FRACTIONATION OF VENOM FROM THE RINGHALS COBRA*

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SUMMARY

1. Venom from the South African ringhals cobra (*Hemachatus haemachates*) has been studied by chromatography on DEAE cellulose and by column electrophoresis, with special regard to the isolation and purification of certain enzymes.

2. In preparations of the venom obtained by commonly used procedures at least two chromatographically different phosphodiesterases have been found, one of them being partly transformed into the other on auto-incubation of the venom.

3. Chromatographic analysis has shown that the method of collecting and drying the venom at room temperature does not give a well-defined material, but that such a material can be obtained by a rapid freeze-drying method.

4. Chromatographic fractionations of 0.5 g of freeze-dried venom have been made showing a complete separation of the phosphodiesterase from acetylcholine esterase and lecithinase A. Toxicity tests have revealed the existence of two well-separated toxins.

5. By electrophoresis all the three enzymes mentioned have been separated from each other.

6. The enzymic properties of the phosphodiesterase are discussed.

INTRODUCTION

Biological studies of snake venoms have been made for several hundreds of years; at the end of the nineteenth century immunologists obtained the first antivenom sera. The protein nature of the venom was demonstrated in 1860, and forty-five years later LÜDECKE recognized the hemolytic agent to be an enzyme which degrades lecithin[1]. References to older work as well as to different properties of venoms can be found in two recent books[2,3].

Most investigators have used venoms from rattle-snakes or cobras. An electrophoretic examination of different cobra venoms has been carried out by POLSON, JOUBERT AND HAIG[4], and GRASSMAN AND HANNIG[5] have compared crude venoms from Viperides and Colubrides by the same method. No extensive study, however, has been devoted to the venom from the South African ringhals cobra, *Hemachatus haemachates* (formerly called *Sepedon haemachates*), although the publications cited contain some important information about it.

Recent chromatographic investigations carried out at this Institute showed

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that the phosphodiesterase activity in the venom of a rattle-snake, *Crotalus adamanteus*, was heterogeneous and that all the enzyme fractions isolated were active toward macromolecular deoxyribonucleic acid (DNA)[6]. It was suggested by LASKOWSKI *et al.*[7] that the diesterase should be an exonucleotidase and this hypothesis has very elegantly been verified by RAZZELL AND KHORANA[8]. The work on snake venom phosphodiesterase has been reviewed by HEPPEL AND RABINOWITZ[9].

It has been found fairly frequently that enzyme preparations from snake venoms are difficult to reproduce from one laboratory to another or even from one batch to another. This can probably be explained by differences in the proteolytic autodigestion which occurs during the collection of the venom (*cf.*[6]). The present work was undertaken in order to study the influence of autodigestion upon the heterogeneity of the phosphodiesterase and if possible to find a reproducible procedure for the purification of phosphodiesterase, lecithinase A and acetylcholine esterase.

MATERIALS AND METHODS

The buffer systems used were tris(hydroxymethyl)aminomethane–hydrochloric acid (THAM–HCl) in the chromatographic experiments, and the volatile buffers triethylammonium carbonate (TEA–CO_2) and triethylammonium acetate (TEA–HAc) in the electrophoresis[10]. Buffer concentrations are given as molarities of THAM or TEA.

The ringhals venom

A number of different preparations of venom from the South African ringhals cobra, *Hemachatus haemachates*, were investigated. For two of them, which had been stored for several years in a refrigerator, no details of the collection procedure were available; they are here called H1 and H2. The other preparations used were obtained from Dr. J. H. MASON, the South African Institute for Medical Research, Johannesburg. One of these, called M1, was prepared in the "ordinary way", that is, the venom was dried over $CaCl_2$ at room temperature (or a higher temperature). The others, designated "freeze-dried venom", were collected in the following way according to Dr. MASON: The snakes were milked over a conical flask which was surrounded by ground dry ice. As the venom ran down the glass, it froze. The flask was immediately transferred to a freeze-drying apparatus and this was run for 18 h. The dried venom was quickly (but only roughly) ground in a mortar (to minimize the uptake of water) and transferred to a dry bottle.

Solutions of the venom were always made up immediately before use. For chromatography, concentrations higher than 2 or 3 % should be avoided. When the venom was dissolved in 0.005 *M* THAM–HCl of pH 8.9, the pH had to be adjusted by the addition of 1 *M* THAM (about 100 μl/100 mg venom). A very small part of the venom did not dissolve and was removed with a glass rod when floating on top of the solution.

The different batches were stored in a desiccator over P_2O_5 in a cold room at about 4°.

Analytical methods

The estimation of protein content, of phosphodiesterase, DNAase, and lecithinase A activities, and of chloride concentration have recently been described in detail[6].

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The *acetylcholine esterase activities* (AChE) were determined with a slight variation of an indicator method (*cf.*[11]) described earlier[12]. The substrate solution had the following composition: 0.01 *M* Na barbital, 0.02 *M* KH_2PO_4, 0.3 *M* KCl, 0.0014 *M* HCl, 0.133 *M* acetylcholine bromide, and 0.005 % bromthymol blue. Of this solution 2 ml were incubated with 25 μl from the different fractions for 2 h at 37°. By the use of a standard curve the difference in optical density at 610 mμ before and after incubation was converted to mmoles of liberated acetic acid per litre of incubation mixture.

The chromatographic technique

Diethylaminoethyl (DEAE) cellulose, prepared according to PETERSON AND SOBER[13] was used as adsorbent. The nitrogen contents were 9.1 and 11.6 mg/g of vacuum-dried ion exchanger, and under identical conditions the same chromatograms were obtained from both preparations. Before packing a column, the DEAE cellulose was suspended in about 20 times its volume of water and allowed to stand for 1–2 h, after which the supernatant was decanted in order to remove the smallest particles; the suspension was then deaerated. The column was packed under a water pressure of about twice its length. Regeneration was carried out by washing the column with 1 % NaOH and then with 20–30 times the dead volume of starting buffer. Before applying the venom solution it was always checked that the effluent from the column had the correct pH.

The chromatograms have been obtained by one-step development[14]. After equilibrating the column with 0.005 *M* THAM–HCl of pH 8.9 (the starting buffer, in which also the venom was dissolved), elution was performed with 0.33 *M* THAM–HCl of the same pH. The fraction volumes were about 1/10 of the dead volume for the 19-ml columns and in the large-scale experiments 1/13–1/35 of the dead volume. Except for the first run on a column with unused ion exchanger, the reproducibility was good, and we used the same column for more than 10 experiments.

A fraction collector working on a time basis was employed, and the abscissa in the figures indicates tube number. All the experiments were carried out in a cold room, maintained at 4°.

Zone electrophoresis was performed in columns (109 × 2 cm and 92 × 2 cm) packed with ethanolysed cellulose, prepared according to FLODIN AND KUPKE[15], using the technique described by POLATH[16]. The columns had dead volumes of about 230 ml and 195 ml respectively. The apparatus was placed in a cold room and in addition cooled with a jacket with circulating ice-water.

RESULTS

Chromatography of different venom preparations

Figs. 1a, 1b and 2a show chromatograms of three different preparations of ringhals venom. All the experiments were performed in the same way with 30 mg of venom on a column (24 × 1 cm) with 19 ml of DEAE cellulose. In these and the following figures, open circles represent extinction at 280 mμ, crossed circles show phosphodiesterase activity, crossed squares stand for DNAase activity and the triangles denote the chloride concentration.

The figures show that the chromatographic behaviour of the phosphodiesterase

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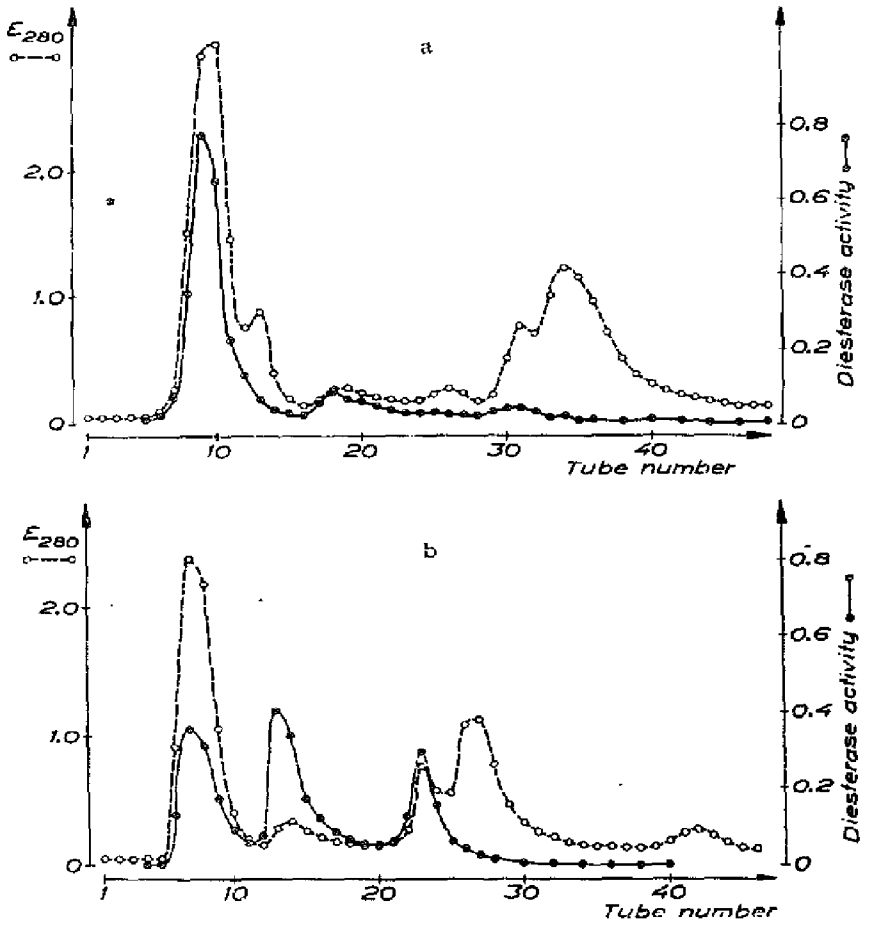

Fig. 1. Chromatograms of two different preparations of ringhals venom, M1 (Fig. 1a) and H2 (Fig. 1b). 30 mg of venom on a 19-ml column of DEAE cellulose. Starting buffer 0.005 *M* THAM–HCl, pH 8.9. Developing buffer 0.33 *M* THAM–HCl of the same pH. Fraction volumes about 0.8 ml in the upper chromatogram, about 1.0 ml in the lower chromatogram. Phosphodiesterase activity (⊙ – – –⊙) is given in mmoles of substrate split/l incubation mixture.

was quite different in these three preparations. In one of the samples (M1, Fig. 1a) most of the diesterase was recovered in the first peak which appeared directly after the dead volume; in the second one (H2, Fig. 1b) three different zones of diesterase were obtained, while for the third one (H1, Fig. 2a) only one zone was obtained, which, however, was well separated from the first zone of ultraviolet-absorbing material. The recovery of the diesterase activity was about 80% in all these experiments.

The hypothesis was made that the differences might be due to an uncontrolled autodigestion occurring during the collection of the venom. For the purpose of testing this possibility, autoincubation was performed with preparation H1. 30 mg of venom was dissolved in 1.5 ml 0.005 *M* THAM–HCl of pH 8.9 and kept at 37° for 4 h. The solution was then dialysed overnight against the starting buffer (at 4°) and chromatographed in the same way as described above (Fig. 2b). When the experiment was repeated under the same conditions but with an autoincubation time of 15 h, the

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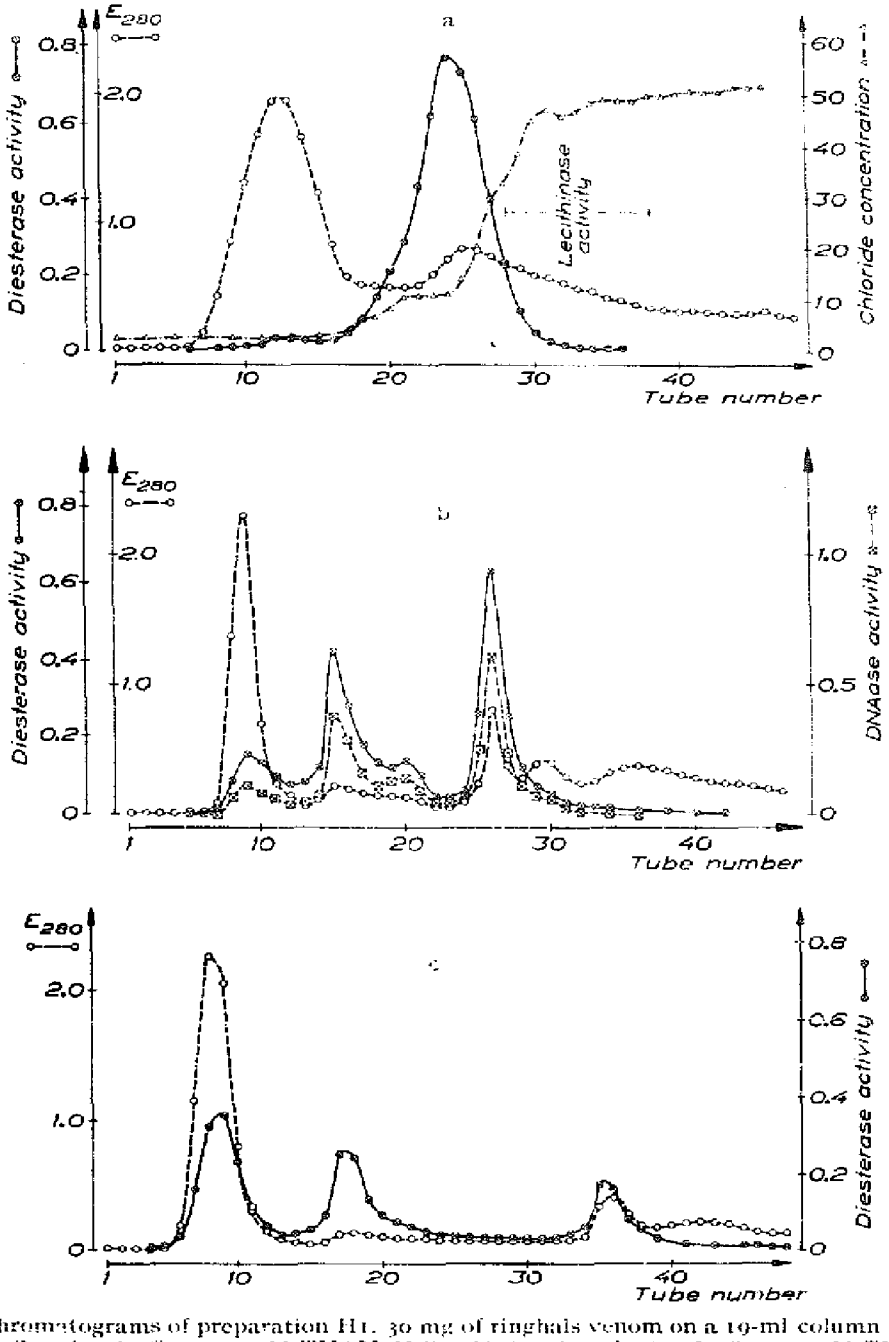

Fig. 2. Chromatograms of preparation H1. 30 mg of ringhals venom on a 19-ml column of DEAE cellulose. Starting buffer 0.005 *M* THAM-HCl, pH 8.9. Developing buffer 0.33 *M* THAM-HCl of the same pH. Phosphodiesterase activity (○——○) is given in mmoles of substrate split/l incubation mixture, DNAase activity (□ - - - □) as liberated extinction at 260 mμ, and chloride concentration (△–·—·–△) in μequiv./ml. Fig. 2a: chromatography without pretreatment of the venom. Fraction volumes 0.6–0.8 ml. Fig. 2b: chromatography after autoincubation of the venom for 4 h. Fraction volumes about 1.0 ml. Fig. 2c: chromatography after autoincubation of the venom for 15 h. Fraction volumes about 1.0 ml.

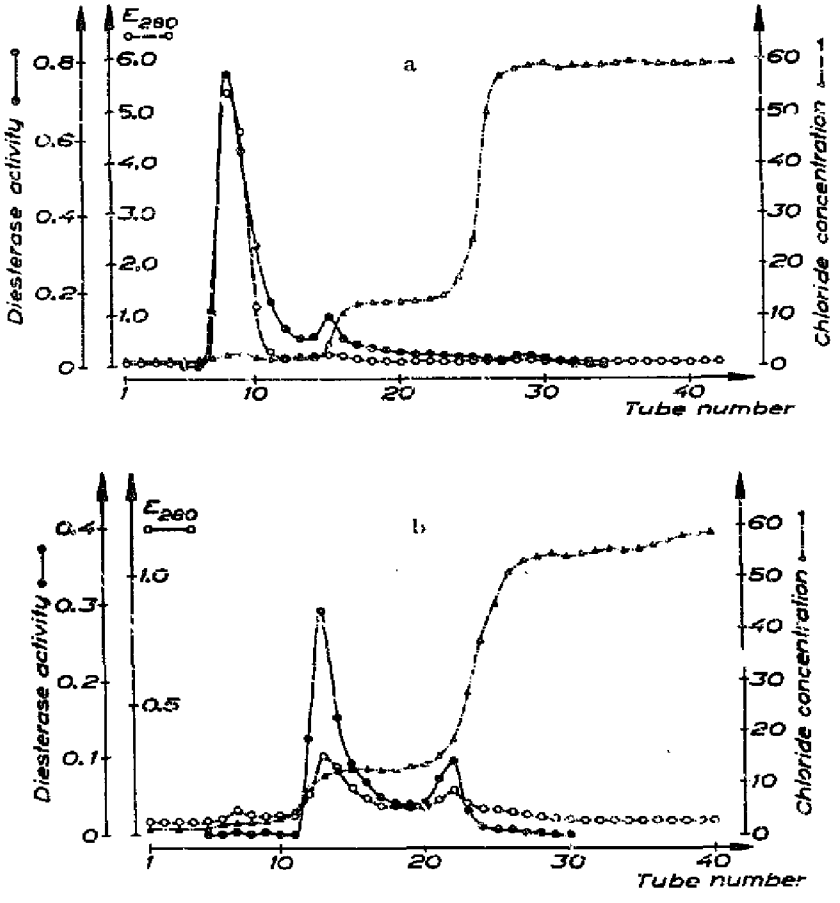

Fig. 3. Rechromatography on a 19-ml column of DEAE cellulose of the first (Fig. 3a) and the second (Fig. 3b) diesterase zones from a large-scale chromatogram of preparation H2. Starting buffer 0.005 *M* THAM–HCl, pH 8.9. Developing buffer 0.33 *M* THAM–HCl of the same pH. Fraction volumes about 1.0 ml. Phosphodiesterase activity (⊗——⊗) is given in mmoles of substrate split/l incubation mixture and chloride concentration (△—·—△) in μequiv./ml.

chromatogram shown in Fig. 2c was obtained. During the dialysis the u.v.-absorbing material decreased with 25–30% and the diesterase activity with about 25% (no diesterase activity was found outside the dialysis membrane). The recovery of the activity in the chromatogram was in both cases about 80% when corrected for the loss during dialysis.

In order to collect sufficient amounts of diesterase from the different peaks a large-scale chromatographic experiment was made with preparation H2. This gave a good yield of the first and the second diesterase zones but only a small amount of the third zone. The total recovery of diesterase activity was about 60%. The tubes from the first and the second diesterase zones were pooled, dialysed, freeze-dried and redissolved, and afterwards chromatographed under the conditions described. The results are given in Fig. 3, where the upper part shows rechromatography of the first diesterase zone, and the lower part rechromatography of the second

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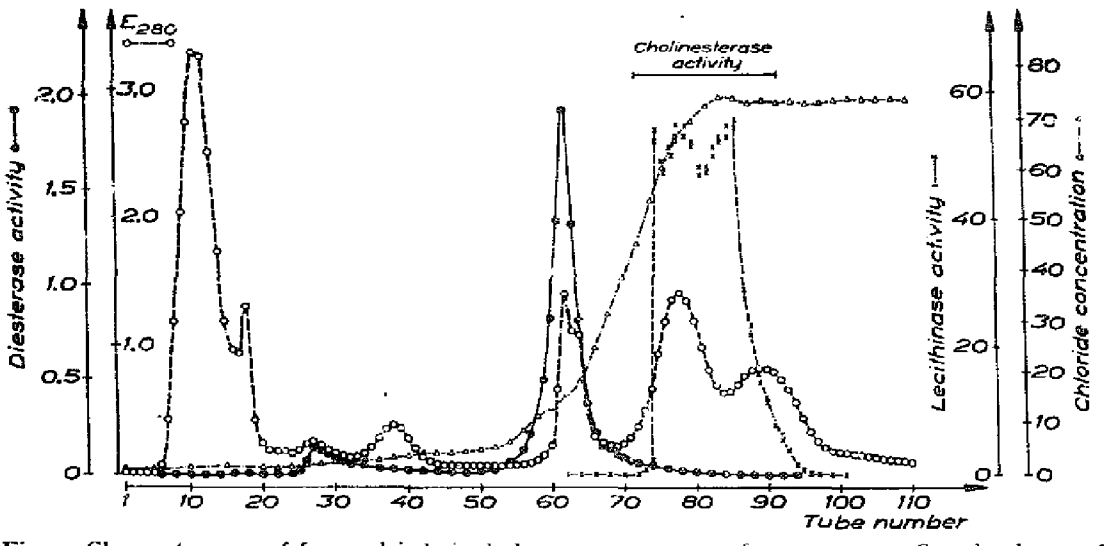

Fig. 4. Chromatogram of freeze-dried ringhals venom. 557 mg of venom on a 380-ml column of DEAE cellulose. Starting buffer 0.005 *M* THAM–HCl, pH 8.9. Developing buffer 0.33 *M* THAM–HCl of the same pH. After 185 ml had passed the column, fractions were collected. Fraction volumes 5.9–6.2 ml. Phosphodiesterase activity (○ — — ○) is given in mmoles of substrate split/l incubation mixture, lecithinase A activity (× — — —×) as liberated extinction at 540 mμ (hemoglobin) and chloride concentration (△ — — △) in μequiv./ml.

diesterase zone. In these experiments the recovery of phosphodiesterase activity was approximately 100 %.

The chromatogram shown in Fig. 4 was obtained with 557 mg of freeze-dried venom on a 380-ml column (84 × 2.4 cm), and most of the diesterase activity appeared as a well-defined zone (tubes 56–68) together with a rather high peak of u.v.-absorbing material. Different batches of freeze-dried venom gave the same results. When 19-ml columns and 180-ml columns were used, however, the protein pattern was about the same as in Fig. 4 (if the lower degree of resolution on the smaller

TABLE I

TOXICITY TESTS ON CHROMATOGRAPHIC FRACTIONS OF RINGHALS VENOM FROM THE CHROMATOGRAM SHOWN IN FIG. 4

The fractions were diluted with 0.005 *M* THAM–HCl of pH 8.9 to an u.v.-extinction of approximately 0.3 and tested by subcutaneous injection on mice. Each test group consisted of 4–10 animals

Tube number (see Fig. 4)	LD_{50} *ml/kg*	*Dilution*	*Symptoms*
11	4–5	1/11	Decreased power of balance
18	2–2.5	1/4	Spasmodic movements
27	10–12	No dilution	Clonic cramp
38	> 25	No dilution	
62	6–7	1/5	As above. In addition, pointwise appearing macroscopic pulmonary haemorrhages
78	15–20	1/5	Pulmonary haemorrhage
90	> 25	2/5	
1 *M* THAM–HCl of pH 8.9	≫ 25		

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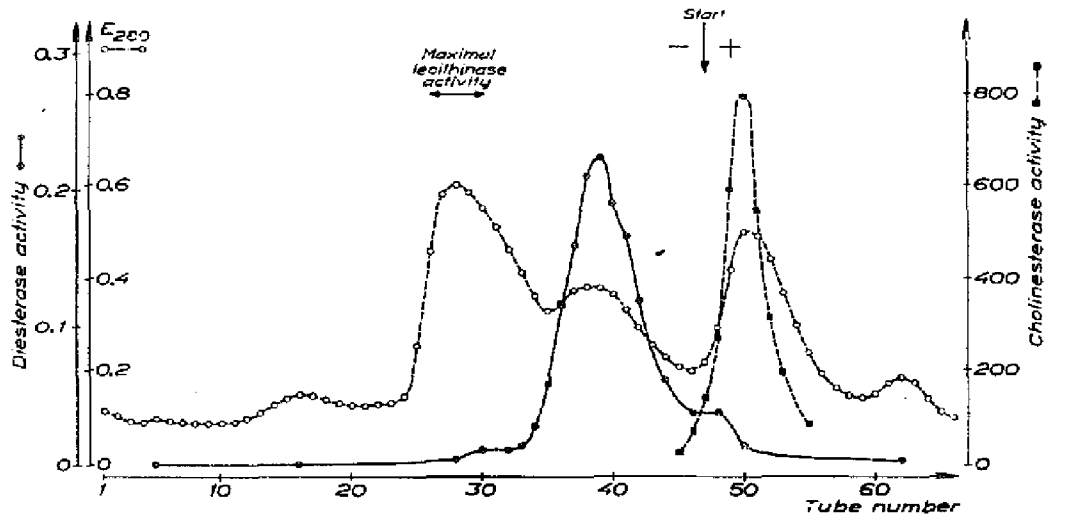

Fig. 5. Electropherogram of 100 mg of freeze-dried ringhals venom in 0.05 *M* TEA–HAc at pH 5.5. Column size 109 × 2 cm. About 840 mA-h passed through the column. Fraction volumes about 3.5 ml. Phosphodiesterase (○——○) and cholinesterase (■- - - -■) activities are given in mmoles of substrate split/l incubation mixture.

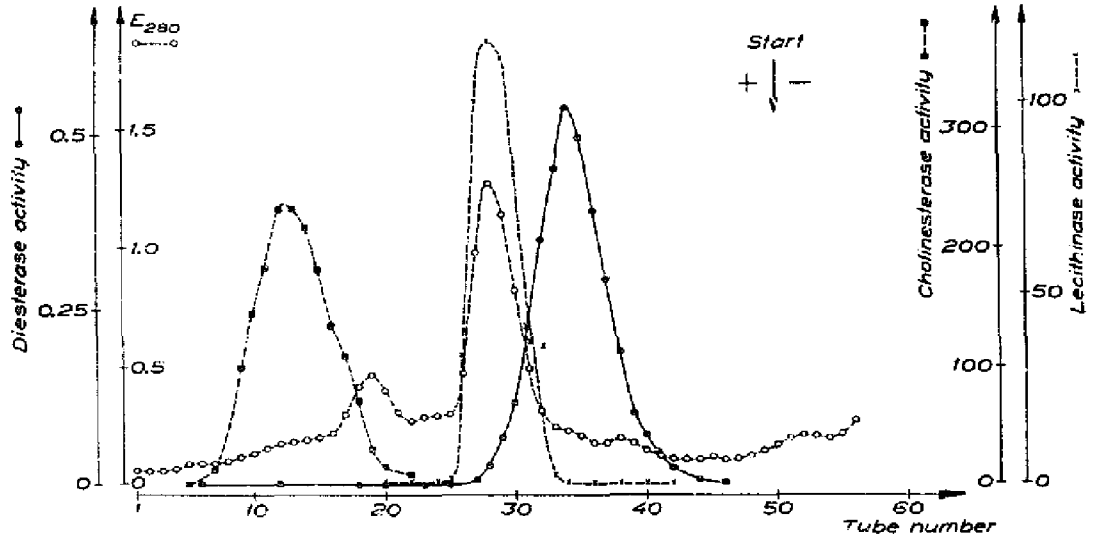

Fig. 6. Electropherogram of 100 mg of freeze-dried ringhals venom in 0.05 *M* TEA-CO_2 at pH 8.7. Column size 92 × 2 cm. About 1350 mA-h passed through the column. Fraction volumes about 3.5 ml. Phosphodiesterase (○——○) and cholinesterase (■- - - -■) activities are given in mmoles of substrate split/l incubation mixture, and lecithinase A activity (×- - - -×) as liberated extinction at 540 mμ (hemoglobin).

columns is taken into account), but 65–70% of the phosphodiesterase recovered was found in the peak corresponding to the tubes 26–28 in Fig. 4 (see DISCUSSION). The figure also shows that the cholinesterase and the lecithinase A appear mixed in the same zone but completely separated from the diesterase activity.

The results of toxicity tests (performed by Dr. R. ELIASSON, Institute of Phys-

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iology, Karolinska Institutet, Stockholm) on the chromatographic fractions in Fig. 4 are shown in Table I.

With Dr. MASON's assistance we have also been able to investigate freeze-dried venom from five different ringhals individuals and from the same individual at different times. With the exception of one case, where the snake died soon after the milking, no significant differences were found in the chromatograms between any of these venom samples.

Zone electrophoresis of freeze-dried venom

Fig. 5 shows an electropherogram of 100 mg of freeze-dried venom in 0.05 *M* TEA–HAc at pH 5.5. The cholinesterase migrated towards the anode, whereas both the phosphodiesterase and the lecithinase A moved towards the cathode, the latter at the higher rate. In addition, at least two protein peaks left the column, one in each direction. In 0.05 *M* TEA–CO_2 at pH 8.7 (Fig. 6) the cholinesterase was still more effectively separated from the other two enzymes. Here these overlapped to some extent, but most of the phosphodiesterase was entirely free from lecithinase A. Two protein components were observed to migrate into the cathode vessel. In no case was the purification of the diesterase better than in the chromatograms.

DISCUSSION

The chromatographic method

When working with anion exchangers at an alkaline pH, one cannot avoid some interaction with the carbon dioxide from the air, unless special precautions are taken. The use of sodium hydroxide in the regeneration cycle increases the difficulties, and under certain conditions this will give rise to a bicarbonate zone in the chromatogram[17]. This zone is accompanied by an increase in the pH and an anomalous step in the concentration of the developing agent (see Figs. 2a and 3a and b). Evidently such a complicated situation may result in the splitting up of a homogeneous compound into two components, one appearing immediately before and the other immediately after the bicarbonate zone. Thus the second and the third phosphodiesterase peaks in the Figs. 1b and 2b and c might represent only one single enzyme form. It should be observed, however, that in some cases this effect may contribute considerably to the purification of a certain component

A tendency to use proportionately more sodium hydroxide in the regeneration of the smaller columns is to be expected. This would lead to a more pronounced bicarbonate zone on the smaller columns than on the bigger ones, and so the differences found between the chromatograms from columns of different sizes could be explained. In Fig. 4 it can be seen that there is only a small "bump" in the chloride concentration of the diesterase zone, while on the 19-ml columns (using the same preparation of venom) the characteristic anomalous chloride step is flanked by two distinct diesterase peaks. One fact, however, which does not fit into the scheme sketched here, is that on the 180-ml columns the separation of two diesterase peaks is even a little better than on the 19-ml columns. Unfortunately, chloride concentration data from these experiments are lacking.

The ringhals venom

A comparison of Figs. 1a, 1b and 2a shows that the composition of venom

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collected and dried at room temperature varies considerably from one batch to another and that consequently this product is not a suitable starting material for a reliable purification procedure. The salt contents of the corresponding preparations were determined by ashing (2.26 %, 2.32 % and 2.28 % for preparations M1, H2 and H1, respectively), and as no significant differences were found this cannot explain the variations between the chromatograms The assumption that the heterogeneity of the phosphodiesterase depends on autodigestion of the venom is strongly supported by the results given in Figs. 2b and c. There it can be seen that the single phosphodiesterase zone in Fig. 2a on autoincubation is split up into three peaks (diesterase *a*, *b* and *c*) and that the first one of them, which was only slightly —if at all—adsorbed on the column, seems to represent the last stage of the transformation. There is an evident similarity between these chromatograms and comparable results from a study of a commercially available rattle-snake venom[6].

Chromatography of dialysed but not incubated venom showed that the diesterase *a* peak was not a product of the dialysis. As regards the other two activity peaks, it has already been pointed out that they might possibly be identical, but there are several facts which are contrary to this supposition. Rechromatography of diesterase *b* from large-scale chromatograms of both preparation H2 (Fig. 3b) and the freeze-dried venom gives predominantly diesterase *b*, while rechromatography of diesterase *c* gives predominantly or exclusively diesterase *c*. Moreover, autoincubation of diesterase *b* seems to transform it into diesterase *c*, whereas incubation of the latter component with or without the addition of crude venom does not change its behaviour. As, however, the chloride concentration was not determined in most of these experiments, we cannot form a definite opinion of their significance. All that can be said at present is that the rechromatography experiments shown in Fig. 3 must be regarded as conclusive evidence for the existence of at least two chromatographically different phosphodiesterases in preparation H2.

On the other hand, chromatograms of different batches of freeze-dried venom showed a very good reproducibility, and no diesterase *a* was obtained (see Fig. 4), which simplifies the purification procedure. Autoincubation of the freeze-dried venom gave a diesterase *a* peak in the chromatogram, but as the incubation time required was much longer than for preparation H1, the original diesterase of the freeze-dried venom seems to be more stable than the active components of the "ordinary" preparations. Furthermore, its specific activity in the crude venom (defined as the ratio of enzyme activity to extinction at 280 mμ) is about 50 % higher than the value for the venom collected and dried at room temperature.

It is thus obvious that for the fractionation and purification of enzymes, freeze-dried snake venom offers considerable advantages when compared with venom collected in the "ordinary" way.

When the venom was exposed to a prolonged dialysis, about 30 % of the material, including a large part of the lecithinase A, passed through the membrane. In the ultracentrifuge the remainder was also shown to be of a rather low molecular weight. This is a disadvantage for the purification of lecithinase A, since the chromatographic fractions cannot be desalted by dialysis. Another difficulty met with is the loss of activity of the phosphodiesterase during dialysis and freeze-drying, which is not so striking for the crude venom but may amount to more than 80 % of the activity for the purified fractions. It would consequently be of great value if favourable

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conditions could be found for fractionation of the venom in volatile buffers. Some preliminary attempts with chromatography in TEA–CO_2 have not been successful, but this work will be continued.

Column electrophoresis with volatile buffers provides a good separation of acetylcholine esterase from phosphodiesterase and lecithinase A, as demonstrated in Figs. 5 and 6. However, the cholinesterase activity is susceptible to a considerable inactivation during experiments in TEA–CO_2 of pH 8.7 (Fig. 6), while the same is true for phosphodiesterase in TEA–HAc of pH 5.5 (Fig. 5). The recovery of cholinesterase activity at pH 8.7 was only 40 % of that obtained at pH 5.5, while for the phosphodiesterase the corresponding value was 230 %. A similar comparison of the specific activities at pH 8.7 and pH 5.5 gives the figures 70 % for the cholinesterase and 420 % for the phosphodiesterase. As no suitable volatile buffers were found in the range between pH 6 and pH 8, the electrophoretic purification of the two enzymes could not conveniently be carried out in the same run.

Fig. 4 shows that chromatography on DEAE cellulose can be used as a suitable step for the fractionation of as much as half a gram of venom. The toxicity tests (see Table I) indicate that two different neurotoxins are present in the ringhals venom; one of them was eluted in the first two peaks, with the highest specific activity in the very narrow zone around tube 18, while the other neurotoxic factor was found in tube 62 (*cf.*[2], pp. 189–196). Our main interest has been concentrated on the phosphodiesterase zone (tubes 56–68), which is well separated from the cholinesterase and the lecithinase A but still contains 5'-nucleotidase and the toxin mentioned above. Freeze-drying of this zone from an experiment similar to that shown in Fig. 4 gave a dry weight as high as about 33 mg/ml. This is in accordance with results from chromatography of rattle-snake venom[6], and probably most of the substance is THAM buffer material.

Several attempts have been made to purify different chromatographic preparations of phosphodiesterase by column electrophoresis. A further two-fold purification was effected at both pH 5.5 and pH 8.7, and it was demonstrated that the diesterase peak corresponding to tubes 56–68 in Fig. 4 is composed of at least four different u.v.-absorbing components. During the dialysis preceding the electrophoresis, however, a large part of the diesterase activity has always been lost. A good separation has also required long experiments resulting in a troublesome zone-broadening.

Chromatography of phosphodiesterase purified by column electrophoresis has only given the same purification as obtained directly with chromatography of the crude venom.

Enzymic properties of the phosphodiesterase preparation

For the use as an analytical tool, phosphodiesterase should not be contaminated with 5'-nucleotidase. Unfortunately, these two enzymes have always appeared together in the chromatograms (the 5'-nucleotidase activity being determined by a semi-quantitative spot test). They have been separated by zone electrophoresis at pH 8.0 and 8.7, but the large zone-broadening has brought about a considerable dilution of the fractions.

The parallelism previously reported for a rattle-snake venom[6] between phosphodiesterase and DNAase activities, the latter being determined as the acid-lanthanum-soluble material liberated, has also been observed in chromatograms of

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the ringhals venom (see Fig. 2b). When the hydrolysis of DNA was followed at different times both by the method mentioned above and by the viscosimetric method, the liberation of low-molecular nucleotides was initially found to be very rapid, while there was almost no change in the viscosity during the same time. This indicates that the phosphodiesterase from the ringhals venom is an exonucleotidase like the other snake-venom diesterases so far investigated[8,9].

Some preliminary experiments have been made in order to study the influence of metals on the digestion of DNA with phosphodiesterase[18]. This work has shown that ethylenediaminetetraacetic acid (EDTA) completely inhibits the enzyme and that both calcium and magnesium may be involved in the enzymic action. An interesting fact is also that all the diesterase fractions tested have had a pyrophosphatase activity toward adenosinetriphosphate (ATP) and diphosphopyridine nucleotide (DPN). It is thus possible that the pyrophosphatase activities earlier found in snake venoms[3] are due to the phosphodiesterase.

ACKNOWLEDGEMENTS

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