4. Although LDH activity per milligram of protein in the developing brain and skeletal muscle did not increase during early development there was a slight increase of LDH in the heart and a threefold temporary increase in the liver between 12 and 16 days of incubation.

5. MDH activity per milligram of protein increased steadily throughout early development in the brain, heart and muscle, but showed a temporary twofold increase in the liver between 12 and 16 days of incubation.

6. The levels of LDH and MDH activity of embryonic organs are compared with those of the adult hen.

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The Separation and Properties of the Neurotoxins from the Venom of the Tiger Snake *Notechis scutatus scutatus*

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The Commonwealth Serum Laboratories prepare antivenenes for the venoms of the Australian tiger snake *Notechis scutatus scutatus* and the taipan *Oxyuranus scutellatus*. These antivenenes provide various degrees of protection against other Australian snake venoms, the extent of which is not readily explained for all in terms of the predominant pathological effect of each venom. For instance, the venoms of *Notechis scutatus* and *Demansia textilis textilis* are both predominantly neurotoxic in action, but the antivenene for

Notechis scutatus provides little protection against the venom of *Demansia textilis textilis*.

As a first approach to the question of which antigens are shared by different species of Australian snake venoms, an attempt was made to isolate the neurotoxin of the venom of *Notechis scutatus* from the components responsible for the other main pathological effects of the whole venom. These components include a haemolysin and a blood-coagulating factor. An effect on the eyes, culminating in a high incidence of blindness among

animals surviving a near-lethal dose (Kellaway, 1929), distinguishes the venom of *Notechis scutatus* from other Australian snake venoms.

Experiments aimed at isolating the main components of venoms reported by Martin (1896), Kellaway, Freeman & Williams (1932) and Holden (1933) were part of the early fundamental studies of Australian snake venoms. New methods of separating proteins have made it possible to extend the isolation of venom components. Further, while the last step in the fractionation procedures about to be described was being developed, namely the chromatographic separation on the Amberlite resin IRC-50, the isolation of crotactin, the acidic neurotoxin from the venom of *Crotalus terrificus terrificus*, was reported by Neumann & Habermann (1955), who used the same resin. Subsequently, Gonçalves (1956) reported the chromatographic separation on this resin of crotamine, the basic neurotoxin, from the same venom. The similarity in the sequence in which the main components of the latter venom and that studied here were separated seems significant. The sequence in each case was the removal of the coagulating factor, the separation of two types of neurotoxins by alcohol fractionation and the chromatography of these on the resin IRC-50.

This paper presents evidence that a number of neurotoxins occur in the venom of *Notechis scutatus*. These have been separated from the enzymes responsible for the other main pathological effects of the venom and include two types of neurotoxins.

MATERIALS AND METHODS

Dried venom. Venom of *Notechis scutatus scutatus*, purchased from E. Worrell, Umina, New South Wales, Australia, had been dried over calcium sulphate as soon as possible after collection. Pooled lots were powdered and re-dried *in vacuo* over phosphorus pentoxide.

Preparation of resin Amberlite IRC-50 (XE-64). The resin (a research gift from Dr J. C. Winter, Rohm and Haas Co., Philadelphia, Pa., U.S.A.) was prepared for chromatography by the method described by Hirs, Moore & Stein (1952) and then equilibrated with the appropriate buffer. The resin was recovered after use by recycling through the sodium form followed by heating for 3 hr. with 2N-HCl on a boiling-water bath, washing and then re-equilibrating as desired.

Paper electrophoresis and elution. The methods were essentially those of Kunkel & Tiselius (1951), Munktel no. 20 filter paper being used. Dextran was used as an indicator of the extent of electro-osmosis. Samples (200–250 μ g.) were examined as 5% (w/v) solutions in the buffer solution selected. After staining with bromophenol blue, elution curves were drawn relating the optical density, at 590 $m\mu$, of the dye eluted from successive segments of the paper strip with the distance of these from the point of application of the sample. Where samples were examined

as adjacent spots on the same strip of paper, the elution curves for each are recorded on the same figure.

Toxicity. White mice in groups of two to five were injected subcutaneously with 1 ml. of solutions of venom over a suitable range of concentrations in 50% steps. Deaths were observed up to 7 days and the LD₅₀ was calculated.

Coagulating factor. This was determined by the coagulation of citrated plasma *in vitro*. The time was recorded in seconds for the coagulation at 37° of 0.1 ml. of human citrated plasma by 0.2 ml. solutions of dried venom and fractions thereof, in tenfold dilution steps. The concentration of the coagulating factor in the venom fractions was recorded as a percentage of that of dried venom by reference to curves relating the logarithm of the concentration to the logarithm of the time of coagulation (Rosenfeld & Lenke, 1935).

Haemolysin. The NaCl-sodium acetate buffer, pH 6.0, used by Holden (1934), was adopted. Rabbit blood was collected and stored in the suspending fluid described by Rous & Turner (1916). Washed rabbit cells were suspended in 20 vol. of the standard buffer as required, and CaCl₂ was added to give a concentration of 2 mg./ml. of suspension. Portions (0.1 ml.) of the suspension of rabbit cells were well mixed with 0.2 ml. lots of the venom solutions in a suitable range of concentrations at 37°. The mixtures were shaken at regular intervals, and the time (min.) for complete haemolysis was recorded (Holden, 1934). From the curves relating log concn. and log time for complete haemolysis, for dried venom and fractions thereof, the concentration of haemolysin in the venom fractions could be determined and expressed as a percentage of that of the dried venom.

When eluates from chromatography columns were examined, samples (0.2 ml.) of these were dialysed in transparent cellulose tubing against the standard NaCl-sodium acetate buffer, with agitation overnight. Samples (0.2 ml.) of a range of concentrations of the same venom solution that had been applied to the column were similarly dialysed and served as controls. After dialysis, the contents of the tubing were quantitatively removed and the haemolysin concentrations determined by the above method.

Estimation of hyaluronidase. This was determined by a method essentially similar to that of Tolksdorf, McCready, McCullagh & Schwenk (1949), a lyophilized preparation of hyaluronidase being used as standard. Hyaluronic acid was prepared from vitreous humour of the ox by the method described by Seastone (1939).

Estimation of phosphatases. The activities of dried venom and selected fractions at concentrations between 0.1 and 0.005% (w/v) were examined against the substrates adenosine 5'-phosphate and adenosine triphosphate at 5 mM-concentrations, an experimental procedure similar to that described by Neumann & Habermann (1955) being used. The activities of venom fractions were expressed as percentages of that of dried venom. Since dried venom showed negligible activity against sodium β -glycerophosphate, the venom fractions were not examined against this substrate.

Estimation of cholinesterase. This enzyme was kindly estimated by Mr de la Lande, of the Nicholas Institute for Medical and Veterinary Research, Sherbrooke, Victoria, with standard manometric procedures with acetylcholine as substrate.

Capillary permeability. Groups of white mice each weighing 22–25 g. were injected intravenously with 0.1 ml. of 1.25% (w/v) solution of the dye Pontamine blue dissolved in 0.85% NaCl. This was followed within 5 min. by the subcutaneous injection in the left abdominal region of 0.1 ml. of the venom solution under test and the mice were killed by coal gas within 30 min. of this injection. Controls were similarly injected with 0.1 ml. of 0.85% NaCl solution. The flap of skin in the region of the injection was turned back and the diameter of any intensified blueing at the site of the injection was measured in two directions and compared with the controls.

Ultrafiltration. A 2% (w/v) aqueous solution of the dried venom was filtered through a membrane similar to that described by Wilson & Holiday (1933). The filtrate was freeze-dried and dialysed in transparent cellulose tubing against distilled water at 6° overnight. All the fractions so obtained were freeze-dried and weighed. These were examined by paper electrophoresis and tested for toxicity.

Fractionation procedures

These are outlined in Scheme 1. The following details are those of a typical experiment.

Removal of the coagulating factor. Adsorption of the venom on BaCO₃ removed the coagulating factor, as Holden (1933) had demonstrated. A suspension of BaCO₃ freshly prepared (Fantl & Nance, 1947) contained 80–100 mg. of BaCO₃/ml. Dried venom (750 mg.) was dispersed in water and centrifuged to remove a small amount of insoluble material. The BaCO₃ suspension was added to the venom solution to give a final concentration of 7–9 mg. of BaCO₃/ml. and 1 mg. of venom/ml., and agitated for 2 hr. and then allowed to stand overnight at 6°. After centrifuging, the supernatant and washings from the BaCO₃ were dialysed against several changes of stirred distilled water at 6° until the pH of the dialysed product fell below 6.0. The resulting solution was freeze-dried and yielded 470 mg. of solid. The coagulating factor was eluted from the BaCO₃ by agitation with 0.2M-sodium citrate. The eluate was then dialysed against distilled water and the product freeze-dried.

Low temperature and 30% methanol precipitation. A 1% (w/v) aqueous solution of the product from which the coagulating factor had been removed (pH 5.9–6.0) was cooled to 2°. Precooled methanol was added slowly with stirring to give a concentration of 30% (v/v), while the temperature was steadily reduced to –5°. After a further stirring for 30 min. at this temperature, the insoluble material was separated in a refrigerated centrifuge and then freeze-dried, yielding 312 mg. of solid (fraction A). The

supernatant was diluted with water to reduce the methanol concentration to below 5% and then freeze-dried, yielding 147 mg. of solid (fraction B).

Chromatography on Amberlite IRC-50 (XE-64). As a guide to the selection of the optimum conditions for chromatography, distribution ratios of fraction A between the resin and a variety of buffers were determined by the procedures recommended by Hirs *et al.* (1952). The distribution ratios for the first equilibration and the re-equilibration recorded in Table 1 indicate that some irreversible adsorption occurred with every buffer system investigated. However, by first eluting with a buffer for which distribution ratios near 1 had been found, and following with M-NaCl as the second eluent, adsorption was largely reversed and a desirable separation resulted. Table 2 records

Table 1. Distribution ratios of fraction A between the resin IRC-50 (XE-64) and a range of buffers

Buffer	pH	Concn. (M)	Distribution ratios	
			1	2
Sodium acetate	5.7	0.2	17.9	26.6
	5.9	0.3	6.8	10.3
Sodium citrate	5.5	0.1	7.7	12.8
	5.5	0.2	7.3	9.6
	5.9	0.2	1.7	3.0
	5.9	0.3	1.2	3.3
	6.0	0.1	4.7	7.0
Sodium phosphate	6.0	0.2	6.4	10.0
	6.3	0.2	2.9	6.6
	6.6	0.2	1.3	3.3
	6.8	0.2	1.25	2.2
	7.0	0.2	0.6	1.3

Table 2. Percentage recovery of fraction A from columns of the resin IRC-50 (XE-64) with 0.2M-phosphate buffer as the first eluent followed by elution with M-sodium chloride

pH	Protein recovered (%)		
	Peak 1	Peak 2	Total
6.0	29	19	48
6.3	44	20	64
6.5	47	29	76
6.6*	66	19	85
6.6*	63	17	80
6.8	61	21	82
6.8	58	25	83
7.0	56	16	72

* Large-scale columns.

Scheme 1. Fractionation procedures.

the percentage recovery of protein from small-scale chromatographic analyses of fraction A, carried out as described in the following section. These results guided the choice of 0.2M-phosphate buffer, pH 6.6, as the first eluent, followed by elution with M-NaCl. The determination of the distribution ratios and all the chromatographic analyses were carried out at 6-8°.

Fraction A. The prepared resin was equilibrated with 0.2M-sodium phosphate buffer, pH 6.6, and two sizes of column were used. For exploratory work 6-7 mg. in 1 ml. of buffer was chromatographed on columns 1 cm. $\times$ 16 cm. with a flow rate of 6-7 ml./hr., and eluates of 1 ml. were collected. On a preparative scale 50-55 mg. in 5 ml. of buffer was applied to a column 2.5 cm. $\times$ 18 cm. and a flow rate of 100-120 ml./hr. provided satisfactory separations, 3 ml. eluates being collected. As soon as the sample had passed into the column, this was followed successively by two equal volumes of buffer, and the buffer reservoir was connected immediately. The elution of the protein was followed by measuring the optical density at 278 $m\mu$ of successive eluates. After emergence of the first protein peak, and when the optical-density values indicated that no further protein was being eluted, the eluent was changed to M-NaCl and a second peak emerged. Elution was discontinued when the optical density returned to the baseline value. Portions of eluates were analysed for toxicity and haemolysin. Successive eluates which concentrated these activities respectively were pooled to form three fractions,

The protein content of each fraction was calculated from the optical density at 278 $m\mu$ relative to that of a known concentration of the starting fraction A. The three fractions from the column were dialysed against distilled water at 6° to remove salts and the products freeze-dried.

Fraction B. The column conditions were the same as those used for the chromatography of fraction A with 0.2M-sodium phosphate, pH 6.6, again as the eluting fluid. Eluates were analysed for toxicity only, the contents of appropriate tubes were pooled, dialysed against distilled water at 6° and the products freeze-dried. This fraction has received limited study.

RESULTS

Paper electrophoresis of the dried venom and fractions thereof are illustrated in Figs. 1, 2 and 5, and provide a guide to the separation procedures.

Considering first the components responsible for the main pathological effects of the venom, Table 3 records the percentage recoveries of the mass, and activity of haemolysin, coagulating factor and the toxicity, together with the LD₅₀ values of fractions taken throughout the procedures already described. Recoveries were determined within each of the three main fractionation steps and these were then related to the original dried venom, which was taken as 100%. By reference to the results of the ultrafiltration experiments reported below, it will be seen that the solids removed during the dialysis of the supernatant from the barium carbonate precipitate would account for a further 11% of the weight of the dried venom. The removal of 90% of the coagulating factor by

Fig. 1. Paper-electrophoresis-elution curve of 250 μ g. of dried venom: 0.04M-veronal buffer, pH 8.6; 300v for 6 hr. D, Position of dextran.

Fig. 2. Paper-electrophoresis-elution curves of ●, 250 μ g. of fraction A; ▲, 250 μ g. of fraction B: 0.04M-veronal buffer, pH 8.6; 300v for 6 hr. D, Position of dextran.

adsorption on barium carbonate, leaving the major part of the toxicity and haemolysin unadsorbed, provided a useful first step. The coagulating factor was eluted from the barium carbonate precipitate with some loss.

The subsequent methanol treatment then concentrated the haemolysin entirely in the insoluble fraction A, and toxicity was spread between both fraction A and the soluble fraction B. However, the difference in type of toxicity, in electrophoretic and chromatographic behaviour of fractions A and B (Figs. 2-4), suggested that different neurotoxins had been separated at this stage.

In animal tests it was shown that fraction A, like the whole venom, affected the eyes and caused paralysis of the hind limbs. Deaths, which were preceded finally with difficulty in breathing, occurred within 1-7 days after injection. Fraction B did not affect the eyes of surviving mice. Deaths occurred within 24 hr. and the action was of a more convulsive nature.

Paper electrophoresis (Figs. 1, 2) showed that fraction A contained the most anionic fraction of the dried venom as well as the component which showed little movement from the origin, whereas the least anionic material of the dried venom had been concentrated in fraction B.

The chromatographic analysis of fraction A, illustrated by a typical elution curve in Fig. 3, resulted in overall recoveries of protein, toxicity and haemolysin in the two peaks of 82, 72-90 and

Fig. 4. Chromatography on IRC-50 XE-64 of 52.5 mg. of fraction B. Eluting buffer, 0.2M-sodium phosphate, pH 6.6; at arrow eluent was changed to M-NaCl. Contents of tubes were pooled to provide the following fractions: tubes 19-25=fraction 1; tubes 26-30=fraction 2; tubes 31-41=fraction 3; tubes 71-75=fraction 4.

Fig. 3. Chromatography on IRC-50 XE-64 of 54 mg. of fraction A. Eluting buffer, 0.2M-sodium phosphate, pH 6.6; at arrow eluent was changed to M-NaCl. ●, Optical density at 278 mμ; ▲, % recovery of haemolytic activity; ■, % recovery of toxicity. Contents of tubes were pooled to provide the following fractions: tubes 18-22=fraction 1; tubes 23-32=fraction 1+; tubes 64-73=fraction 2.

Fig. 5. Paper-electrophoresis-elution curves of fractions (250 μg.) from chromatography of fraction A. ●, Fraction 1; ■, fraction 1+; ▲, fraction 2. 0.04M-Veronal buffer, pH 8.6; 300v for 6 hr. D, Position of dextran.

Table 3. *Recovery of mass, haemolysin, coagulating factor and toxicity as percentages of dried venom*

Fraction	Mass	Haemolysin	Coagulating factor	Toxicity	LD ₅₀ (μg.)
Dried venom	100	100	100	100	11
Insol. aqueous	3	—	—	—	—
Supernatant from BaCO ₃ ppt.					
Freeze-dried	64	90	4	89	—
Eluate of BaCO ₃ ppt.	15	—	75	6	—
Low-temperature methanol treatment of supernatant from BaCO ₃ ppt.					
Fraction A	43	86	1.9	57	8
Fraction B	20	<2	<1	27	8
Chromatography of fraction A on IRC-50					
Fraction 1	21	71	—	9	24
Fraction 1 +	7	4	—	15	4
Fraction 2	8	3	<1	21	4

90 % respectively of the material applied to the column. The small amount of trailing protein eluted after the first peak was not included in these recoveries. The three fractions selected, referred to as 1, 1+ and 2, contained the haemolysin which emerged sharply in the first peak at the elution volume of the column, the neurotoxin which immediately followed it with some overlap and the neurotoxin of peak 2 respectively (Fig. 3 and Table 3). The last neurotoxin, which was free of significant amounts of the haemolysin and coagulating factor, is now referred to as neurotoxin A. Fig. 5 shows the paper-electrophoresis-elution curves of the freeze-dried preparations of these three fractions. Fraction 2, which was then incompletely soluble, was applied to the paper as a suspension. The most anionic fraction contained an enriched haemolysin, the least anionic fraction, a neurotoxin, and the one which shows little movement from the origin contains neurotoxin A. On dialysis of these fractions against distilled water at 6° overnight, followed by freeze-drying of the product, no loss of protein could be detected in any of the above three fractions, whereas losses of 30 % of the haemolytic activity of fraction 1, and 27 and 40 % of the toxicity of fractions 1+ and 2 (neurotoxin A) respectively occurred. Fractions 1 and 1+ were thereafter soluble in water, whereas approx. 30 % of neurotoxin A was insoluble but dissolved in 2 % sodium chloride. However, toxicity tests failed to detect any significant difference in toxicity between the soluble and insoluble forms.

All three fractions produced the typical effect of the unfractionated venom on the eyes of mice surviving a near-lethal dose.

The chromatographic analysis of fraction B, the fraction which was soluble in 30 % (v/v) methanol, is illustrated by a typical elution curve in Fig. 4. Overall recoveries of 88 % of the protein and 94 %

Table 4. *Recovery of protein and toxicity in fractions from chromatography of fraction B on IRC-50 (XE-64)*

Fraction	Protein recovery (%)	Toxicity (%)
1	64	55
2	16	25
3	5	13
4	3	2

of the toxicity resulted. The eluates in the main protein peak which eluted sharply with the first eluent, and at the elution volume of the column, were divided into three fractions according to the slope of the elution curves. With the second eluent, m-sodium chloride, a further 2–3 % of both the protein and the toxicity was eluted. Table 4 shows the recovery of the protein and toxicity in each fraction, expressed as a percentage of that of the material applied to the column. On dialysis against distilled water at 6° overnight, neither the protein nor the toxicity of fraction 1 was dialysable, and 80 % of both the protein and toxicity were found in the diffusate of fraction 2. Fractions 3 and 4, available in only small quantities, were not examined in this manner. The small concentration of toxic material isolated in fraction 4 affected the eyes of surviving mice in the same manner as the neurotoxin A (Fig. 3). It was assumed on the basis of both its properties and position on the chromatographic column to be a small amount of this fraction incompletely precipitated by the 30 % methanol.

Table 5 shows the distribution of the other enzymes which were studied. No significant activity of either the hyaluronidase or the phosphatases remained after the barium carbonate treatment. At this stage 41 % of the cholinesterase activity also was lost, the remainder being concentrated in the chromatographic component of

fraction A which contained the haemolysin, with some overlap into the adjacent fraction.

In the ultrafiltration experiment the freeze-dried filtrate contained 17 % of the mass of the venom applied to the filter and, on dialysis, 11 % of this passed through the membrane, the remaining 6 % being non-dialysable. The last portion, when examined by paper electrophoresis, proved to correspond in position to the least anionic component of the original venom. Toxicity tests showed that it contained neurotoxic material, which represented 5 % of the toxicity of the venom applied to the filter, with properties indistinguishable from those associated with the fraction B separated from the unfiltered dried venom. The portion retained by the membrane, when fractionated by the procedures outlined above, still contained a fraction with all the properties of fraction B from unfiltered dried venom. Inorganic salts and small organic molecules, among which purine compounds have been identified (Doery, 1957), no doubt account for the 11 % of the venom which was dialysable. Thus although no great separation of protein components was achieved by this technique, material with the properties of fraction B was found in both the portion retained by the ultrafiltration membrane and that portion of the filtrate which was non-dialysable.

The experiments designed to detect increased capillary permeability showed that the dried venom, the neurotoxin A and the fraction containing the majority of both the haemolysin and the cholinesterase were all highly active. Mice were sensitive to less than 0.02 µg. of each of the above preparations, i.e. approx. 1/200–1/800 of the LD₅₀ dose for these samples. The average diameter of the area of intensified blueing observed was 10–20 mm. Whereas fraction B, containing the least anionic neurotoxins, was definitely active, a dose of more than 0.2 µg. was required to cause a response. This dose is approx. one-fortieth of that of the LD₅₀ for this fraction.

DISCUSSION

Although primarily concerned with the neurotoxins of the venom of *Notechis scutatus*, this study has provided methods for obtaining the haemolysin and the coagulating factor in separate fractions.

From the results presented it may be concluded that two types of neurotoxin were present. One type, represented by neurotoxin A, when tested in mice first caused paralysis of the hind limbs. Breathing became progressively more difficult before death, which occurred in 1–7 days after injection. Mice surviving a near-lethal dose showed the effect on the eyes typical of that produced by the unfractionated venom.

The paper-electrophoresis pattern of neurotoxin A (Fig. 5) and the absence of significant amounts of the main enzymes of the venom (Tables 3 and 5), encourage the belief that neurotoxin A is substantially free from the other main components of the venom. However, while approximately a three-fold concentration of toxicity from the crude venom has been demonstrated in this fraction, on the basis of activity: mass ratio, the concentration of 3 % of the total haemolysin in 8 % of the mass indicates that the isolation of neurotoxin A is incomplete. The property which affects the eyes of mice surviving a near-lethal dose is shared by three chromatographic fractions, including neurotoxin A. Although the chromatographic peak containing neurotoxin A is separated from that containing the haemolysin and the less anionic neurotoxin adjacent to it on the column (Fig. 3), the paper-electrophoresis patterns of all three fractions (Fig. 5), and also the distribution of the other enzymes reported in Table 5, show that the latter two are by no means homogeneous. Both include definite amounts of the stationary component which on paper electrophoresis occupies the same position as neurotoxin A.

Lysolecithin, when injected into the anterior chamber of the rabbit's eye, produces an effect on

Table 5. *Distribution of hyaluronidase, cholinesterase and phosphatase in fractions, expressed as a percentage of that of dried venom*

Fraction	Hyaluronidase	Cholinesterase	Phosphatases	
			AMP	ATP
Supernatant from BaCO ₃ ppt.	Nil	—	—	—
Eluate of BaCO ₃	Nil	—	—	—
Fraction A	—	59	<5	10
Fraction B	—	Nil	<10	<10
Chromatography of fraction A on IRC-50				
Fraction 1	—	46	—	—
Fraction 1 +	—	7	—	—
Fraction 2	—	Nil	<5	<5

the cornea (Feldberg & Kellaway, 1938) which is similar to that described by Kellaway (1929) as characteristic of mice surviving the venom of *N. scutatus* as distinct from the venoms of the other Australian snakes. Since all these venoms which contain haemolysins produce lysolecithin, it would appear that the observed effect on the eyes can be produced by some other venom component. The fractions containing neurotoxin A have been found to affect the eyes of test mice, and it seems probable therefore that this is an inherent property of this neurotoxin.

The other type of neurotoxin, represented by two or more proteins, produced a more convulsive action in mice. No effect on the eyes was observed and death, which was more rapid, occurred within 24 hr. The evidence for two or more neurotoxins within this group is found in the distribution of this type of activity in both the filtrate and the remainder of the venom separated by the ultra-filtration experiments, also in two of the fractions which behaved differently on dialysis, obtained from the chromatography of fraction B. Without further study, the identity of neurotoxins within this group cannot be established, but we may conclude that two or more neurotoxins are represented here, which may differ from one another only in molecular size.

It is probable that the neurotoxic activity of the chromatographic fraction 1+ is modified by the presence of traces of the haemolysin and neurotoxin A, the presence of which may be due to adsorption of traces of fraction B on fraction A during precipitation (Figs. 3, 5). The small losses in toxicity during the three main separation procedures suggest that no other type of neurotoxin makes a significant contribution to the toxicity of the unfractionated venom.

Reference to Table 3 indicates that no great concentration of toxicity has been achieved in the neurotoxins isolated in comparison with that of the unfractionated venom. This fact is to be expected when the toxicity is shared between a number of components and is in keeping with the observations of Kellaway (1929) that the venom of *N. scutatus* was predominantly neurotoxic in action.

Boman & Kaletta (1957) and Boman & Westlund (1957) drew attention to the frequency with which enzymes from a wide variety of sources, originally considered to be homogeneous, have subsequently been resolved by chromatography and electrophoresis into two or more enzymes of the same type. The venom of *Notechis scutatus* may now be included among the snake venoms in which groups of neurotoxins have been reported. These are the venoms of the cobra species *Naia naia* and *N. flava* (Micheel, Dietrich & Bischoff, 1937), and of *Crotalus terrificus terrificus* (Gonçalves & Vieira, 1950; Neumann & Habermann, 1955).

Enzymes variously reported in snake venoms other than those listed in Tables 3 and 5 have not been studied. However, the paper-electrophoresis-elution curve of the eluate from the barium carbonate precipitate which was not recorded here indicated that it contained a mixture of proteins which were not identified. The enzymes shown to be lost at this stage must be either inactivated or adsorbed along with any inert protein in the venom. Hence a variety of proteins could be anticipated in the eluate of the barium carbonate precipitate, of which the coagulating factor is only one.

Considering the chromatographic separations, the results of the preliminary studies of the resin IRC-50 recorded in Tables 1 and 2 show that the distribution ratios of fraction A between the resin and various buffers are strongly influenced by the nature of the buffer salt, the pH and the concentration. At pH 6.6 the total protein eluted, as well as that eluted in peak 1, were a maximum. The buffer at this pH was chosen because it provided the greatest selection of material separating as peak 2.

The use of this resin for the isolation of the neurotoxins crotoxin (Neumann & Habermann, 1955) and crotoamine (Gonçalves, 1956), from the venom of *Crotalus terrificus*, and also for the isolation of the two types of neurotoxin reported here, emphasizes the wide applications that are possible in problems of protein fractionation. In the course of isolating these neurotoxins, crotoxin, the acidic neurotoxin, was eluted with phosphate buffer, pH 6.87, leaving the haemolysin from the same venom strongly adsorbed. With the venom studied here, at pH 6.6 the anionic haemolysin and the less anionic neurotoxins were eluted from the column, whereas neurotoxin A, which was stationary during electrophoresis, was strongly adsorbed. It should be noted that flow rates which operated in each case were considerably greater than those normally required for sharp separations in protein chromatography. Boardman & Partridge (1953), Boardman (1955) and Neumann & Habermann (1955) have proposed probable operating mechanisms in the separation of proteins which they studied. Without considerably more data on the behaviour of the individual components concerned here under various operating conditions no further contribution can be made on this point. However, the separations reported may serve to emphasize the difficulty of forecasting the most selective adsorbent for proteins which are alike in respect of their basic units only but differ in molecular size, structure and net charge.

The pharmacological action of the neurotoxins crotoxin, predominantly paralytic, and crotoamine, predominantly convulsive, which Neumann & Habermann (1955) described, and the two types of

neurotoxin reported here, show some broad similarities in respect of these properties. The increase in capillary permeability shown by both the last-named two types of neurotoxins and crotactin extends this similarity. Since the neurotoxins in question may be considered to be free from significant amounts of the main enzymes of the venoms, the demonstration of properties common to neurotoxins from two different sources stimulates interest in the problem of their mode of action.

The response of the isolated jejunum of the guinea pig suspended in Tyrode's solution shown by various Australian snake venoms has been described by Feldberg & Kellaway (1937) and Trethewie (1939). Dr E. R. Trethewie (unpublished data) demonstrated response, with the typical 'slow-reacting substance' type of reaction, after the injection of 100–200 µg. of both neurotoxin A and fraction B, both being less active than the whole venom. The question whether any or all of the following properties, namely the increase in capillary permeability, the 'slow-reacting substance' activity and the effect on the eyes of animals surviving a near-lethal dose, are intimately associated with the mechanism of the action of the neurotoxins studied here, cannot be answered conclusively within the scope of this report.

A further paper, relevant to this report, by Neumann (1957), describing improved methods for the isolation of crotactin, phospholipase A, crotoamine and a third toxin, 'toxin III', from the venoms of Brazilian rattlesnakes, appeared as this paper left for the press.

SUMMARY

1. Two types of neurotoxin have been separated from the venom of the tiger snake *Notechis scutatus scutatus* by means of adsorption on barium carbonate, low-temperature methanol-fractionation and chromatography on the Amberlite resin IRC-50.

2. One type of neurotoxin is stationary during paper electrophoresis at pH 8.6; the other type is represented by a group of weakly anionic proteins.

3. Both types of neurotoxin have been separated from significant amounts of the components responsible for the other main pathological effects of the venom, i.e. the coagulating factor and the haemolysin, as well as the enzymes hyaluronidase, phosphatases and cholinesterase.

4. The properties of the neurotoxins are described and discussed in relation to those of the neurotoxins of the venom of *Crotalus terrificus terrificus*.

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