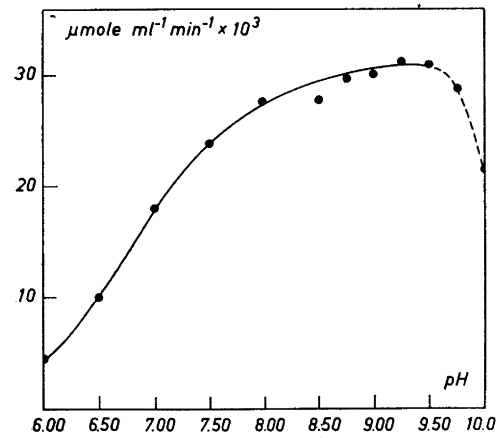

Fig. 2. pH-activity curve for butyrylcholine esterase at 25°C. Substrate: 10^{-2} M acetylcholine iodide.

tyrylcholine iodide (final concentration). The activity was recorded during a 5 min period immediately after addition of the substrate and after a small correction of the pH had been made. (This correction was necessary since the substrate was somewhat acid.) Fig. 1 shows the result. The activities are expressed in per cent of an initial enzyme activity obtained at pH 8.00. Irreversible denaturation was found to be slowest around pH 6.00 and increases with the pH and with the incubation time.

The automatic recording titrator was then used to reinvestigate the pH-activity curve for BuChE. A 10^{-2} M solution of acetylcholine iodide was used as substrate. The obtained values of the activity were corrected for spontaneous hydrolysis as described earlier [2]. Fig. 2 shows the curve, which agrees well with the experimental data published earlier by Wilson [3] and a curve published by Hase [4]. (Hase, however, used equine serum as source for the enzyme). The curve obtained in this work is, however, different from curves published by many others and is also different from two curves obtained in this laboratory. [5,6] The present curve and those published by Wilson and Hase were obtained with an automatic titrator, thus without buffer solutions, and in two cases without incubation times for the enzyme (Hase seems to have incubated for 15 min). The curves were determined with a 10^{-2} M or $10^{-2.4}$ M (Hase) concentration of the substrate. Wilson, furthermore, used a 0.1 % solution of gelatine to protect the enzyme. An earlier published curve by Heilbronn [5] was obtained in buffer solutions and at a lower substrate concentration. The different buffer ions may have caused denaturation of the enzyme during the incubation time of 30 min and may also have enhanced hydrolysis in different ways. Tammelin [6] used unpurified human serum, and also a lower substrate concentration. Obviously, the shape of the pH-activity curve depends on the method used.

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Fractionation of Snake Venom by the Gel-Filtration Method

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The use of gel-filtration as a fractionation method has recently been reported [1]. Attempts to apply the procedure to the fractionation of crude biological extracts have been successful [2,3], but in these cases only for separating proteins and protein-complexes from low-molecular weight material. It was shown, however [2], that proteins of low molecular weight like ribonuclease and cytochrome c could be retarded on a gel of suitable crosslinking, and Gelotte has recently obtained a separation of enzymes from pancreatic juice (private communication). In earlier work carried out at this institute [4] most of the UV-absorbing components of the venom from the ringhals cobra, *Hemachatus haemachates*, including lecithinase A, had been found to have a rather low molecular weight, and thus we thought it would be of great value to in-

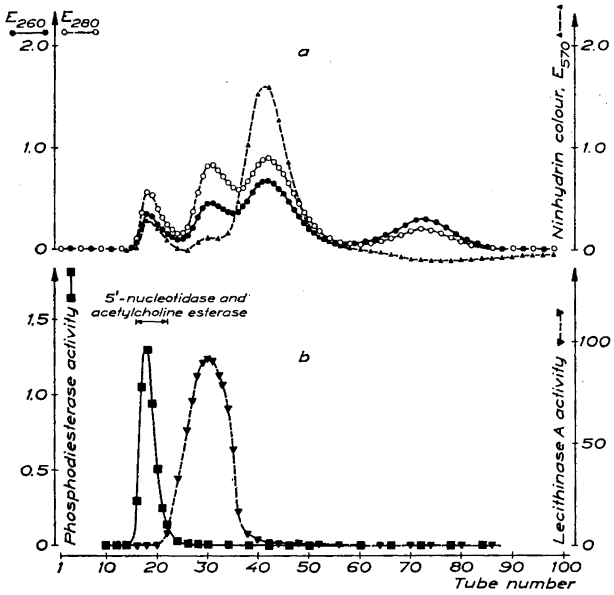

Fig. 1. Gel-filtration of 30 mg of freeze-dried ringhals cobra venom on a 60 ml column (1.9 × 21 cm) of dextran gel. The water regain of the gel was about 8 g/g. The venom was dissolved in 1 ml 0.05 M THAM-HCl of pH 7.6, and elution was performed with the same buffer. Fraction volumes about 1.0 ml. For the ninhydrin tests (▲ — — — ▲) 0.2 ml aliquots were taken. Phosphodiesterase activity (■——■) is given in mmoles of substrate split in 30 min per litre of incubation mixture, and lecithinase A activity (▼ — — — ▼) as increased extinction in 30 min at 540 mμ (hemoglobin).

vestigate the behaviour of this venom on gels of a low degree of crosslinking.

Freeze-dried ringhals venom was obtained from The South African Institute for Medical Research, Johannesburg. In order to compare it with another type of snake venom, we also used the venom of a rattle-snake, *Crotalus adamanteus;* this venom was bought from Ross Allen's Reptile Institute, Silver Springs, Florida.

Starch gel with a water regain of 5.1 g/g and dextran gel with a water regain of about 8 g/g were supplied by A.B. Pharmacia, Uppsala, Sweden. The buffer system used was tris(hydroxymethyl)aminomethane-hydrochloric acid (THAM-HCl). Before packing a column the gel was allowed to swell in the buffer overnight, and then it was deaerated in a suction flask. All the experiments were performed in a cold room at 4°.

The extinctions at 280 and 260 mμ were measured in a Beckman DU spectrophotometer using a 1-cm semi-micro cell. Phosphodiesterase, lecithinase A, L-amino acid oxidase and acetylcholine esterase activities were determined as described earlier [4,5]. For the estimation of 5'-nucleotidase activity a substrate solution of the following composition was used: 0.01 M adenosine-5'-phosphate, 0.01 M magnesium sulphate and 0.05 M THAM-HCl of pH 8.9. Of this solution 0.5 ml was incubated for 30 min at 37° with 0.05 ml of each fraction to be tested. The reaction was stopped with 1 ml of 2.5 % H_2SO_4, and the inorganic phosphate liberated was determined by a modification of King's method [6].

The ninhydrin curve was obtained according to Moore and Stein [7]. In the figures the colour given by the filtration buffer has been subtracted.

Fig. 1 shows the result of a gel-filtration of 30 mg of ringhals venom on a 60 ml column (1.9 × 21 cm) of dextran gel. The venom had been dissolved in 1 ml THAM-HCl of pH 7.6, and after applying the solution to the

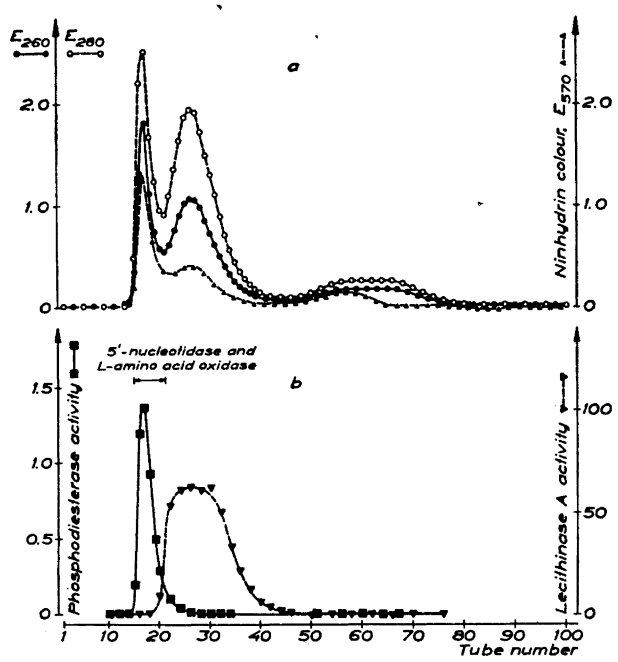

Fig. 2. Gel-filtration of 30 mg of rattle-snake venom on the same column and under the same conditions as in Fig. 1.

column, elution was performed with the same buffer. The rate of elution was about 6 ml/h, and 1 ml fractions were collected. In the upper part of the figure it can be seen that the venom was fractionated into four different zones, and that 90 % of it was retarded on the column. Three of the enzymes tested for, phosphodiesterase, acetylcholine esterase and 5'-nucleotidase, appeared together in the first peak (lower part of the figure), whereas the lecithinase A was eluted in the second peak, well separated from the other enzymes. In all the experiments performed the recovery of UV-absorbing material was about 100 %.

By chromatography on diethylaminoethyl (DEAE) cellulose the phosphodiesterase has been separated from cholinesterase and lecithinase A[4], but then the latter two enzymes left the column in a single zone. Gel-filtration would therefore be a useful step in the purification of the three enzymes, especially as a transference to another buffer could be achieved at the same time. Unfortunately, the recovery of cholinesterase from the dextran gel was very low, 10—20 %, probably depending on impurities in the gel. Washing with *ortho*-phenanthroline in order to remove traces of heavy metals had no significant effect, but after treating the gel with 10 % hydrochloric acid the recovery of cholinesterase was increased considerably. The inactivation could be ascribed neither to removal of a cofactor nor to reversible adsorption.

The recoveries of phosphodiesterase and lecithinase A were about 90 % and 100 %, respectively. In the experiments made on starch gel we also got a very high recovery of cholinesterase activity, but in that case the fractionation was not so good. One third of the UV-absorbing material was eluted in the first peak and more than half of it in the second peak, while the third peak had disappeared completely. However, with a more weakly cross-linked starch gel the separation properties would certainly be improved.

The quotient E_{260}/E_{280} was found to be higher than unity in the most retarded zone (tubes 60—85 in Fig. 1), which may be taken as an indication of a large proportion of nucleotides in this zone. It should also

be observed that the corresponding ninhydrin values are below zero. This might be caused by the formation of complexes between the substances eluted and the THAM buffer, and such a binding of THAM by some components of snake venom has already been suggested as an explanation of a phenomenon encountered in ion exchange chromatography of venom [4,5]. By gel-filtration of the chromatographic fractions in question we have indeed shown that they are partly identical with the fourth zone in Fig. 1.

Gel-filtration of the rattle-snake venom (Fig. 2) was performed on the same dextran-gel column and under the same experimental conditions as described above. This venom does not contain any cholinesterase but has a much higher concentration of L-amino acid oxidase and a considerably stronger UV-absorption than the cobra venom. As in the former case lecithinase A was the only enzyme retarded to any appreciable degree. The ninhydrin and UV-extinction patterns, however, were quite different from those obtained with the ringhals venom. About 85 % of the material applied appeared in the first two peaks, and the large ninhydrin zone corresponding to the tubes 35—50 in Fig. 1 was missing. The most retarded zone had a very flat and elongated shape, and the quotient E_{260}/E_{280} was lower than unity in all fractions. As the ringhals and the rattle-snake belong to different families (*Elapidae* and *Crotalidae*, respectively), it would be of great interest to know to what extent these differences are typical. In the general mapping of the components of snake venoms gel-filtration may prove to be a valuable tool.

We have also compared gel-filtration experiments carried out at different pH values and with buffers of varying concentrations. The only deviation found was a slightly more pronounced tailing of the lecithinase A at low ionic strengths. This might be interpreted as a weak adsorption tendency, but at present no definite opinion can be formed on this question.

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Quantitative Determination of Carotene by Paper Chromatography

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It has been shown previously [1] that carotenes can be quantitatively separated from the other chloroplast pigments by chromatography on a filter paper with a kieselguhr filler. On the basis of this observation a method for the quantitative determination of carotene in dried grass and seaweed meal has been worked out.

Procedure. Approximately 1 g of the finely ground sample is moistened * with 2 ml of water in a 100 ml Erlenmeyer flask. After standing for 15 min (to allow swelling), acetone (25 ml) is added and the sample left over night under nitrogen in the dark. The solution is then decanted through a sintered glass funnel, and the residue is extracted with another 25 ml of acetone. The extraction is repeated until the filtrate remains colourless. Then the combined extracts are concentrated *in vacuo* to a small volume, transferred quantitatively to a volumetric flask (10 or 25 ml according to the content of carotene) and made up to the mark with acetone.

An aliquot of the extract (0.2—0.8 ml) is applied onto the centre of a circular filter paper; (Schleicher & Schüll, No. 287, Kieselguhr-filter, 18 cm diameter) the solvent being continuously evaporated by a stream of nitrogen. When the spot is dry, the paper is placed between similar halves of 15 cm petri dishes, and the chromatogram is developed with pure petroleum ether (b.p. 60—80°C) according to the technique of Rutter [2]. As soon as the carotenes, which migrate close to the solvent front, are separated from the other pigments,

* Essential for quantitative extraction of carotene from the seaweed meal.