5. A new method is proposed for the identification of N-terminal arginine where it occurs as the DNP-derivative in presence of large amounts of ε-DNP lysine.

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# CHROMATOGRAPHY OF RATTLESNAKE VENOM

## A SEPARATION OF THREE PHOSPHODIESTERASES

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### INTRODUCTION

Snake venoms have been studied for more than twenty years (see reviews by SLOTTA[1] and ZELLER[2]) but so far most of the enzymes from this biologically interesting material have not been obtained in pure states. Also, with the exception of *l*-amino

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*References p. 630/631.*

acid oxidase[3], comparatively little information is available concerning their enzymic properties.

Venom from rattlesnakes has been utilized in a large number of the investigations reported on. GRASSMANN AND HANNIG[4] have compared the electrophoretic behavior of unfractionated venom from Vipers and Colubrides, and the same method, together with the ultracentrifuge, has been used by GONCALVES AND DEUTSCH[5] for the characterisation of venoms from different species of the Crotalidae. NEUMANN AND HABERMANN[6] have purified the specific toxin of the Brazilian rattle snake. Special interest has been devoted to the phosphodiesterase, and partial purifications of this enzyme have been described by HURST AND BUTLER[7] and by SINSHEIMER AND KOERNER[8], while a more intensive purification has been reported by PRIVAT DE GARILHE AND LASKOWSKI[9].

During recent years, different chromatographic systems have been tried for the fractionation and purification of enzymes (see review by MOORE AND STEIN[10]). At this Institute we have found that one-step-elution chromatography from anion exchangers[11,12] is a rapid and convenient way to achieve a preliminary fractionation of a complex protein mixture. When this technique was applied to venom from *Crotalus adamanteus*, two unexpected findings were made. First, a striking parallelism was found between the activity curves for phosphodiesterase and deoxyribonuclease (DNAase), a fact which, together with heat inactivation experiments, indicated that the same enzyme is responsible for both of the activities. A report about this has recently been published[13]. Secondly, it was found that this activity was multiple, which indicated the existence of several components with the same activity. The present paper shows a separation of three diesterases (designated *a*, *b* and *c*) all with DNAase activity. Rechromatography has firmly established that the enzymes differ from each other, and in order to facilitate the interpretation, the effluent has been analysed for the eluting chloride ion. The heterogeneity has been further confirmed with zone electrophoresis.

## MATERIALS AND METHODS

*The buffer system* used in all chromatographic experiments and for substrate solutions has been tris(hydroxymethyl)aminomethane-hydrochloric acid[14] (THAM-HCl), obtained from Sigma Chemical Co., St. Louis 18, Mo. For the electrophoresis experiments, the volatile buffer, triethylammonium carbonate[15] (TEA-$CO_2$), was used. A concentrated stock solution of this buffer was prepared by mixing a given amount of TEA with water (to a suspension) and then bubbling $CO_2$ into the mixture until the desired pH was reached. Buffer concentrations are given as molarity of THAM or TEA.

*Dried venom* of the Eastern Diamondback Rattlesnake, *Crotalus adamanteus* was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida. Solutions for the different experiments were always made up immediately before use. When a 2% solution was made with a 0.02 *M* buffer, some less soluble material had to be removed by centrifugation, but no diesterase activity was lost in this way. This precipitation did not occur when a 5% solution was made, a fact which may indicate the presence of some salt in the venom. The usual concentration was 2 or 3%, and a higher concentration will produce increasing fluctuations in the pH of the effluent. Dialysis has been avoided since it results in a rapid fall of the diesterase activity.

### *Analytical methods*

*The protein content* was estimated as the extinction at 280 mμ using a Beckman DU spectrophotometer and 1-cm semi-micro cell. The fractions from small scale experiments were diluted; usually 300 μl of protein solution was added to 1 ml of water. Water was also used as a blank in all experiments.

*The phosphodiesterase activity* was measured as the amount of nitrophenol liberated from

*References p. 630/631.*

calcium(bis(*p*-nitrophenyl)phosphate)$_2$, which was prepared as described[9] except for the fact that the recrystallization of tris(*p*-nitrophenyl)phosphate was made in glacial acetic acid. The substrate solution was 0.01 *N* with respect to Ca(bis(*p*-nitrophenyl)phosphate)$_2$, 0.01 *M* to $MgSO_4$, and 0.05 *M* to THAM-HCl of pH 8.9. Two hundred micro-liters of this solution were incubated with 10 $\mu$l from the test fractions for 30 min at 37°. One blank without enzyme was included within each set of determinations. The reaction was stopped with 2 ml of 0.1 *N* NaOH and the nitrophenol liberated was estimated near the wavelength of maximum absorption, 400 m$\mu$ (earlier investigators have for unknown reasons used 440 m$\mu$), and converted to millimoles by use of a standard curve constructed from measurements with pure *p*-nitrophenol. The activities given in the figures are expressed as millimoles of substrate split per liter of incubation mixture during 30 min at 37°, using the mentioned proportion of enzyme solution and substrate.

*The DNAase activity* was determined as the acid-lanthanum-soluble nucleotide material liberated from high molecular weight deoxyribonucleic acid (DNA). Two different types of DNA have been used, one prepared with detergent according to KAY *et al.*[16], the other by salt precipitations as described by HAMMARSTEN[17] and by SEVAG AND LACKMAN[18]. A solution of 0.6% DNA was made by dissolving DNA in 0.05 *M* THAM, and afterwards adjusting the pH to 8.9 with 1 *N* HCl. This substrate solution was compared with DNA, dissolved in 0.05 *N* NaOH and then dialyzed against 0.05 *M* THAM-HCl at pH 8.9. A standard enzyme solution showed no difference in DNAase activity with respect to these dialysed or undialyzed DNA preparations. Of the substrate solution, 100 $\mu$l was incubated with 10 $\mu$l of the test samples for 60 min at 37°. The reaction was stopped with 1 ml (in some experiments 2 ml) 0.01 *M* $La(NO_3)_3$ in 0.1 *N* HCl[19], the precipitated DNA was spun down in a centrifuge and the extinction of the supernatant fluid at 260 m$\mu$ was taken as the DNAase activity. This was measured against a blank of substrate incubated without enzyme. The relation between the DNAase activity so obtained and the amount of enzyme is not linear but has a lag part in the beginning. The extinction at 260 m$\mu$ of the blank measured against water, has been regarded as a control of the amount of low molecular weight nucleotides contaminating the DNA solution and has varied between zero and 0.03. Substrate solutions older than 2 days have not been used.

*Amino acid oxidase activity* is usually determined with the Warburg apparatus[3]. As this was too time-consuming for the present investigation, a semi-quantitative technique was developed. A 0.09% solution of *l*-leucine in 0.05 *M* borate buffer of pH 8.2 was used as substrate. Of this solution, 100 $\mu$l was incubated with 10 or 25 $\mu$l of the test samples at 37°, usually for 1 hour. Afterwards 5 or 10 $\mu$l of the incubation mixture were placed on a piece of Whatman 54 paper and dipped in a acified 0.02% ninhydrin solution (in acetone) to develop the colour of the remaining leucine. Fig. 1 shows such a paper with spots from a leucine solutions incubated with fractions from a chromatographic experiment. The first line of spots was applied after 15 min of incubation and the second one after 30 min. The missing ninhydrin colour around tube 28 shows the location of the *l*-amino acid oxidase. It is possible to stop the reaction with 25 $\mu$l 5 *N* NaOH, vacuum-dry the samples in a desiccator and quantitatively determine the amounts of leucine left with the ninhydrin reaction of MOORE AND STEIN[20]. With less accuracy, the incubation can also be made on a paper, impregnated with leucine or sprayed with leucine after the application of the enzyme.

*Lecithinase activity* (or phospholipase A; *cf.* a review by ACKER[21]) was followed indirectly as the hemolyzing power of the incubation mixture. The substrate solution had the following composition: 10 volumes of 0.15 *M* phosphate buffer of pH 7.0, 1 volume of packed human red blood corpuscles and 1 volume of egg yolk, mixed as a suspension. Of this solution, 200 $\mu$l was incubated with 10 $\mu$l test sample for 30 min at 37°. Then, 2 ml of ice-cold 0.9% NaCl was added, and the solution was mixed and centrifuged. The extinction at 540 m$\mu$ of the supernatant fluid (the amount of hemoglobin liberated) was read in a Beckman B spectrophotometer and taken as a measure of the activity. The method has been used in the present study as a semi-quantitative test only, principally to check *the absence* of lecithinase activity in other components and to localize the main part of the enzyme in a chromatogram.

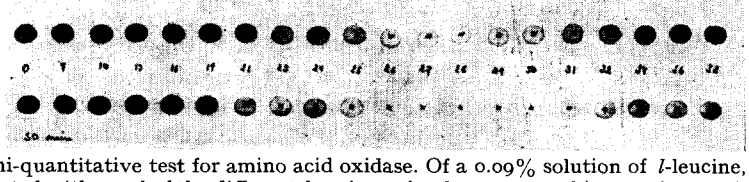

Fig. 1. Semi-quantitative test for amino acid oxidase. Of a 0.09% solution of *l*-leucine, 100 $\mu$l has been incubated with 25 $\mu$l of the different fractions of a chromatographic experiment. After 15 and 30 min, 10 $\mu$l was removed from the reaction mixture, put on a paper, dried and developed with ninhydrin. Around tube 28, all leucine has been oxidized, indicating the localization of the enzyme.

*References p. 630/631.*

*The chloride analyses* were made as titrations with mercuri nitrate[22]; *cf* also [11]. The chloride concentrations in the figures are given in microequivalents per ml ($\mu$equiv./ml).

*The chromatographic technique*

The adsorbent used was diethylaminoethyl (DEAE) cellulose, prepared according to PETERSON AND SOBER[23]. The nitrogen content of the preparation was 5.5 mg/g of vacuum-dried cellulose. Before packing, the ion exchanger was deaerated and then decanted in about 20 times its volume of water (1–2 hours sedimentation time) in order to remove very small particles. The packing was done under a water pressure of about twice the length of the column. The column was regenerated by washing with 1% NaOH and afterwards with 10–20 times the dead volume of the starting buffer for the next experiment. It was usually checked that the effluent from the column had the correct pH before applying of the venom solution.

The chromatograms have been developed by one-step elution[11,12]. At the start of the experiment the column was in equilibrium with a dilute buffer (the starting buffer) in which also the venom was dissolved. Elution was then directly carried out with a 12–30 times more concentrated buffer of the same pH. As demonstrated earlier[11], the best pH control on an anion exchanger is obtained with a cation buffer such as THAM and this is of importance when the alternations in ionic strength are large. Despite this arrangement, the pH of the effluent may vary 0.2–0.4 units in small-scale experiments (*cf.* also Fig. 6). We have attributed these fluctuations to the large differences shown by the components in their tendency to complex with buffer ions (*cf.* also DISCUSSION). In order to utilize possible displacement effects, the fraction volume ought to be as small as is convenient. For small columns (23 × 1 cm) it was about 1/10 of the dead volume; for the large one (66 × 1.8 cm) it was about 1/30. The same column has been used more than 10 times. We have, however, observed that the first chromatogram from a column with unused ion exchanger may differ slightly from the subsequent ones; otherwise the reproducibility was good.

The fraction collector was worked on a time basis and the abscissa in the figures is given in tube number. This has been preferred to effluent volume since the latter alternative, with small fraction volumes, leads to a summarizing of errors which will never be evident in the figures.

All experiments were carried out in a cold room, maintained at 4°.

*The zone electrophoresis* were performed in a column (100 × 2 cm) with ethanolysed cellulose, prepared according to FLODIN AND KUPKE[24], and using the technique described by PORATH[25]. The column had a dead volume of 225 ml. Its lower part was cooled by an electrode vessel, the upper part by a fan. The apparatus was placed in a cold room and it was checked that the temperature on the surface of the column, during a run, did not exceed 5°. During elution, fractions were shifted every third minute with volumes around 2.5 ml.

## RESULTS

### *Chromatography at different pH and ionic strength*

All experiments described in this section were made on columns (23 × 1 cm) with 19 ml of the anion exchanger, DEAE-cellulose[23], which were in equilibrium with the starting buffer, 0.02 *M* THAM-HCl. Different pH has been used in the separate experiments. The amount of venom was constant (40 mg) and was dissolved in 2 ml of the starting buffer (except for the experiment shown in Fig. 3 when 1 ml was used). The experiments were developed with a 12–20 times more concentrated buffer of the same pH. After 2 ml had passed through the column, fractions were collected every 8 min with volumes of 0.5–0.8 ml.

Fig. 2 shows an experiment at pH 7.3 developed with a 0.33 *M* THAM-HCl buffer of the same pH. In this as well as the following figures, phosophodiesterase activity is denoted with crossed circles and DNAase activity with crossed squares; open circles represent extinction at 280 m$\mu$ and triangles stand for the chloride concentration. The figure shows that, under the conditions used, only a first zone of diesterase and DNAase activity (maximum in tube 9) has separated from the rest of the enzymes, which are eluted together by the chloride front. This gives rise to a complex zone (tubes 14–25) containing both diesterase, DNAase, lecithinase (phospholipase A) and *l*-amino acid oxidase. The total recovery of the phosphodiesterase activity was 86%.

*References p. 630/631.*

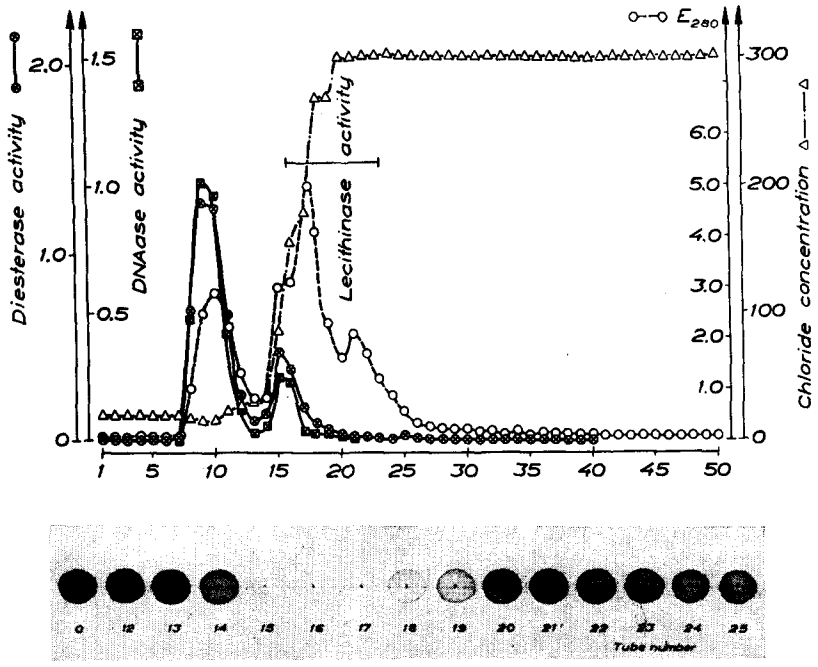


Fig. 2. Upper part: Chromatogram of 40 mg of venom from *Crotalus adamanteus* on a 19 ml column of DEAE cellulose at pH 7.3. Fraction volumes 0.8–9.9 ml. Phosphodiesterase activity (⊗—⊗) given in mmoles of substrate split. DNAase activity (⊠—⊠) given as liberated extinction at 260 mμ. Chloride concentration (△—·—△) given in μequiv./ml. Lower part: *l*-amino acid oxidase test of some fractions from the experiment shown in upper part.

Figs. 3 and 4 show experiments made at pH 8.2 but with different concentrations of the developer, 0.40 and 0.25 *M*, respectively. In Fig. 3 it can be seen that when the more concentrated developer was used, the second diesterase zone tends to split (a minimum in tube 21). Compared with Fig. 2 the lecithinase has been pressed slightly backwards in the chromatogram. If the experiment instead is developed with a more dilute buffer, all the three diesterases can be separated as seen in Fig. 4. A similar result was obtained at pH 8.9 when a 0.4 *M* THAM-HCl buffer of the same pH was used as developer (see Fig. 5). Also in this experiment there is a coincidence between the curves for the phosphodiesterase and DNAase activities. The effluent was analyzed also for lecithinase and *l*-amino acid oxidase and no activity was found before fraction 45 but further on both of the enzymes appeared together in a very elongated zone. The total recovery of the phosphodiesterase activity in this experiment was about 90%.

*Large-scale experiments*

In order to obtain sufficient amounts of enzyme for rechromatography of the different zones of diesterase a few large-scale experiments have been performed. A column (66 × 1.8 cm) with 170 ml of DEAE-cellulose was used in equilibrium with 0.02 *M* THAM-HCl buffer, pH 8.9. Of the venom, 300 mg was dissolved in 10 ml of this buffer. The pH obtained was 8.5 but the addition of 0.3 ml of 1 *M* THAM adjusted it to 8.9. As earlier, undissolved material was removed by centrifugation. Elution was

*References p. 630/631.*

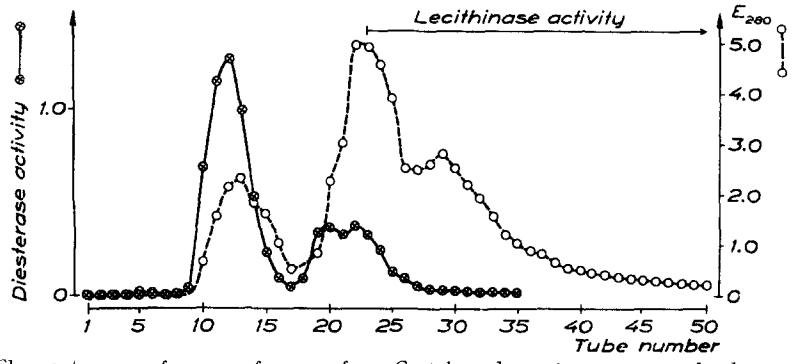


Fig. 3. Chromatogram of 40 mg of venom from *Crotalus adamanteus* on a 19 ml column of DEAE cellulose at pH 8.2 developed with 0.4 *M* THAM-HCl buffer. Fraction volumes 0.7–0.8 ml. Phosphodiesterase activity (⊗—⊗) is given in mmoles of substrate split. The position of the chloride front was not determined in this experiment.

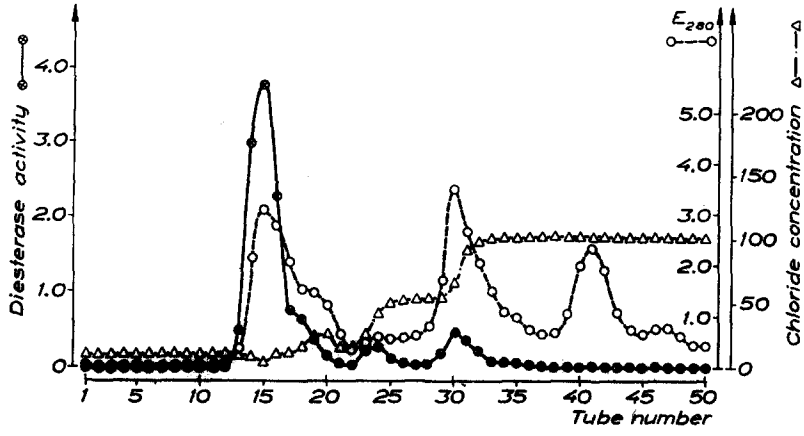


Fig. 4. Chromatogram of 40 mg of venom from *Crotalus adamanteus* on a 19 ml column of DEAE cellulose at pH 8.2 developed with 0.25 *M* THAM-HCl buffer. Fraction volumes around 0.5 ml. Phosphodiesterase activity (⊗--⊗) is given in mmoles of substrate split. Chloride is given in $\mu$equiv./ml.

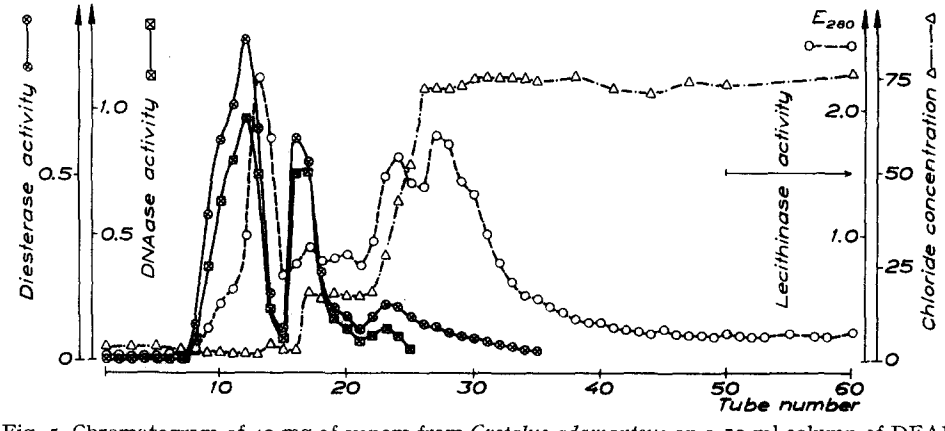


Fig. 5. Chromatogram of 40 mg of venom from *Crotalus adamanteus* on a 19 ml column of DEAE cellulose at pH 8.9. Fraction volumes around 0.7 ml. Phosphodiesterase activity (⊗—⊗) is given in mmoles of substrate split. DNAase activity (⊠—⊠) is given as liberated extinction at 260 m$\mu$. Chloride is given in $\mu$equiv./ml.

here carried out first with 0.33 *M* THAM-HCl of pH 8.9; after 80 ml of effluent passed a change was made to 0.60 *M* buffer of the same pH and finally, after another 25 ml a shift was made to 0.33 *M* THAM-HCl of pH 7.3. As can be seen in Fig. 6, three zones of diesterase have separated from each other and travel well before the chloride gradient which elutes a large zone containing both amino acid oxidase (with maximum in tube 75) and lecithinase. From the most active fractions of each zone, tubes 8–12, 28–29 and 51–52, 2 ml were pooled and freeze-dried. The first zone contained about 3.5 mg dry weight/ml, the second 15.2 mg/ml, while the third contained as much as 60 mg/ml. The pH of the effluent varied more than in the small-scale experiments, as demonstrated by the crosses in Fig. 6. The total recovery of the phosphodiesterase activity was 54%.

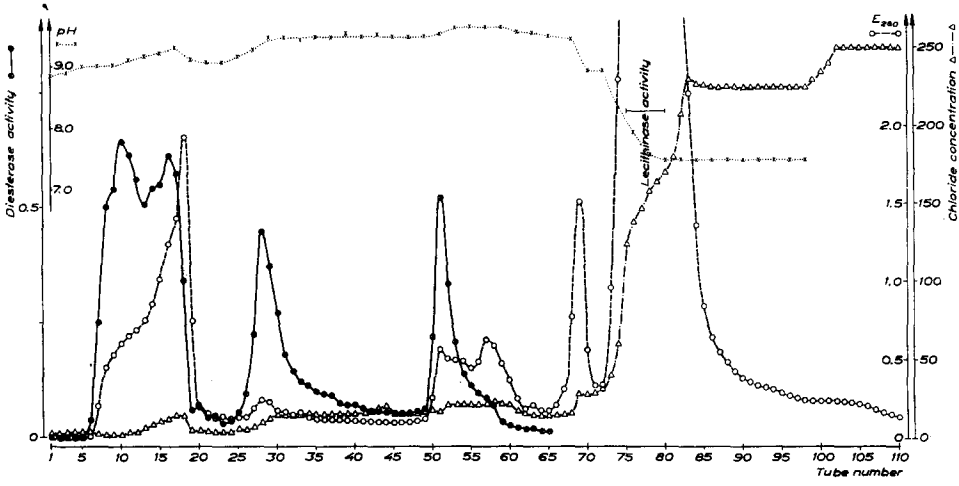


Fig. 6. Chromatogram of 300 mg of venom from *Crotalus adamanteus* on a 170 ml column of DEAE cellulose at pH 8.9. Fraction volumes around 2.5 ml. Phosphodiesterase activity (⊗—⊗) is expressed as mmoles of substrate split. Chloride given in μequiv./ml.

*Rechromatography of the diesterases*

Material from the three different diesterases obtained in the large-scale experiment shown in Fig. 6 have been subjected to rechromatography on a smaller column (22 × 1 cm) with 18 ml of DEAE-cellulose. These three experiments were performed in a similar way and with a very slow rate of flow through the column in order to permit the enzymes to be in contact with the adsorbent for the same time as in the preliminary run. The starting buffer was 0.02 *M* THAM-HCl, pH 8.9, and the developer was 0.33 *M* buffer of the same pH. After 2.5 ml of effluent had passed through the column, fractions were collected every 24 min with volumes of about 0.7 ml (water pressure: 10 cm). The material from the diesterase *a* and *b* zones was dissolved in 2 ml of starting buffer. For diesterase *b* the pH had to be adjusted with HCl from 9.5 to 9.0. As the protein content in the third zone was estimated to be of the order of a few per cent only, 239 mg were dissolved in 2 ml of starting buffer and dialyzed for 1 hour, during which time 20% of the activity was lost; after pH adjustment the rest was applied to the column. The results of these experiments are shown in Fig. 7 (diesterase *a*), Fig. 8 (diesterase *b*) and Fig. 9 (diesterase *c*). Only one single diesterase peak was

*References p. 630/631.*

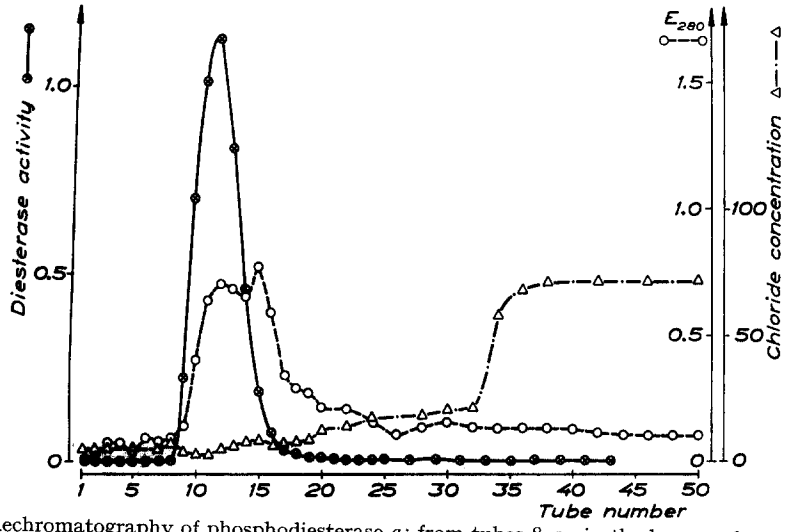


Fig. 7. Rechromatography of phosphodiesterase *a*; from tubes 8–12 in the large-scale experiment (see Fig. 6). Fraction volumes around 0.7 ml. Phosphodiesterase activity (⊗—⊗) expressed as mmoles of substrate split. Chloride given in μequiv./ml.

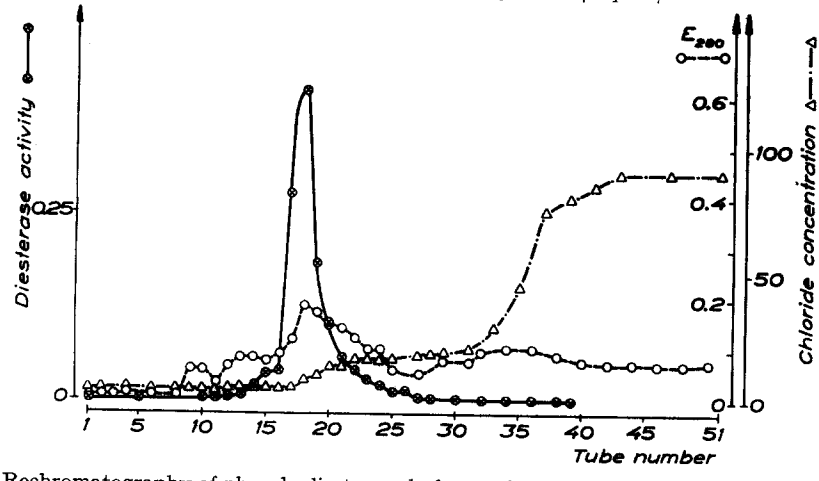


Fig. 8. Rechromatography of phosphodiesterase *b*; from tubes 28–29 in the large-scale experiment (see Fig. 6). Fraction volumes around 0.7 ml. Phosphodiesterase activity (⊗—⊗) expressed as mmoles of substrate split. Chloride given as μequiv./ml.

obtained in each run and all of them showed activity toward DNA. The retention volumes (counted to the center of the zone) were 10.3 ml for the first zone, 14.2 ml for the second and 15.0 for the third. The first zone travelled 14.5 ml before the middle of the chloride gradient, the second one 9.9 ml and the third one 8.1 ml. The recovery of the activities (excluding losses during freeze-drying and dialysis) were 73% for diesterase *a* and 77% for diesterase *c*.

*Column electrophoresis*

In order to see whether the heterogeneity of the diesterase activity could be confirmed by another method, crude venom was investigated by zone electrophoresis in a column

*References p. 630/631.*

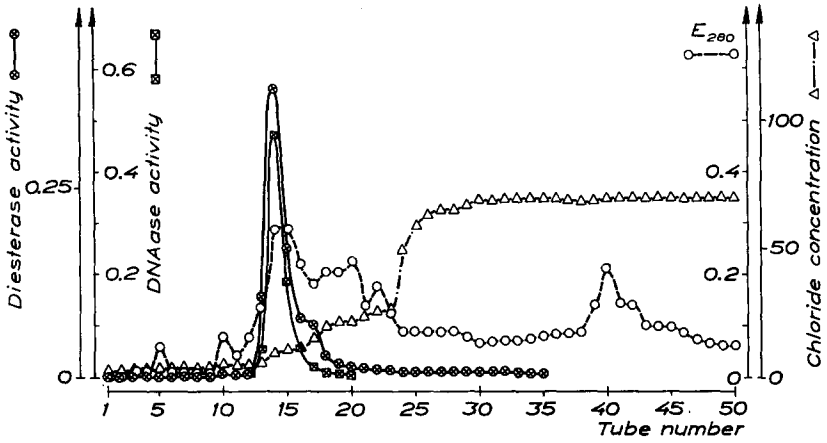


Fig. 9. Rechromatography of phosphodiesterase *c*; from tubes 51–52 in the large-scale experiment (see Fig. 6). Fraction volumes around 0.8 ml. Phosphodiesterase activity (⊗—⊗) expressed as mmoles of substrate split. DNAase activity (⊠—⊠) is given as liberated extinction at 260 mμ. Chloride given in μequiv./ml.

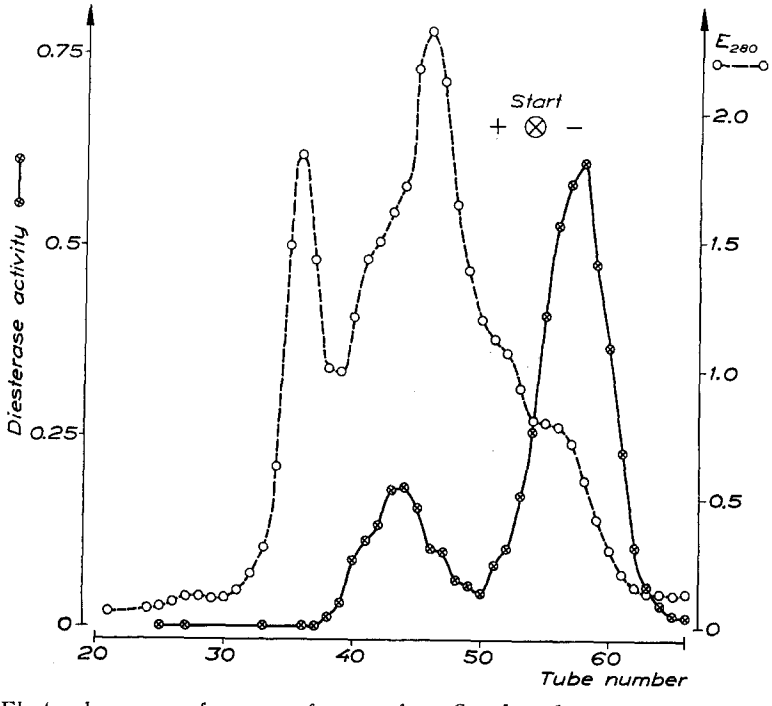


Fig. 10. Electropherogram of 100 mg of venom from *Crotalus adamanteus* on a cellulose column (100 × 2 cm). The run was performed at 20 mA for 46 hours in 0.1 *M* triethylamine-carbonate buffer of pH 9.1. Fraction volumes around 2.5 ml. Phosphodiesterase activity (⊗—⊗) expressed as mmoles of substrate split. Lecithinase activity was found in all tubes after 40 with maximum around tube 46.

(100 × 2 cm) of ethanolysed cellulose[24,25]. Fig. 10 shows the electropherogram from such an experiment with 100 mg of venom in 0.1 *M* TEA-$CO_2$ buffer of pH 9.1. The current was 20 mA (440 V) and the time was 46 hours (temp. 4°). The figure shows that

*References p. 630/631.*

the diesterase activity was split into two rather unsymmetrical peaks. Lecithinase activity was found in all fractions after number 40 with a maximum around tube 46. Some material was adsorbed to the column and eluted with 0.5% NaOH. The total recovery of the phosphodiesterase activity was 74%.

In another zone-electrophoresis experiment the freeze-dried diesterase *a*, collected from four chromatographic experiments, was run under the same conditions. This experiment gave only one narrow and symmetrical activity peak with the same mobility as shown by the slow component in Fig. 10 (with maximum in tube 58).

## DISCUSSION

### *Interpretation of the chromatograms*

Figs. 2–5 show that an increase in pH and a decrease in the concentration of the developing buffer will produce an elongation of the chromatograms and give gradually better separations. As the THAM concentration of the starting buffer has been constant, the chloride concentration has been decreasing with increasing pH. The purification of the first diesterase zone appears not to be significantly influenced by this variation. Comparable experiments with 0.01 *M* and 0.005 *M* starting buffers of pH 8.2 showed no improvement in the resolution. That the concentration of the developer is a very important factor is demonstrated by the differences between Figs. 3 and 4. It is not only the diesterases that separate better with a more dilute developer, but also the second protein zone (tubes 20–35 in Fig. 3) is resolved into two separate fractions with maxima in tube 30 and 41 (see Fig. 4).

The first part of the large-scale experiment (Fig. 6) exhibits a still better resolution than obtained earlier. This may partly be attributed to the fact that the fraction volumes are about 1/30 of the dead volume of the column, while the corresponding figure is 1/10 for the small-scale experiments. The later part of the chromatogram was compressed by the use of buffers of increasing chloride concentration and decreasing pH. The last buffer with pH 7.3 was able to overtake the two earlier and gave rise to the large zone (tube 72–90) containing both amino acid oxidase and lecithinase. The purpose of this procedure was to recover these two enzymes within a limited number of tubes.

The re-running of the three diesterases (Figs. 7–9) shows that the first two components, *a* and *b*, differ considerably in retention volume as well as in distance from the chloride front. The rechromatography of the third diesterase, *c*, (Fig. 9) did not show large differences with respect to diesterase *b* but these seem to be significant in supporting the conclusion that the three peaks in Fig. 6 correspond to three different proteins (or protein complexes) with the same activity. An additional confirmation of this statement is given by the result of the zone electrophoresis shown in Fig. 10. Even if this experiment does not show more than two diesterase zones, the unsymmetrical shape of the activity peaks indicates a further heterogeneity. It cannot at present be decided whether the shape of the peak of diesterase *a* in Figs. 5 and 6 is an indication of a further heterogeneity of this component.

In earlier studies of horse-radish enzymes on different anion exchangers[11,12], it was found that if one-step elution was used a doubling of the load gave a marked increase in the zone width of weakly adsorbed components. This relation was interpreted as an indication of displacement. In the present study, we have not been able to find any similar relations.

*References p. 630/631.*

*Heterogeneity of the enzymes*

During the last year several enzyme preparations have been shown to yield two or more components with the same activity[26–28], and references to older literature on this subject are given in a review of COLVIN *et al.*[29] It appears that chromatography and zone electrophoresis are particularly suitable for the detection and separation of such active components since these methods permit the continuous assay of protein content and enzyme activity over a separated zone.

The chromatographic study of PIERPOINT[27] is of special interest since it deals with phosphoesterases of another type which show multiplicity as well as activity toward rather different substrates.

When the material from the three diesterase zones of the large-scale experiment (Fig. 6) was freeze-dried, it was found that the dry-weight content per ml for diesterase *b* was 4–5 times, and for diesterase *c* 17 times, that of diesterase *a*. As the ultraviolet extinction shows that the protein content was of the order of 0.1–0.2% a large part of the material may be salt. However, the chloride content was rather low in all three of the diesterases (see Fig. 6) and the remaining explanation is that most of the material was THAM.

The two forms of pancreatic ribonuclease were recently shown to differ in their number of carboyxlic groups[30], and a similar explanation is suggested by PIERPOINT[27] for the pea phosphatases. The hypothesis that the diesterases studied here may also differ with respect to their number of acidic groups is supported by the following observations. The zone electrophoresis experiments show that diesterase *a* is identical with the slow diesterase component in Fig. 10. The faster component, which is more negatively charged, is thus probably made up of diesterase *b* and *c*. If these two forms are more acid one would expect that their retention volumes on an anion exchanger would be larger than for diesterase *a* and also that they would have an increasing tendency to complex THAM ions, which is in agreement with our findings.

Venom of *Crotalus adamanteus* is known to digest hemoglobin and to have clotting activity[31]. The simplest explanation for the existence of the three diesterases may thus be that they are produced by proteolytic enzymes. However, a venom solution stored during 24 hours at 4° showed the same chromatographic behavior as a fresh solution. It is therefore unlikely that action has occurred during the time of our experiments. Unfortunately, no information is available about the procedure for the collection of the venom and for this reason further speculations are to no purpose.

*Enzymic properties of the diesterases*

We have previously suggested that the phosphodiesterase and the DNAase activities are due to the same enzyme[13]. During the course of the present study we have tried to separate the two activities on both anion and cation exchangers as well as zone electrophoresis but we have not even been able to change the ratio between the activities. A similar parallelism between the phosphodiesterase and DNAase activities has been found also for enzyme from a cobra venom (observations by BJÖRK AND BOMAN).

The name "DNAase" has been used here for an enzyme that digests DNA, regardless of the type of action. The exact definition of such an enzyme will naturally depend on the methods used for the determination of undigested DNA. It is, however, obvious that the venom enzyme is different from the pancreatic DNAase (*cf.*

*References p. 630/631.*

SCHMIDT[32]). While the latter enzyme can be described as a depolymerase, LASKOWSKI has suggested (private comunication) that the enzyme from rattlesnake venom, may liberate 5'-terminal mononucleotides, in analogy to the splitting of proteins by carboxypeptidase (*cf.* also [33]). Such a difference in the action on DNA would explain why previously no "DNAase" activity was found in a diesterase from venom[9] when the determinations were carried out with a viscosimetric method.

If the phosphodiesterase has an exonucleotidase activity, the simplest explanation for the rather alkaline pH optimum of the enzyme (between 9 and 10) is that a number of hydrogen bonds in the DNA are broken by the high pH and thus provide a substrate molecule, which is more easily attacked by the enzyme.

The recovery of the phosphodiesterase activity has been calculated for most of the experiments and has been found to vary between 54 and 90%. However, the activity appears to be fairly sensitive to the ionic strength, which naturally varies in the effluent. Furthermore, it is somewhat unsatisfactory to use a calcium salt of the substrate for a magnesium-activated enzyme. For these reasons the activity values (and recoveries) given here are unsuitable for detailed interpretations. We hope, however, to continue the purification of at least one of the enzyme forms, and to return to the question of the specificity, which can only be solved with an enzyme preparation which fulfills reasonable purity criteria.

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SUMMARY

1. Venom from rattlesnake (*Crotalus adamanteus*) has been studied by chromatography on DEAE cellulose. Special interest has been devoted to phosphodiesterase and DNAase, while lecithinase and *l*-amino acid oxidase have been followed by semi-quantitative tests only.

2. Under optimal conditions three different forms of phosphodiesterases have been separated from lecithinase and *l*-amino acid oxidase. The heterogeneity has been confirmed by rechromatography and zone electrophoresis.

3. All experiments have shown a parallelism between the phosphodiesterase and DNAase activities.

4. A new test for *l*-amino acid oxidase activity is given, using *l*-leucine as substrate and ninhydrin to determine remaining amino acid.

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# Short Communications

## On the acylation of the carcinogen 2-aminofluorene by rat liver *in vitro**

A previous investigation disclosed that rat liver *in vitro* acetylated and deacylated the carcinogen 2-aminofluorene (AF) repeatedly in a cyclic manner[1]. Conceivably, other intermediates of the citric acid cycle or of fatty acid synthesis could also serve as acyl group donors for aromatic amines such as AF. JOHNSON AND QUASTEL[2] have indicated that in the system, pigeon liver extract-rat brain homogenate, the increased rate of conjugation of the aromatic amino group of sulfanilamide with added succinate may be due, in part, to direct acylation by succinate. We have investigated whether this reaction occurs in the liver with AF as the acyl group acceptor. Liver slices were incubated with AF, labeled with carbon-14 in the 9 position, and sodium succinate, and the isotope content of the expected product, N-2-fluorenylsuccinamic acid (SAF), was determined. Analysis by the method of inverse isotope dilution[3] revealed that 0.86% of the available AF-9-$^{14}$C was acylated to SAF-9-$^{14}$C (Table I). When AF-9-$^{14}$C and sodium succinate were incubated under similar conditions, but in the absence of liver slices, 0.89% of the radioactivity appeared as SAF-9-$^{14}$C. Thus, in contrast to acetate utilization in the acetylation of AF[1], rat liver appears to be incapable of utilizing succinate for *direct* enzymic succinoylation. Likewise, butyrate is not directly transferable to AF since incubation of liver slices with AF-9-$^{14}$C and sodium butyrate did not yield the acylated derivative, N-2-fluorenylbutyramide, as determined by the unequivocal carrier technique (Table I). As judged from these slice experiments, rat liver acetyl kinase exhibits a high degree of acyl group specificity which is quite similar to that of the purified acetyl kinase of pigeon liver[4].

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