tubes 44–77 were pooled for further purification.

M buffer, *p*H 6, on the descending part of the peak. The contents of the tubes containing endonuclease with improved potency were combined, lyophilized to one third the original volume, and dialyzed against water. The lyophilization and dialysis were repeated and the enzyme solution was reduced to a volume of 1–2 ml.

Step 6. Second Chromatography on DEAE.—The enzyme solution from step 5 was dialyzed against 2 liters of 0.075 M Tris-HCl buffer, *p*H 7.15, for 12 hours and was applied to a 0.5 × 25 cm DEAE column, previously adjusted to the same buffer. As soon as all the enzyme solution passed into the column, the latter was connected with Peterson and Sober's "Varigrad" (1960) containing a linear gradient of 0.075 M and 0.150 M Tris-HCl buffer, *p*H 7.15. If all of the protein had not emerged by the time the solutions in the Varigrad were exhausted, the elution was continued with 0.15 M buffer until no more enzyme appeared (Fig. 3).

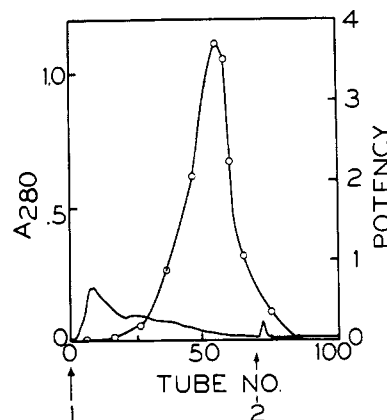

FIG. 3.—Step 6 of the purification procedure. Elution pattern of the second chromatography on DEAE-cellulose. Column 0.5 × 25 cm, flow rate 3 ml per tube, 2–3 tubes per hour at 4°. Elution: at arrow 1 with linear gradient, between 0.075 M and 0.15 M Tris-HCl buffer, *p*H 7.15 (125 ml in each of two chambers of the gradient); at arrow 2 with 0.15 M Tris-HCl buffer, *p*H 7.15. ——Protein concentration expressed as absorbancy at 280 mμ; O——O potency of venom endonuclease. The contents of tubes 33–66 were pooled for further purification.

Step 7. Ammonium Sulfate Precipitation.—The portion of the peak containing the enzyme in step 6 was lyophilized to one third of its volume, dialyzed against water for 12 hours, lyophilized to approximately 5 ml, dialyzed against water once more, and finally reduced by lyophilization to 1 ml. A saturated solution of ammonium sulfate was added dropwise until the first cloudiness appeared, which after the solution had stood for several hours changed to a semicrystalline precipitate. The precipitate was centrifuged off and discarded. The supernatant was saturated with solid ammonium sulfate. The enzyme was separated from the mother liquor by centrifugation, dissolved in water, dialyzed against water, and kept frozen. Table I summarizes the purification procedure.

The potency of the final product was of a similar order of magnitude (2.5 U/A_{280}) as that of the crystalline DNase I, measured under identical conditions (8 U/A_{280}). Even in the deep-freeze (−20°) the enzyme lost 70% of its activity after 6 months of storage. The yield was about 3 mg (3 A_{280} units) from 40 g of the "42% acetone fraction" obtained from 100 g venom. In view of this, only a limited characterization was possible, and physical studies were excluded.

Table I
Yield and Extent of Purification of Venom Endonuclease

Purification Step	Potency (units/A_{280})	Total Activity (units)	Yield (%)
42% acetone precipitate of venom	0.002	5.4	100
Step 1. Heating to 60°	0.007	6.4	118
Step 2. Calcium gel adsorption	0.008	4.7	87
Step 3. Ammonium sulfate fractionation	0.015	4.1	76
Step 4. DEAE, first chromatography	0.14	1.5	28[b]
Step 5. Amberlite IRC-50, chromatography[a]	1.0	1.2	22
Step 6. DEAE, second chromatography	2.0	1.0	18
Step 7. Ammonium sulfate fractionation	2.5	0.9	16

[a] From step 5 onward the values given are calculated as if one prepaiation was carried all the way to step 7. In reality three or more preparations from step 4 were combined and carried through the next three steps. [b] The low yield in this step is due to the fact that only the contents of tubes with potency higher than the one charged were combined and processed further.

Properties of Venom Endonuclease.—The optimal *p*H of the nuclease action was determined in sodium acetate buffer of constant ionic strength (0.2) and varying *p*H values. The Kunitz assay method was employed. Activity was calculated from the values obtained in the first 9 minutes of the reaction. Figure 4 illustrates the results.

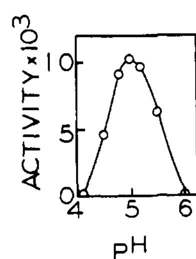

Fig. 4.—Effect of *p*H on activity of venom endonuclease. Kunitz's spectrophotometric method was used. The reaction cells contained 3 ml DNA solution (0.04 mg/ml in H_2O), 0.05 ml enzyme solution (0.01 unit), 0.15 ml H_2O, and 0.8 ml 1 M sodium acetate of the indicated *p*H.

The effects of buffer concentration and Mg^{++} were measured by the method of Kunitz. Sodium acetate buffers of varying concentrations and constant *p*H (5.0) were tested. Concentrations lower than 0.15 M and higher than 0.4 M were inhibiting. Magnesium chloride introduced into the system in the presence of 0.2 M sodium acetate, *p*H 5.0, proved inhibiting at concentrations higher than 5×10^{-3} M (Fig. 5).

Even though purified preparations have not been

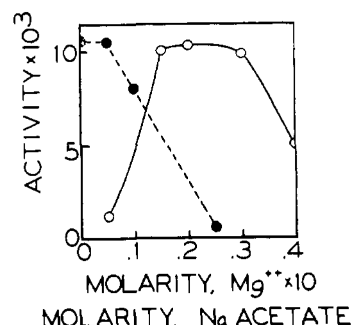

Fig. 5.—Effect of buffer and Mg^{++} concentrations on the activity of venom endonuclease. Kunitz's spectrophotometric method was used. For the determination of the optimal buffer concentration, the reaction cells contained 3 ml DNA (0.04 mg/ml in H_2O), 0.05 ml enzyme (0.01 unit), a calculated quantity of 1 M sodium acetate buffer, *p*H 5.0, to attain the indicated molarity, and water to a total volume of 4 ml. For the determination of the effect of Mg^{++} the reaction cells contained 3 ml DNA (0.04 mg/ml), 0.05 ml enzyme, a calculated volume of 0.25 M $MgCl_2$ to attain the indicated molarity, and water to a total volume of 4 ml. O——O Effect of buffer, ●—● effect of Mg^{++}.

used for heat and *p*H stability studies, the crude enzyme was tested under various conditions. It was found that, in the presence of 0.1 M sodium acetate buffer, the enzyme is stable at *p*H 6.0 but loses more than half of its activity at *p*H 4.0, both when it is boiled for 30 seconds and when it is left at 4° for 72 hours. At *p*H 8.0 in the presence of 0.1 M Tris-HCl buffer the enzyme is stable under both of the previously mentioned conditions.

The purification procedure for the endonuclease was devised on the basis of its DNase activity. However, the purified enzyme exhibits toward RNA an activity of a similar order of magnitude (Fig. 6), except for the "RNA core" which was resistant to the enzyme.

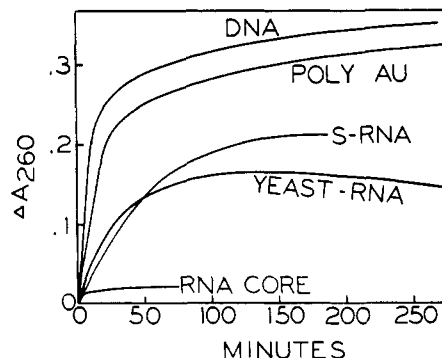

Fig. 6.—Digestion of various substrates with venom endonuclease. Kunitz's spectrophotometric method was used. The reaction mixtures contained 0.8 ml 1 M sodium acetate buffer, *p*H 5.0, 0.2 ml enzyme solution (potency toward DNA 1.8), 3.0 ml substrate. The concentrations of the various substrates in A_{260} units per ml of final reaction mixture were: DNA 1.96, Poly AU 1.75, yeast RNA 2.05, S-RNA 2.19, RNA core 2.7.

Products Formed.—Three large-scale experiments were performed with calf thymus DNA. In the first experiment, which was aimed at complete digestion, 100 mg of DNA was hydrolyzed with 4 units of endonuclease in 0.2 M sodium acetate buffer, *p*H 5.0, total volume 24 ml. After 6 hours at 37°, the reaction mixture was diluted to 1000 ml, adjusted to a *p*H of about 8 with 2 drops of concentrated ammonia, and charged on a 20 × 1 cm Dowex 1-X2 column previously washed with 500 ml each of 2 M ammonium acetate and water. The second experiment was identical except that 0.4 units of enzyme (one tenth of the previous amount) was used. The third experiment was aimed at elucidating the specificity in the very early stages of the reaction. Fifty milligrams of DNA were digested with 0.1 unit of nuclease in a total volume of 24 ml, in the presence of 0.2 M sodium acetate, *p*H 5.0. After 14 minutes, at which time the viscosity of the solution fell to one tenth of its original value, the reaction was stopped by immersing the tube in boiling water for 5 minutes. The reaction mixture was then centrifuged, dialyzed to eliminate excess salt, and digested with a massive dose of phosphodiesterase. The results of chromatographic analysis of experiments 1 and 2 are shown in Figures 7 and 8, respectively, and the relative distribution of products among different fractions is shown in Table II.

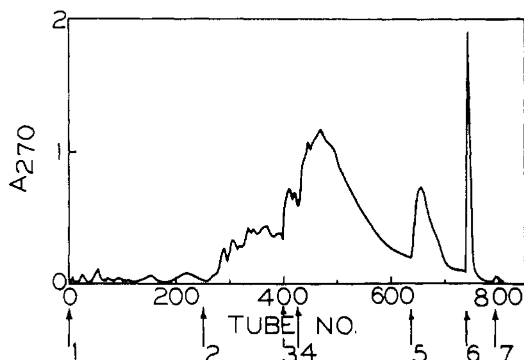

Fig. 7.—Elution pattern of the "exhaustive" digest of DNA by venom endonuclease, chromatographed on a Dowex 1-X2 column (20 × 1 cm). Flow rate 8 ml per tube, 6 tubes per hour, at room temperature. Elution was carried out with ammonium acetate buffer, *p*H 4.5. The arrows indicate changes in buffer concentration. The numbers below the arrows indicate the molarities of the buffers used, which are as follows: 1, gradient between 0.1 and 1.0 M [chambers 1 and 2 of the "Varigrad" contained the low molarity buffer (250 ml each) and chambers 3 and 4 the high molarity buffer (250 ml each); the column was connected to chamber 2]; 2, linear gradient between 1.0 and 2.0 M; 3, 2.0 M; 4, linear gradient between 2.0 and 3.0 M; 5, 3.0 M; 6, 4.0 M; and 7, 5.0 M. ——Concentration of split products expressed in absorbancy units at 270 mμ.

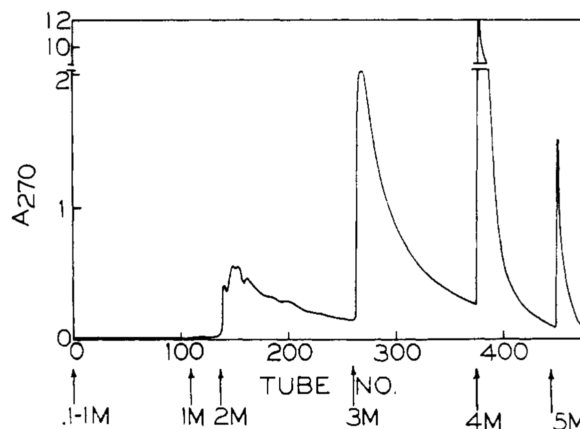

Fig. 8.—Elution pattern of the "limited" digest of DNA by venom endonuclease chromatographed on a Dowex 1-X2 column (20 × 1 cm). Flow rate 8 ml per tube, 6 tubes per hour, at room temperature. Elution was carried out with ammonium acetate buffer, *p*H 4.5, of the molarity indicated by arrows. ——Concentration of split products expressed in absorbancy units at 270 mμ.

Table II

Relative Distribution of Products Expressed in Per Cent of Absorbancy Units (A_{270}), in Respect to the Strength of Buffers Required for Elution from Column (Length of the Chains)

	Molarity of Eluting Buffer					Recovery[a] (% of total A_{270} units charged)
	0.1–1	2	3	4	5	
Experiment 1	4.0	19.5	72.1	4.1	0.2	108
Experiment 2	0.1	14.0	40.3	41.2	3.8	104

[a] Recovery higher than 100% is caused by the UV absorbing substance derived from the resin.

In order to characterize the products in respect to the end-groups, several experiments have been performed (Fig. 9 and Table III). An aliquot of 250–300 A_{270} units of the exhaustively lyophilized "3 M fractions" of experiments 1 and 2 were incubated at 37° with 8 units of venom phosphodiesterase in 0.05 M Tris-HCl buffer, *p*H 8.9, in the presence of 5 × 10^{-3} M magnesium, total volume 2 ml. After 6 hours, 8 more units of phosphodiesterase were added and the incubation was continued for 18 more hours. Paper chromatography of the products revealed in both cases all four nucleosides, all four nucleotides, and only two nucleoside diphosphates: deoxyadenosine 3′,5′-diphosphate and deoxyguanosine 3′,5′-diphosphate (Fig. 9). The diphosphates of deoxycytidine and

Table III

Frequency of Occurrence of Different Bases in Both Terminal Positions of Deoxyribo-oligonucleotides Formed by the Action of Venom Endonuclease

Substance Isolated	Exper. 1 (% of A_{270})	Exper. 2 (% of A_{270})	Exper. 3 (% of A_{270})
	Percentage Composition of Nucleosides and Nucleoside Diphosphates[a]		
A	25.5	21.2	14.7
G	29.9	45.8	51.0
C	31.7	19.8	9.7
T	11.9	13.1	11.9
pAp	20.6	16.1	
pGp	38.2	54.0	
pCp	21.5	13.5	
pTp	19.5	16.3	

[a] Calculated from experiments performed on Dowex 1-X2 columns.

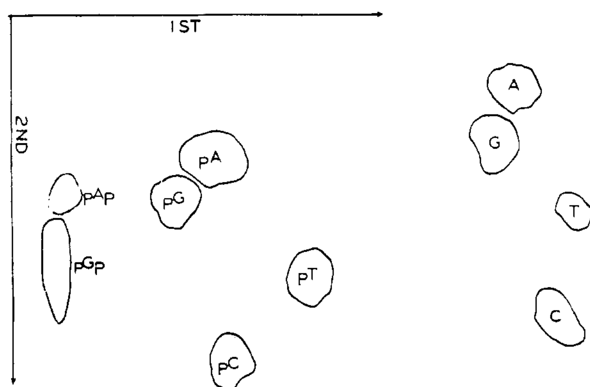

Fig. 9.—Two-dimensional paper chromatogram according to Felix *et al.* (1960). First direction: ethanol, 75, 1 M ammonium acetate, *p*H 7.5, 30; second direction: saturated ammonium sulfate–isopropanol–water (80:2:18). The "3 M fractions" (Fig. 7 and 8) were lyophilized exhaustively and an aliquot of 250–300 absorbancy units at 270 mμ from each fraction were incubated with 8 units of venom phosphodiesterase at 37° in the presence of 0.05 M Tris-HCl buffer, *p*H 8.9, and 5×10^{-3} M $MgCl_2$. After 6 hours 8 more units of venom phosphodiesterase were added and the reaction was continued for an additional 16 hours. A sample of 0.3 ml was placed on Whatman No. 3 MM paper and chromatographed.

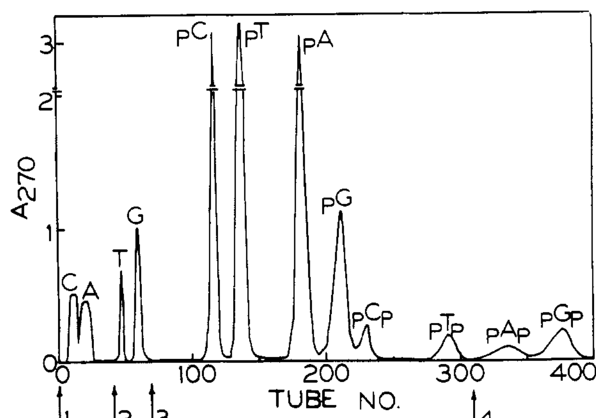

Fig. 10.—Elution pattern of a venom phosphodiesterase digest of the "3 M fraction" in Figure 7. Chromatography on a Dowex 1-X2 column, 20 × 1 cm. Flow rate 3 ml per tube, 6 tubes per hour at room temperature. The arrows indicate change of the eluting buffer. The numbers have the following connotations: 1, H_2O; 2, 0.1 M ammonium acetate buffer, *p*H 6.2; 3, 1000 ml gradient between 0.1 and 1.0 M ammonium acetate buffer, *p*H 4.5 [chambers 1 and 2 of the "Varigrad" contained the low molarity buffer (250 ml each) and chambers 3 and 4 the high molarity buffer (250 ml each); the column was connected to chamber 2]; 4, 500 ml linear gradient between 0.75 and 1.5 M ammonium acetate buffer, *p*H 4.5. —— Concentration of split products in absorbancy units at 270 mμ.

thymidine could not be seen on the paper.[6] However, elution and quantitative estimation of the visible spots revealed an excess of nucleosides over nucleoside diphosphates. The remaining phosphodiesterase digests were chromatographed on a Dowex 1-X2 column, and the elution patterns are illustrated in Figures 10 and 11. Column chromatography revealed the presence of all four nucleoside diphosphates, the total amount of which, however, was about 25% less than the total amount of nucleosides.[7] These, and other experiments in which 2 M and 4 M fractions were used, unequivocally established that all products were terminated in 3′-monophosphate.

The endonuclease digest of experiment 3 was treated with a massive dose of phosphodiesterase (45 units). Column chromatography of the digest under the conditions described in Figures 10 and 11 resulted in the recovery of nucleosides and mononucleotides in the ratio 1:68, but neither diphosphates nor any undigested residue could be recovered.[8] The technique described in the legends to Figures 10 and 11 allows the quantitative determination of nucleosides in the presence of a 400-fold excess of mononucleotides.

Table III records the relative frequencies of nucleosides and nucleoside diphosphates in the terminal positions of oligonucleotides. It will be noted that the chains begin and terminate predominantly in deoxyguanosine. The predominance of guanosine is most pronounced during the early stages of the reaction. Therefore, the most sensitive linkage is de-Gp-Gp. As the reaction progresses, there is an increase in the relative amounts of other linkages cleaved. The enzyme, therefore, shows only a limited preference toward adjacent bases.

Discussion

The enzyme has been purified by following its ability to hydrolyze DNA. Later it was found that the ability to hydrolyze RNA was of a similar order of magnitude. Although this is not definite proof that the same enzyme is responsible for both activities, the alternative that the second activity is caused by an impurity is rather unlikely for three reasons. The activity appears too high to represent contamination by a few per cent, while a 50:50 mixture is unlikely after so many steps. Second, the extent of purification (determined by the increase in absorbancy at 260 mμ) of RNase activity from step 3 to step 7 was approximately 90-

[6] The discrepancy between the results obtained on columns (all four nucleoside diphosphates) and on paper (only de-pAp and de-pGp) cannot be explained satisfactorily. One possibility is that de-pCp and de-pTp were present on the paper but were overlooked because, under the mineral light lamp with a maximum light intensity at 254 mμ, pyrimidines do not show as well as purines.

[7] The explanation for the discrepancy between the amounts of nucleosides and nucleoside diphosphates recovered rests on the quality and quantity of phosphodiesterase used. In order to digest chains bearing 3′-monophosphate groups large quantities (several units) of phosphodiesterase are required. In an effort to purify phosphodiesterase from the last traces of contaminating 5′-nucleotidase, a preparation has been obtained which in high concentrations is capable of hydrolyzing 3′,5′-mononucleoside diphosphates (see footnote 8).

[8] In this laboratory, latest experiments by E. Sulkowski (unpublished) have shown that de-pCp is hydrolyzed by a massive dose of highly purified phosphodiesterase into de-pC and inorganic phosphate.

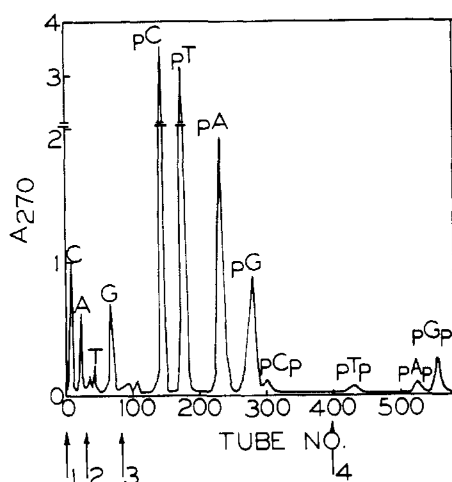

FIG. 11.—Elution pattern of a venom phosphodiesterase digest of the "3 M fraction" in Figure 8. Chromatography on a Dowex 1-X2 column, 20 × 1 cm. Flow rate 3 ml per tube, 6 tubes per hour at room temperature. The arrows indicate the change of the eluting buffers. The numbers have the following connotations: 1, H_2O; 2, 0.1 M ammonium acetate buffer, *p*H 6.2; 3, 1000 ml gradient between 0.1 and 1.0 M ammonium acetate buffer, *p*H 4.5 [chambers 1 and 2 of the "Varigrad" contained the low molarity buffer (250 ml each) and chambers 3 and 4 the high molarity buffer (250 ml each); the column was connected to chamber 2]; 4, 500 ml gradient between 0.75 and 1.5 M ammonium acetate buffer, *p*H 4.5. ——Concentration of split products in absorbancy units at 270 mμ.

fold, and that of DNase activity 300-fold (Table I). This is considered a fair agreement, since such factors as contaminating phosphodiesterase in the early stages of purification and differences in substrate concentration are expected to interfere with the accuracy of the determinations. Third, the deterioration of both activities during storage is parallel.

The enzyme is an endonuclease. This is supported by the fact that the viscosity of the reaction mixture disappears very early during the reaction. It is also supported by the patterns reproduced in Figures 7 and 8, which are indicative of the size of products. Thus in experiment 1 (Fig. 7 and Table II) only 4% of the total digest appears below the 2 M fraction. This 4% represents mono- and dinucleotides present in the exhaustive digest. The great majority, 72% of the total, is recovered in the 3 M fraction and corresponds to fragments varying in size probably from 3 to 7 nucleotide units. From the pattern it may be concluded that no more than 20% of the total number of internucleotide bonds have been broken during this exhaustive digestion, and that the predominant size of the products is in the range of the 3 M fraction.

A different pattern is seen in Figure 8 (see also Table II), representing digestion with a moderate amount of enzyme. Neither mononucleotides nor dinucleotides are present. The 3 M fraction is quantitatively smaller than that observed in Figure 7. Higher oligonucleotides constitute the majority of the products formed.

The pattern of Figure 7 is shifted to the left relative to the pattern shown in Figure 8 (see also Table II). This suggests an analogy to the behavior of pancreatic DNase I (Vanecko and Laskowski, 1961). Oligonucleotides formed during the earlier phase of the reaction may serve as substrates in the later phase, but a large excess of enzyme is required. With a tenfold excess of venom endonuclease only 4% of fragments shorter than 2 M fraction were formed. However, with the same tenfold excess of enzyme the 3 M fraction increased pronouncedly. It appears, therefore, that degradation of oligonucleotides proceeds fairly easily until some critical length is reached. From there on the formation of shorter fragments proceeds very reluctantly.

The evidence presented indicates that the purified enzyme from venom should be classified tentatively (Laskowski, 1961) as follows: (1) it is nonspecific toward the sugar moiety, (2) it is an endonuclease, (3) it forms 3′-monoesters, (4) it preferentially splits the de-Gp-Gp bond, at least during the early stages of reaction. No definite statement can be made concerning the recently suggested (Sung and Laskowski, 1962) additional criterion, the ability of the enzyme to hydrolyze dinucleotides. A new interesting property of this enzyme emerges, namely, a tendency to form products of comparatively uniform length (predominance of products eluted with 3 M acetate buffer). An attempt has been made to investigate the preferential length among the products with use of uniform substrates[9]: poly A and poly U. However, the substrates were comparatively resistant to the action of the enzyme and the results were inconclusive.

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[9] We are greatly indebted to Prof. S. Ochoa for this gift.

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The Action of Trypsin on Ribonuclease-*S**

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S-Protein and *S*-peptide, either separately or combined in the active enzyme RNase-*S*, can be digested by trypsin, whereas the native enzyme, RNase-*A*, is not attacked. In *S*-peptide the bond between residues 10 and 11 is hydrolyzed much more rapidly than the one between numbers 7 and 8. Cleavage of the first bond results in a loss of all demonstrable interaction with *S*-protein. Residue 11 appears to be a glutamyl (or glutaminyl) group, in agreement with the results of White and Anfinsen (1959). The tryptic attack on *S*-protein produces at least three chromatographically separable intermediates which all bind *S*-peptide and produce complexes with varying catalytic activity but in all cases less than that observed with *S*-protein itself. These intermediates appear to contain most, if not all, of the residues present in *S*-protein, but additional amino-terminal groups are found. The maximum number of bonds hydrolyzed in *S*-peptide is 2, in *S*-protein 7 to 8, but in RNase-*S* only 6 to 7. From this discrepancy and the kinetics of the activity loss, it is concluded that trypsin can attack RNase-*S* without dissociation of the parts, but that the course of proteolysis and the final products are not the same as in the sum of the digests of the separated components. *S*-Protein polymerizes in the neutral *p*H range but appears to be a monomer at *p*H 2, with a sharp transition between *p*H 2 and 3. The polymers are dissociated easily by *S*-peptide. The complex, RNase-*S*, does not show any tendency to polymerize. No evidence for polymerization of the tryptic digestion intermediates was found. In the very early stages of digestion of *S*-protein and RNase-*S* by trypsin, an increase in the RNase activity was observed. This was not seen with *S*-peptide or RNase-*A*. No correlation was established with extent of hydrolysis or amount of trypsin. Chromatograms of partial digests have not shown any material with specific activity higher than the starting material and the activity recoveries were low. No explanation has been found for this activation effect.

Proteolytic enzymes vary widely in their ability to attack "native" protein substrates, with the plant enzymes in general being much less fastidious than those of animal origin. In almost all cases the rate of proteolysis is increased by prior denaturation of the substrate. In earlier studies such denaturation was usually effected by exposure of the protein to high temperatures or extremes of *p*H, resulting in gross changes in physical properties (Green and Neurath, 1954). More recently it has been noted in many studies that altered susceptibility of enzymes used as substrates occurs in the presence or absence of their activators, coenzymes, or competitive inhibitors (see, for example, Fischer and Stein, 1960). The extent of the structural change in a substrate protein required to effect digestion by a proteolytic enzyme may be very subtle indeed. Such a case is reported in this paper.

Ribonuclease-*S* is produced from the native bovine pancreatic enzyme by cleavage of the peptide bond between residues 20 and 21 (Richards and Vitayathil, 1959). In dilute aqueous buffer solutions in the *p*H range 5 to 9 this modified enzyme is qualitatively and quantitatively indistinguishable from the native enzyme: in its catalytic activity toward a variety of substrates; in its ability to precipitate antisera against the latter (Singer and Richards, 1959); and in its sedimenta-

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