STUDIES OF THE PHOSPHODIESTERASE FROM RATTLESNAKE VENOM*

by

M. PRIVAT DE GARILHE** AND M. LASKOWSKI

Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis. (U.S.A.)

The increasing interest in phosphodiesterase stems from the usefulness of this enzyme in the study of nucleic acids or their fragments. During the past few years, HURST AND BUTLER[2], and SINSHEIMER AND KOERNER[3], described methods leading to partially purified preparations of phosphodiesterase from snake venom. In both procedures the emphasis was made on obtaining a preparation of phosphodiesterase free from a significant contamination by 5'-nucleotidase.

In our recent study[4] of enzymic degradation of deoxyribonucleic acid (DNA) by two different deoxyribonucleodepolymerases (DNases) numerous fragments of different molecular size and composition were obtained. In order to study further the composition of these fragments it became desirable to obtain a highly purified preparation of phosphodiesterase.

The present paper describes the method of chromatographic purification of phosphodiesterase from rattlesnake venom, and some applications of this enzyme to the degradation studies of fragments obtained from the digest of DNA by pancreatic deoxyribonuclease (DNase I).

METHODS

1. Determination of phosphodiesterase activity was routinely performed on Ca [bis (*p*-nitrophenyl) phosphate]$_2$, prepared from tris (*p*-nitrophenyl) phosphate according to the method of YOSHIDA[5]. The tris (*p*-nitrophenyl) phosphate was prepared according to RAPP[6]. The details of the method were as follows:

Step 1. 110 g (0.7 *M*) of phosphorus oxychloride was cooled in an ice-bath with stirring. 200 g (2.1 *M*) of phenol was added in several portions in order not to allow the temperature to rise above 10°. After the addition was completed, the mixture was heated over a Bunsen burner until no HCl could be detected in the vapor (3–4 h). The mixture was cooled, and poured into 1 liter of 2 *M* NaOH with stirring. A heavy precipitate of triphenyl phosphate formed. It was left over-night in a cold room to crystallize. The crystalline precipitate was collected on a Büchner funnel and was washed with water, until neutral, m.p. 45–46°.

Step 2. 100 g of triphenylphosphate were dissolved with stirring in 500 g of fuming nitric acid (sp.gr. 1.5–1.6), previously precooled in an ice-bath. The reaction was allowed to proceed for 1 hour. Tris (*p*-nitrophenyl) phosphate was precipitated with 1 liter of distilled water, and was filtered with suction. The crude material was recrystallized from acetone-water (1:1), m.p. 130°.

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** Fulbright Scholar, on leave of absence from Laboratoire de Chimie Biologique de la Faculté des Sciences, Paris, France.

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Step 3. 10 g of tris (*p*-nitrophenyl) phosphate were suspended in 100 ml of absolute alcohol and the suspension was warmed to boiling. 20 ml of 10% NaOH were added, which resulted in a clarification of the suspension. The mixture was filtered and diluted with 500 ml of water. After cooling the mixture was extracted 3 times with ether. The aqueous layer was acidified with 50% HCl to pH 4.0, and was concentrated *in vacuo* to half volume. A saturated solution of $CaCl_2$ was added until no more precipitate of Ca [bis (*p*-nitrophenyl) phosphate]$_2$ formed. The mixture was allowed to stand overnight and was filtered with suction. The precipitate was dried in a vacuum desiccator and was used as substrate.

The principle of the assay was that of YOSHIDA[5]. The liberation of *p*-nitrophenol was measured directly in a Beckman DU spectrophotometer with a temperature controlled chamber at 37°, at 440 mμ. Into a Beckman Corex cell 1 cm wide were pipetted: 1 ml of 0.3 *M* glycine-NaOH buffer, pH 9.0, 1.2 ml of 0.001 *M* solution of Ca [bis (*p*-nitrophenyl) phosphate]$_2$, and 0.3 ml of 0.3 *M* $MgSO_4$. A solution of enzyme containing 0.01 to 0.05 units (see below) per ml was warmed to 37°. After 5 minutes of equilibration, 0.5 ml of the enzyme solution was pipetted into the Beckman cell. Readings were taken at 3-minute intervals for a period of 15 to 30 minutes. Under these conditions a zero order reaction persisted for at least 30 minutes (Fig. 1). A direct proportionality between the activity and the amount of enzyme (within the recommended range) was observed (Fig. 2).

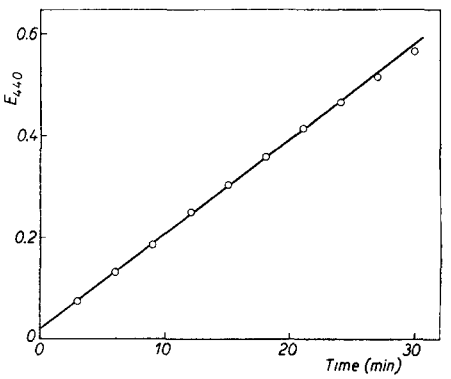

Fig. 1. The effect of time on the liberation of nitrophenol by the crude venom. Readings were taken every 3 minutes, the conditions of assay, as described in METHODS. The enzyme used was a solution of crude venom, having an optical density at 280 mμ of 1.5.

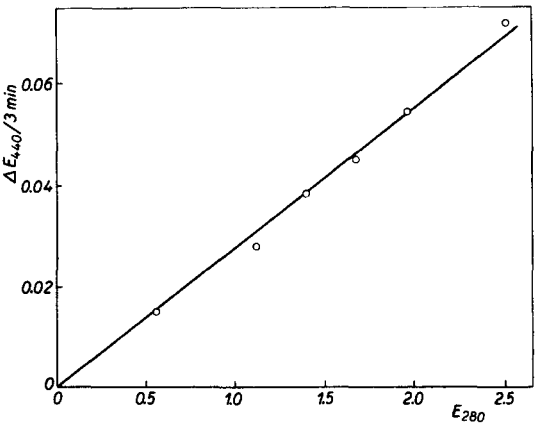

Fig. 2. The effect of enzyme concentration on activity. The activity was expressed as ΔE_{440}/3 minutes. The enzyme was a solution of crude venom. The concentrations of the added enzyme were expressed as 280 mμ absorbing material.

The activity of phosphodiesterase was expressed as the increase in optical density at 440 mμ per 3 minutes. A unit of phosphoesterase was defined as that amount of enzyme, which under the described conditions would result in $\Delta E_{440}/3$ min = 1.0. The protein concentration of the enzyme solution was expressed in terms of 280 mμ absorbing material. The potency of the enzyme preparation at any stage of purification was expressed as $\Delta E_{440}/3$ min/E_{280}.

2. Determination of 5′ nucleotidase was carried out on adenosine-5′-phosphate (AMP) purchased from Pabst Laboratories, by a slightly modified method of SINSHEIMER AND KOERNER[3]. 0.2 ml of enzyme solution, 0.1 ml of 0.3 *M* $MgSO_4$, and 0.4 ml of 0.3 *M* glycine-NaOH buffer, pH 9.0 were pipetted into a test tube, and placed in a 37° water bath. After 5 minutes of equilibration, 0.3 ml of substrate solution (157 mg per 50 ml) were added, and the mixture was incubated for 15 minutes. The tubes were then removed from the water bath, and immediately treated with 10 ml of molybdate reagent, and 1 ml of reducing reagent, according to the method of FISK AND SUBBAROW[7], for determination of inorganic phosphorus.

The deoxyribonucleic acid (DNA) was prepared according to the method of KAY, SIMMONS AND DOUNCE[8]. The determinations of deoxyribonuclease were carried out essentially by the viscosimetric procedure previously described[9], using microviscosimeters.

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The oligonucleotide fractions were prepared according to the previously described procedure[1] from DNA after digesting it with crystalline pancreatic deoxyribonuclease, purchased from the Worthington Biochemical Corporation.

Venom of the Eastern Diamondback Rattlesnake (*Crotalus adamanteus*) was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, as a lyophilized powder. It was chromatographed on Amberlite IRC-50, XE-64, mesh 200–400, and on carboxymethyl cellulose. Amberlite was prepared as described by HIRS, MOORE AND STEIN[10], with the exception that 0.2 *M* sodium acetate buffer, pH 6.2, was used instead of phosphate.

Carboxymethyl cellulose (CM-cellulose) was a gift from Dr. E. A. PETERSON, to whom we express our gratitude. Prior to use the CM-cellulose was washed for 24 hours with 0.005 *M* sodium acetate buffer, pH 5.5.

RESULTS

The purification of phosphodiesterase was achieved by 3 successive chromatographies. *Step 1* was performed on Amberlite IRC-50, XE-64. The size of the column was 30 × 2 cm. The rate of flow was about 3 ml per hour. 0.8 g of crude venom was dissolved in 5 ml of 0.2 *M* acetate, pH 6.2, and was placed on the column, which was then washed with the same buffer.

The column was purposely overloaded in such a manner that 30 to 50% of phosphodiesterase came through, whereas the remainder of the phosphodiesterase plus all of the 5′-nucleotidase were retained. As shown in Fig. 3 about 100 ml of 0.2 *M* buffer were usually required to obtain the first peak, consisting of the non-retained material. The adsorbed material was then eluted with 1 *M* acetate, pH 6.5.

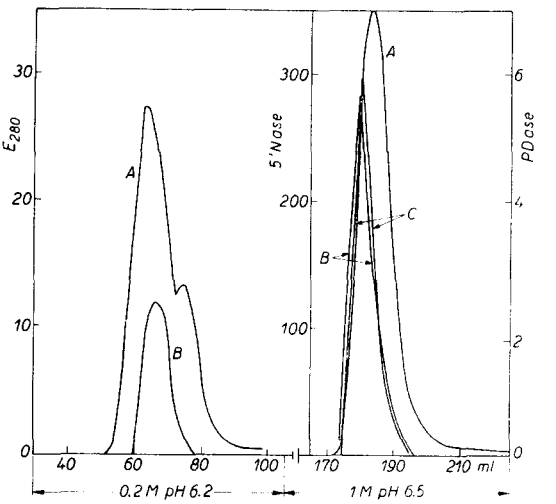

Fig. 3. *Step 1*. Resolution pattern of crude venom (0.8 g). Column 2 × 30 cm, Amberlite IRC-50, XE-64, 200–400 mesh. Eluent sodium acetate buffer. Volumes, molarities, and pH values as indicated. A – protein concentration, B – activity of phosphodiesterase (PDase), C – activity of 5′-nucleotidase.

In spite of a low yield and small improvement in potency, this step was useful, since it led to a preparation of phosphodiesterase virtually devoid of 5′-nucleotidase. So far, no more efficient way of separating these two enzymes has been found.

Step 2. Only the first peak of the previous chromatography was used for further purification. It was lyophilized, dissolved in a minimum amount of water, and dialyzed against 0.2 *M* acetate buffer, pH 6.2. The total volume, after dialysis was between 10 and 20 ml. The solution was placed on a column of the same size, containing the same quantity of the same resin, as the column used in *Step 1*. This time phosphodiesterase was retained, whereas most of the inactive material came through in approximately 100 ml of 0.2 *M* acetate, pH 6.2, as shown in Fig. 4. Phosphodiesterase having a potency about 30 fold higher than the crude venom was then eluted with 1.0 *M* acetate, pH 6.5.

Step 3. The active fraction from the second step was lyophilized, dissolved in

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a minimum amount of water and dialyzed against 0.005 *M* acetate, pH 5.5, containing 0.001 *M* $MgSO_4$. Unfortunately, heavy losses were encountered during this procedure. Addition of $MgSO_4$, although helpful, did not prevent the loss of activity. The dialyzed extract, about 5 ml, was placed on a column of CM-cellulose 10 × 0.8 cm and equilibrated with 0.005 *M* acetate, pH 5.5, as described in METHODS. The column was then successively perfused with acetate buffers of increasing molarity and pH values. The results are illustrated in Fig. 5. The curve representing activity almost parallels the curve representing the total 280 mμ absorbing material.

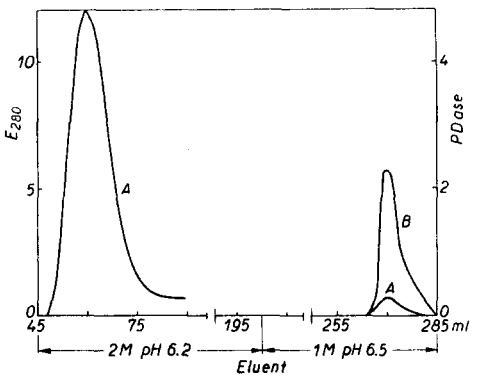

Fig. 4. *Step 2*. Chromatographic pattern of the first peak of *Step 1*. Conditions as in Fig. 3. A – protein concentration, B – activity of phosphodiesterase.

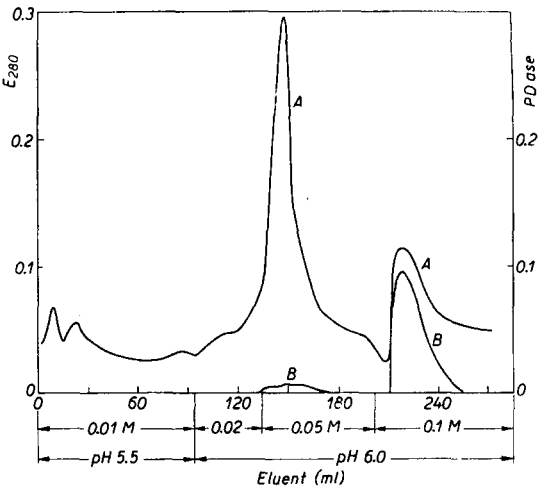

Fig. 5. *Step 3*. Chromatographic pattern of the active fraction of *Step 2*. Column 0.5 × 10 cm, of CM-cellulose. Eluent was sodium acetate buffer, volumes, molarities and pH values as indicated. A – protein concentration, B – phosphodiesterase activity.

The repetition of *Step 3* resulted in a further improvement of chromatographic purity, as shown in Fig. 6. Again the losses between steps were heavy. For most of the further work the phosphodiesterase from *Step 3* was used.

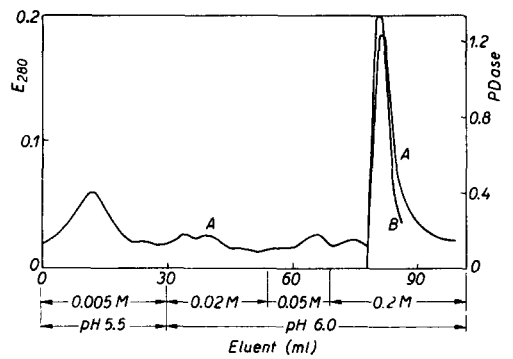

Fig. 6. *Step 3* bis. Chromatographic pattern of the active fraction obtained from *Step 3*. All conditions identical to those in Fig. 5.

The yield and the extent of purification are summarized in Table I. Whereas the recovery of phosphodiesterase activity during each chromatography was over 90%, the small total yield was caused by: (a) discarding a large part of the active material in *Step 1*; and (b) treatment of enzyme between *Steps 2* and *3*. Purified phosphodiesterase can be kept in 0.1 *M* NaCl at refrigerator temperatures for several weeks with little loss of activity. When kept frozen in a deep-freeze, the activity is preserved for a few months.

Some of the properties of the purified enzyme have been investigated. The effect

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TABLE I

YIELD AND EXTENT OF PURIFICATION OF PHOSPHODIESTERASE

Data from the exp. #31

Fraction	*Total* E_{280}	*Total Activity Units*	*Potency* U/E_{280}	*Increase in Potency*	*Yield* %
Crude Venom 1.0 g	1400	47.00	0.032	1	100
Step 1	389	23.25	0.060	1.9	49
Step 2	23.8	19.55	0.820	26.0	42
Step 3	2.44	5.70	2.35	74.0	11

of pH upon the activity toward the Ca [bis (*p*-nitrophenyl) phosphate]$_2$ is illustrated in Fig. 7. The purification of phosphodiesterase did not affect the shape of the curve when measured in the same buffer. However, it was observed that the nature of the buffer was important, since a different type of curve was obtained with borate buffer.

The purified phosphodiesterase was then tested on several substances in order to delimit its specificity and to ascertain that it was free from significant amounts of contaminating enzymes.

The presence of DNase and RNase has been reported in snake venom[11,12]. In crude venom the greatest activity of DNase was found at pH 5.0, in the absence of Mg*. When the purified phosphodiesterase was tested for DNase activity at the level of 0.06 U/ml, no activity was found under the above conditions. The purified enzyme was also tested at the level of 0.006 U/ml, in glycine buffer, pH 9.0, in presence of Mg. No drop in viscosity could be detected after 1 hour incubation**.

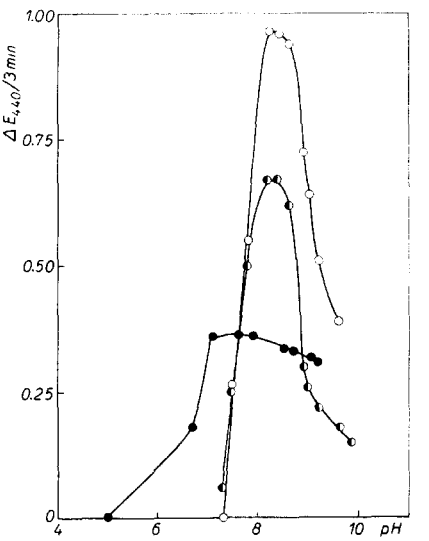

Fig. 7. Activity of phosphodiesterase at different pH values. ○ – crude venom, 0.064 units, glycine-NaOH buffer. ● – crude venom, 0.064 units, borate buffer. ◐ – purified phosphodiesterase, 0.026 units, glycine-NaOH buffer.

In addition an experiment was performed in which 10 mg of DNA were incubated with 0.5 unit of phosphodiesterase in the glycine — NaOH buffer, pH 9.0, for 16 hours at 37°. The products of the reaction were analyzed by paper chromatography and column chromatography. No movement of the initial spot was observed on paper in the *iso*propanol-water-ammonia system of MARKHAM AND SMITH[13]; and no significant amounts of oligonucleotides of any size could be eluted from the column with 3 *M* buffer by the previously described procedure[4]. It was concluded therefore that during the purification procedure DNase was eliminated, and that the purified phosphodiesterase is not capable of hydrolyzing a highly polymerized molecule of DNA.

* L. CUNNINGHAM, unpublished experiments.

** We are indebted to Dr. L. CUNNINGHAM for these determinations.

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The purified enzyme had no action on commercial RNA* as measured by a titration of liberated acidic groups. Deoxyadenosine-5'-phosphate, thymidine-5'-phosphate, and thymidine-3'-5'-diphosphate purchased from the California Foundation for Biochemical Research were tested. No formation of inorganic phosphorus was observed with either of the three substances after 24 hours incubation with purified phosphodiesterase. Also the purified enzyme was virtually free from ophioadenosinetriphosphatase, when tested on crystalline ATP, purchased from Pabst Laboratories.

The oligonucleotides obtained from the digest of DNA by DNase I, according to the method previously described[4], were then studied as substrates for purified phosphodiesterase. Since it is not yet possible to describe oligonucleotides in more exact terms, they were labeled as 0.5 *M*, 1.0 *M* and 2.0 *M* fractions, respectively. The designation refers to the molarity of ammonium formate buffer, necessary to elute the fraction from a Dowex 1-2x column[4]. Probably, each of these fractions is a mixture of numerous oligonucleotides of different composition and different molecular weight. However, by analogy with the already identified lower oligonucleotides it has been assumed that the larger the oligonucleotide, the higher the molarity of the solvent necessary for its elution. Therefore the 2.0 *M* fraction represents the largest oligonucleotides resistant to the action of DNase I, and (as in the case of peptides) probably is less heterogeneous than the lower fractions.

As an example of this type of experiment the results of the digestion of "0.5 *M* fraction" are illustrated in Figs. 8 and 9. The lyophilized "0.5 *M* fraction" containing approximately 30 mg of oligonucleotides was dissolved in 2.0 ml of 0.2 *M* glycine NaOH buffer, pH 9, and adjusted to pH 9 with 10% NaOH. 0.1 ml of 1 *M* $MgSO_4$ was added and then 0.1 Unit of purified phosphodiesterase. The mixture was incubated at 37° and the progress of the digestion was followed by paper chromatography in *iso*propanol-water-ammonia, according to MARKHAM AND SMITH[13]. The results shown in Fig. 8 indicate that the hydrolysis of oligonucleotides proceeded quite rapidly.

After three hours of incubation, the digest was transferred to the Dowex 1-2x column 6 × 0.8 cm, and was chromatographed as previously described[4]. The results are shown in Fig. 9. Neither dinucleotides, nor higher oligonucleotides were found. All four mononucleotides were present.

The mononucleotides were preceded by a small peak in the region characteristic for nucleosides. A more detailed analysis of this peak indicated that it contained considerable amounts of 260 mμ absorbing material of a different nature than any of the known derivatives of nucleic acid. A substance with a similar spectrum has been previously observed[4]. It has been postulated[4] that this substance originated from the resin itself during the collection of fractions of DNase I digest. The remainder of the material in the first peak represented a nucleoside.

It was surprising that only one nucleoside was found. The "0.5 *M* fraction" and "2.0 *M* fraction" contained only thymidine, while the "1.0 *M* fraction" contained only deoxyadenosine. The experiment with the "2.0 *M* fraction" was repeated twice, using DNase I digests. In both cases only thymidine was found, which was identified by paper electrophoresis according to MARKHAM AND SMITH[13], and by paper chromatography according to HOTCHKISS[14]. The relative quantities of thymidine in two different "2.0 *M* fractions", however, differed considerably.

* We are indebted to Dr. P. M. ROLL for this experiment.

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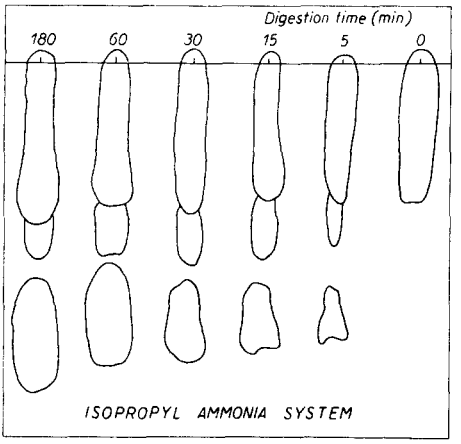

Fig. 8. Progress of the digestion of oligonucleotides. "0.5 *M* fraction" was subjected to the action of purified phosphodiesterase (see text). The reaction was followed by withdrawing samples at indicated time intervals. These samples were then chromatographed on paper according to MARKHAM AND SMITH[13].

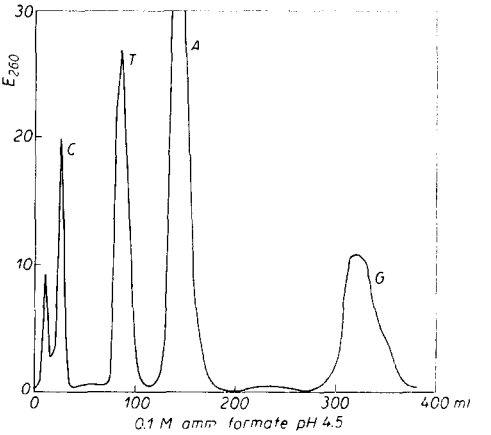

Fig. 9. Elution pattern of oligonucleotides ("0.5 *M* Fraction") digested with purified phosphodiesterase (see text). Column Dowex 1–2x, 0.8 × 6 cm, equilibrated with 0.1 *M* ammonium formate, pH 4.5. Unlabeled peak thymidine (see text), C = deoxycytidylic acid, T = thymidylic acid, A = deoxyadenylic acid, G = deoxyguanylic acid.

In view of the negative results with standard nucleotides as substrates for the phosphodiesterase preparation, and in view of the fact that only one nucleoside was recovered from each fraction, it seems highly improbable that the nucleoside resulted from the action of contaminating 5′-nucleotidase. This finding, therefore, appears significant, although not readily explainable at present.

The results of the experiments performed on degradation of 3 different oligonucleotide fractions are summarized in Table II. As would be expected from the findings of SINSHEIMER[15] and our own[4], indicating that pyrimidine nucleotides predominate in the low fractions, the higher fractions contained a predominance of purine nucleotides.

TABLE II

PRODUCTS OF DIGESTION OF DIFFERENT OLIGONUCLEOTIDES FRACTIONS WITH PURIFIED PHOSPHODIESTERASE

All figures are in per cent of the micromoles of each constituent of total number of micromoles recovered.

Oligonucleotides	*Products Obtained*					
	Nucleotides				*Nucleosides*	
	A	*C*	*G*	*T*	*A*	*T*
"0.5 *M* Fraction"	35.5	12.4	19.4	28.0	0.0	4.35
"1.0 *M* Fraction"	30.0	14.2	21.0	32.2	2.37	0.0
"2.0 *M* Fraction"	36.6	0.0	22.8	24.5	0.0	16.6
"2.0 *M* Fraction"	36.0	0.0	27.0	33.0	0.0	3.6

A very interesting finding was a lack of cytidylic acid in the "2.0 *M* fraction". This experiment was repeated on 2 different "2.0 *M* fractions", Table II. In addition an aliquot of the "2.0 *M* fraction" was hydrolyzed with concentrated formic acid

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in sealed tubes at 175°, according to VISCHER AND CHARGAFF[16], and the hydrolysate was chromatographed on paper according to HOTCHKISS[14]. No cytosine was found.

DISCUSSION

Two different views concerning the specificity of phosphodiesterase from venom may be found in recent literature. BRAWERMAN AND CHARGAFF[17] assayed their barley phosphodiesterase on oligonucleotide fractions, because they considered that no definite proof had been supplied that the same enzyme is responsible for the hydrolysis of oligonucleotides and synthetic diesters of phosphoric acid. On the other hand SCHMIDT[18] referred to venom phosphodiesterase as an "unspecific phosphodiesterase".

The results presented in this paper, showing that a few micrograms of the purified enzyme are capable of hydrolyzing several milligrams of either substrate with high velocity, strongly suggest that the same phosphodiesterase is responsible for both reactions. The recovery of each component base as a mononucleotide demonstrates that qualitatively the enzyme possesses no specific base requirement. Whether it exhibits a quantitative preference cannot be answered at present. A study now in progress may shed some light on the order of cleavage of the internucleotide linkages by phosphodiesterase. On the other hand some limits for "unspecificity" have also been observed. The purified phosphodiesterase hydrolyzed neither a highly polymerized DNA, nor a commercial RNA. The cause for the resistance of these substances appears highly intriguing.

SUMMARY

Phosphodiesterase from rattlesnake venom has been purified by 3 successive chromatographies, two on Amberlite IRC-50, XE-64, and one on CM-cellulose. The total purification achieved was over 70 fold, and the total yield was about 10%. The final product approached chromatographic purity.

The purified phosphodiesterase was free from 5'-nucleotidase, ophio-adenosinetriphosphatase, DNase and RNase. It rapidly hydrolyzed the oligonucleotide fractions, obtained from the digest of DNA by DNase I, producing almost exclusively mononucleotides. A small amount of a nucleoside, however, has been detected in some fractions. The origin of this nucleoside can not yet be explained.

RÉSUMÉ

La phosphodiestérase du venin de Crotale a été purifiée par trois chromatographies successives; les deux premières sur Amberlite IRC-50 XE-64 et la troisième sur carboxyméthylecellulose. La purification obtenue est de 70 fois et le rendement total est de 10%. Le produit final est chromatographiquement à peu près pur.

La phosphodiestérase ainsi purifiée est exempte de 5'-nucléotidase, d'ophio-Adénosinetriphosphatase, de DNase et de RNase. Elle hydrolyse rapidement les oligonucléotides obtenus après digestion de l'acide desoxyribonucléique par la DNase I en fournissant presque exclusivement des mononucléotides. Cependant, une petite quantité d'un nucléoside a été décelée dans certaines fractions; l'origine de ce nucléoside ne peut pas encore être expliquée.

ZUSAMMENFASSUNG

Die Phosphodiesterase des Klapperschlangengiftes wurde durch drei aufeinanderfolgende Chromatographien gereinigt. Bei zweien wurde Amberlit IRC-50 XE-64, und bei einer CM-Zellulose angewandt. Eine mehr als 70 fache Gesamtreinigung wurde erzielt, und die Ausbeute war ungefähr 10%. Das Endprodukt kam chromatographischer Reinheit nahe.

Die gereinigte Phosphodiesterase war frei von 5'-Nucleotidase, Ophioadenosintriphosphatase, DNase und RNase. Sie hydrolisierte rasch die oligonucleotiden Fraktionen, die durch die Verdauung von DNA mit DNase I gewonnen wurden und bildete fast ausschliesslich Mononucleotide In einigen Fraktionen wurde jedoch ein kleiner Anteil eines Nucleosids festgestellt. Der Ursprung dieses Nucleosids kann noch nicht erklärt werden.

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