

## Separation of Protein and Enzyme Components of Venom of the Indian Cobra (*Naja naja*) under Disaggregating Conditions

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Strong protein-protein interactions have been detected among components of venom of the Indian cobra (*Naja naja*). Such interactions lead to "apparent" heterogeneity being observed upon column chromatography and disc electrophoresis. Especially the toxic proteins appear to show a marked tendency to form aggregates (homoaggregates), with the aggregates possessing different lethal potencies. Even the enzymes form aggregates (heteroaggregates). Triton X-100 abolishes most of such aggregation and yields nearly electrophoretically homogeneous proteins, without impairing the lethal potency of the neurotoxin. Nearly a dozen enzyme activities have been screened in crude venom and fractions thereof. Significant protease activity is associated with cobra venom and some of its fractions. Fractions that apparently correspond to cardiotoxins/cobramines, which have hitherto been not known to be enzymatically active, display pyrophosphatase activity.

**Key Words:** *Naja naja*, Enzymes, Proteins, Aggregation, Triton X-100

### Introduction

The chemistry and biology of snake venoms have been extensively reviewed (Lee 1979, Tu 1977a). Several snake venom toxins have been purified and the amino acid sequence of over 80 toxins has been determined (Yang 1978). Phenomena of aggregation among snake venom proteins leading to difficulty of obtaining clean separations and pure proteins have been noted earlier (Meldrum 1965, a, b, Fischer & Kabara 1967, Kabara 1971, Henriques & Henriques 1971). Aggregation of identical molecules, with formation of homoaggregates to varying extents, leads to "apparent" heterogeneity that can be very misleading.

We report in this paper modification of an ion-exchange column chromatographic procedure (Achyuthan et al. 1980) for the separation of Indian cobra (*Naja naja*) venom proteins involving the use of the non-ionic detergent Triton X-100 (polyethyleneglycol -p- isooctylphenylether) to overcome problems posed by aggregation. Nearly a dozen enzyme activities have been screened for in cobra venom and fractions obtained therefrom.

### Materials and Methods

Commercial samples of Indian cobra (*Naja naja*) venom (Batch No. 195-213)

were obtained from Haffkine Institute, Bombay. For assay of phospholipase A, egg yolk homogenate from hen's egg was used as substrate. All other chemicals and reagents used were commercial samples of analytical grade. All solutions were made in glass distilled water. Folin's reagent was prepared (Lowry et al. 1951) in the laboratory.

Protein was estimated by Folin-Lowry method (Lowry et al. 1951). Inorganic phosphate was determined (Fiske & Subbarow 1923) colorimetrically. Colorimetric measurements were made using a Coleman Junior Spectrophotometer. Samples were lyophilized in a Toshniwal Lyophilizer. Absorption spectra were taken on a Toshniwal Model Spectrophotometer Type RL O2.

Lethality of the samples was determined after intra-peritoneal administration of varying doses of the proteins to a set of forty albino mice (18-20 g body weight) of either sex, divided into five groups of eight animals each. The  $LD_{50}$  and slope function values were calculated as described by Litchfield and Wilcoxon (1949), slope function being defined as  $1/2(LD_{84}/LD_{50} + LD_{50}/LD_{16})$ . Preliminary screening of fractions for toxicity was done by i.p. administration of 10  $\mu$ g or less of the sample to a group of four albino mice (18-20 g body weight) of either sex. Although mortality was observed over a period of 72 hr in no case did death occur beyond 24 hr.

Disc electrophoresis was done with continuous and/or discontinuous systems. Continuous electrophoresis was done on polyacrylamide gels (7.5%) using 0.2M acetate buffer, pH 4.2 and a current of 4 mA/tube. Discontinuous electrophoresis was done on 7.5% gels using 0.2M acetate buffer pH 4.2 with tris-glycine pH 8.5 as the matrix buffer. The gels were stained in 1% Amido Black 10 B in 7%

acetic acid and destained with 7% acetic acid. SDS-electrophoresis of samples incubated with 0.2% SDS at 40° for 2 hr was carried out in a continuous system using 0.2M acetate buffer, pH 4.2 or 0.2M citrate-phosphate buffer, pH 6.0, each buffer containing 0.2% SDS, with a current of 8 mA/tube. The gels were stained with 0.25% Coomassie Brilliant Blue for 2 hr and the excess dye was washed off in a solvent system containing acetic acid; methanol : water (7.5 : 5 : 87.5). All fractions had cathodic mobility.

*Enzyme assays:* The following enzyme assays were carried out on crude venom and fractions therefrom using previously described assay procedures: protease (caseinolytic) (Anson 1938), 5'-nucleotidase (Heppel & Hilmoe 1955), deoxyribonuclease I and II (DNase I and II) (McDonald 1955), alkaline phosphomonoesterase (Heppel 1955a), ribonuclease (RNase) (Takahashi 1961), phosphodiesterase (Butler 1955), inorganic pyrophosphatase (Heppel 1955b), ATPase (Kielley 1955), acetylcholinesterase (Bockendahl & Ammon 1963), and phospholipase A (Tu & Passey 1971).

Inorganic pyrophosphatase activity was assayed by incubating 1.1  $\mu$ moles of sodium pyrophosphate, 1-5  $\mu$ moles of  $MgCl_2$  and 500  $\mu$ g of enzyme protein (crude venom or peak fractions) in a final volume of 1 ml. of 0.05M tris-HCl buffer of pH 7.2. At the end of 15 min the inorganic phosphate liberated was estimated by the method of Fiske and Subbarow (1923).

Protease activity was also assayed according to the method of Kunitz (1947) as follows: 1 ml of a 1% casein solution was mixed with 1 ml of 10.05-0.1% enzyme (peak fractions or crude venom) and kept at 35° for exactly 20 min. Then 3 ml of 5% trichloroacetic acid was added to terminate the reaction and the

mixture was left at room temperature for 1 hr. It was then centrifuged at 3000 g for 20 min and the OD (280 nm) of the supernatant was read against a suitable blank. The specific activity of the enzyme was calculated according to Kunitz (1947) as modified by Friederich and Tu (1971).

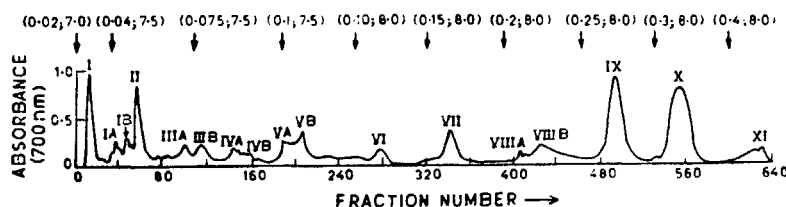
### Experimental and Results

**Ion-exchange chromatography of cobra venom:** Cobra venom was fractionated on CM-Sephadex C-25 columns according to a procedure reported earlier (Achyuthan et al. 1980). The elution profile obtained is shown in figure 1. Cobra venom was then fractionated on CM-Sephadex C-25 column (0.9 × 70 cm) using phosphate buffers containing the non-ionic detergent Triton X-100 at concentrations of 1% and 0.1% (v/v). The elution profile obtained at either concentration of the detergent was similar. The elution profile shown in figure 2 was obtained when cobra venom was fractionated on CM-Sephadex C-25 column using eluents containing 0.1% (v/v) Triton X-100. The venom was dissolved in the starting buffer containing 0.1% Triton X-100 and applied to the column. Protein recovery was 67%. Fractionation was carried out at room temperature.

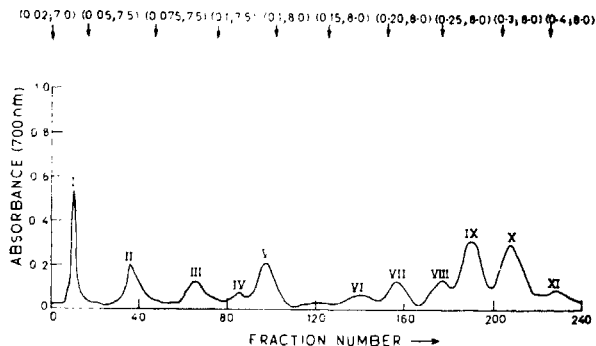
**Desalting:** Fractions corresponding to different peak materials (figure 2) were pooled separately and lyophilized. Lyophilized samples were desalted on Sephadex G-10 columns of size 3.8 × 40 cm using 0.01N acetic acid as eluent. Two ml fractions were collected at a flow rate of 40 ml/hr. Protein got eluted in tubes 75-95, while phosphate emerged from tube 100 onwards. The desalted fractions were pooled, lyophilized and stored at 4°C.

**Rechromatography:** Fraction V-A (figure 1) was rechromatographed on a DEAE-cellulose column (0.9 × 70 cm) using 0.01M phosphate buffer pH 8.5 without incorporation of Triton X-100. A single peak emerged unadsorbed soon after the hold up volume. Fraction V-A was then further rechromatographed on a column (2.5 × 76 cm) of CM-Sephadex C-25 using 0.05M phosphate buffer, pH 6.7, that also was devoid of Triton X-100. Two non-symmetric peaks—peak V-A<sub>1</sub> and V-A<sub>2</sub>—were obtained in approximately equal yields (figure 3).

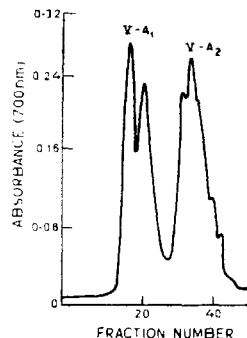
**Disc Electrophoresis:** Fraction V-A (from figure 1) revealed (figure 4A, 2) four cathodically migrating bands upon electrophoresis at pH 4.2. Fraction V-B (from figure 1) under similar conditions gave 8 bands (figures 4A, 3). Fractions V-A<sub>1</sub> and V-A<sub>2</sub> (from figure 3) each got



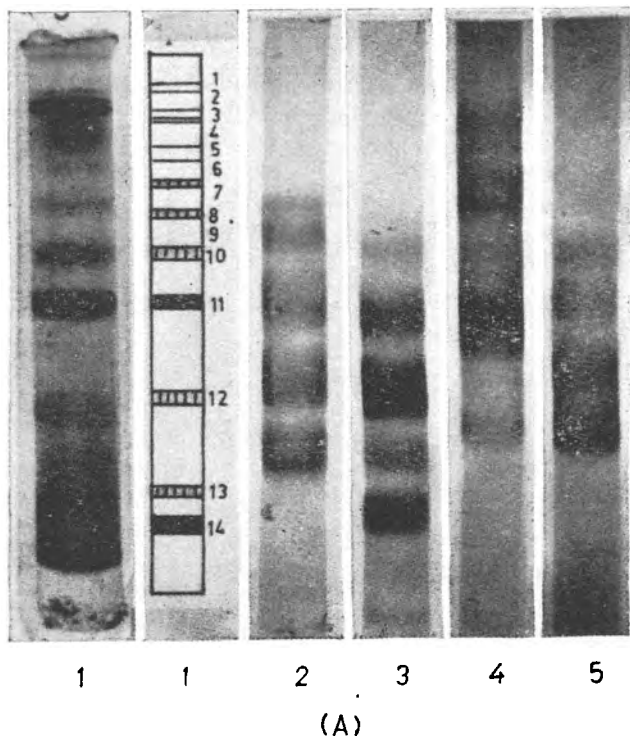
**Figure 1** CM-Sephadex C-25 column chromatography of *Naja naja* venom. Haffkine Batch Nos. 195-203; load: 7.5 g/18 ml of 0.02M phosphate buffer, pH 7.0; dimensions of column packing: 4 × 110 cm; flow rate: 340 ml/hr; fraction vol: 40 ml; elution was carried out stepwise with phosphate buffers of molarities and pHs as indicated. The values in parentheses on top of the figure refer, respectively, to the molarity of the eluent and its pH. Recovery: 83%.



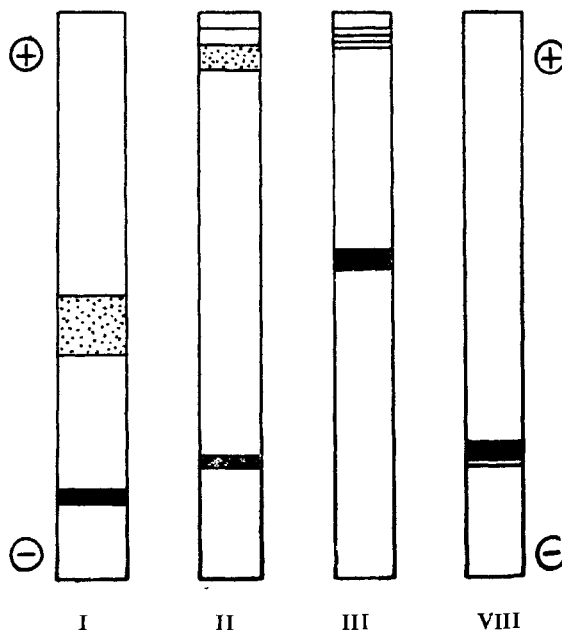
**Figure 2** CM-Sephadex C-25 column chromatography of *Naja naja* venom. Haffkine Batch No. 200; load : 100.80 mg in 1.0 ml of 0.02M phosphate buffer, pH 7.0 containing 0.1% (v/v) Triton X-100; dimensions of column packing : 0.9 × 70 cm; flow rate: 20 ml/hr; fraction volume: 2 ml; elution was carried out with phosphate buffers of molarities and pHs as indicated. All buffers contained 0.1% (v/v) Triton X-100. The values in parentheses on top of the figure refer, respectively, to the molarity of the eluent and its pH. Recovery: 67%.



**Figure 3** Rechromatography of fraction V-A obtained from column run (figure 2) on CM-Sephadex C-25 column; load : 43.1 mg fraction V-A material in 0.5 ml of 0.05 M phosphate buffer, pH 6.7; dimension of column packing : 2.5 × 76 cm; flow rate : 100 ml/hr; fraction volume : 5 ml; elution was carried out at room temperature with 0.05 M phosphate buffer, pH 6.7



**Figure 4 A** Disc electrophoresis (pH 4.2) of cobra venom (1 and 1) and of fractions V-A (2), V-B (3), V-A<sub>1</sub> (4) and V-A<sub>2</sub> (5) obtained from column runs shown in figures 1 and 3. Time of run: 1.5 hr (for experimental details see text)



**Figure 4 B** Disc electrophoretic patterns (pH 4.2) of fractions I, II, III and VIII obtained from column run shown in figure 2. Time of run: 1.5 hr (for experimental details, see text)

**Table 1** Yield and toxicity of fractions from cobra venom (Reference to figure 2)

Peak No.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Weight yield of fraction %	5.7	5.3	4.8	2.3	8.0	3.0	4.9	4.7	12.3	12	4.0
Toxicity* µg/mouse	NT**	10	10	10	5	10	NT	10	NT	NT	NT

\*Fractions that cause death of albino mice (18-20 g wt.) at dose levels of 10µg or less per mouse are considered toxic; cobra venom was toxic at 15 µg/mouse.

\*\*NT—Non-toxic

separated into four non-overlapping bands (figures 4 A, 4 and 5) at pH 4.2. Seven out of eleven fractions obtained from CM-Sephadex C-25 chromatography (figure 2) using 0.1% Triton X-100 did not resolve into several components on electrophoresis at pH 4.2, while four fractions (fractions I, II, III & VIII) revealed more than a single band on electrophoresis at pH 4.2 (figure 4 B). Crude venom revealed 14 bands upon disc electrophoresis using the continuous system (figures 4 A, 1 & 1).

**SDS-Electrophoresis:** Fraction V-A (from figure 1) was subjected to SDS-electrophoresis as described under Methods. It migrated as a single band at pH 4.2 and pH 6.0 (figures 4 C, 1 & 2 respectively). Similarly, fraction V (from figure 2) appeared monodisperse at pH 4.2 and pH 6.0 (figures 4 D, 1 & 2) respectively.

**Toxicity of fractions:** Among the fractions obtained after Triton X-100 chromatography of crude venom (figure 2), fractions II, III, IV, V, VI & VIII were lethal to mice at dose levels (i.p.) of 10 µg or less per mouse. Fraction V was lethal even at a dose level of 5 µg per mouse (table 1). The toxic fractions accounted for 28% of total venom protein.

**LD<sub>50</sub> determinations:** The LD<sub>50</sub> of cobra venom and of fractions V-A and V-B (from figure 1), V-A<sub>1</sub>, V-A<sub>2</sub> (from figure 3) and of fraction V (from figure 2)

are given in table 2. The LD<sub>50</sub> for Triton X-100 is known (Schwartz et al. 1958) to be 0.4 to 1.5 g/kg. and any contaminating detergent in protein fractions would not materially affect LD<sub>50</sub> values measured for protein fractions.

**Table 2** LD<sub>50</sub> values of cobra venom and fractions thereof

Sample	LD <sub>50</sub> (mg/kg)	slope func- tion	Potency ratio**	Ref. to column run
Cobra venom	0.46 (0.58-0.37)*	1.50	1.0	—
Peak V-A	0.19 (0.25-0.14)	1.65	2.5	Fig. 1
Peak V-B	0.30 (0.34-0.26)	1.26	1.5	„ 1
Peak V	0.18 (0.22-0.15)	1.39	2.6	„ 2
Peak V-A <sub>1</sub>	0.30 (0.24-0.20)	1.75	1.5	„ 3
Peak V-A <sub>2</sub>	0.19 (0.25-0.14)	1.65	2.5	„ 3

\*Values in parentheses represent 95% confidence limits

\*\*The potency ratio of crude venom in mice is taken as 1

**Spectral studies:** The absorption spectra of fractions V-A, V-B (from figure 1), V-A<sub>1</sub> and V-A<sub>2</sub> (from figure 3) were taken in distilled water and 0.1N NaOH. Broad maxima were obtained. The E<sub>1%<sup>1</sup>cm</sub> at 280 nm was also calculated (table 3). The spectrum of fraction V (from figure 2) was identical to the spectrum of Triton X-100 both when taken in

**Table 3** Spectral data for cobra venom fractions

Fraction (Peak)	$\lambda$ Max. (water) (nm)	$\lambda$ Max. (0.1N NaOH) (nm)	$E^{1\%}_{1cm}$ (280 nm) (water)	$E^{1\%}_{1cm}$ (280 nm) (0.1N NaOH)	Ref. to figure
V-A	275-280	280-284	7.8	8.8	1
V-B	274-230	278-284	8.6	10.0	1
V-A <sub>1</sub>	272-280	280-283	6.6	8.8	3
V-A <sub>2</sub>	272-280	278-283	7.4	10.0	3

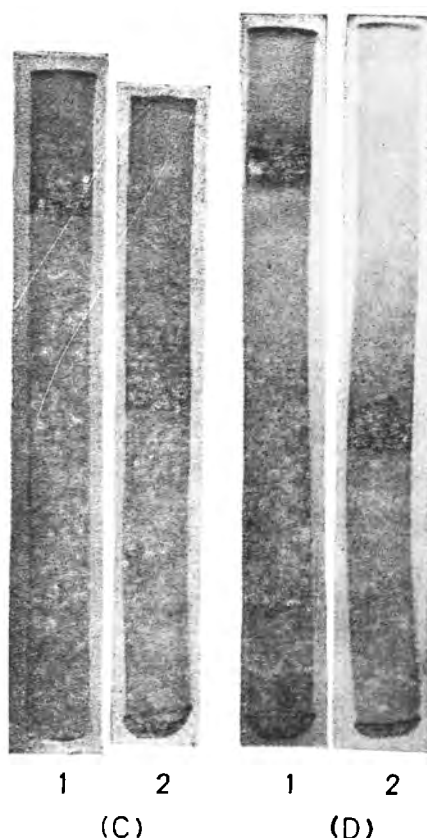
distilled water and in 0.1N NaOH. Even at very low concentrations, Triton X-100 showed high absorbance around 280 nm, thus obliterating the absorbance due to protein at this wave length. Hence reliable  $E^{1\%}_{1cm}$  value at 280nm for fraction V (from figure 2) could not be obtained.

**Enzyme assays:** Nearly a dozen enzyme assays (tables 4 & 5) have been carried out on crude venom and fractions thereof (figures 1 & 2). The specific activity of each enzyme in unfractionated cobra venom has been taken as 1 and the activity of peak fractions are expressed on a relative basis.

### Discussion

During the course of the present investigation the occurrence of strong protein-protein interactions among some of the proteins of the venom of the Indian cobra has come to light. The multiple bands obtained on electrophoresis of fraction V-A (figures 4A, 2) and their non-identity with any of the bands obtained on electrophoresis of crude venom (figures 4A, 1 & 1) suggested that the apparent inhomogeneity may probably be due to aggregation. It was surprising that fraction V-A (figure 1) which constitutes only 3.3% of venom protein could separate into two dissymmetric peaks (figure 3) and still reveal four components each upon electrophoresis (figures 4A, 4 & 5). It is conceivable

that intermolecular aggregation occurs leading to differences in size and charge of the aggregates (homoaggregates) and making them behave on column chromatography and electrophoresis as though they were different components. This is borne out by the fact that incorporation of Triton X-100 in the eluents results in most of the fractions obtained, appearing monodisperse upon disc electrophoresis.



**Figure 4 C** SDS-electrophoresis pH 4.2 (1) and pH 6.0 (2) respectively of fraction V-A obtained from column run shown in figure 1. Time of run: 1 hr (for experimental details see text)

**Figure 4 D** SDS-electrophoresis [pH 4.2 (1) and pH 6.0 (2) respectively] of fraction V obtained from column run shown in figure 2. Time of run: 1 hr. (for experimental details see text)

Table 4 Enzyme activities of fractions obtained from column run (figure 1)

Peak No.	I	I-A	I-B	II	III A	III B	IV A&B	V-A	V-B	VI	VII	VIII A	VIII B	IX	X	XI
Enzyme Activity*																
Protease	—	—	—	1.7	0.36	—	1.2	—	1.6	—	—	—	—	—	—	—
5' - Nucleotidase	0.65	—	—	1.0	—	—	—	—	1.0	0.65	1.8	—	1.2	0.8	±	0.65
Deoxyribonuclease I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Deoxyribonuclease II	0.5	1.2	—	1.3	0.5	0.5	0.84	—	0.5	—	—	—	0.8	0.9	—	—
Alkaline phosphomonoesterase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Phosphodiesterase	—	—	—	—	—	—	0.5	—	0.41	—	0.6	—	0.4	—	—	—
Ribonuclease	—	—	—	0.5	—	—	—	—	—	—	—	—	—	—	—	—
Inorganic pyrophosphatase	—	—	—	3.0	—	—	—	—	—	—	4.2	—	19	2.2	10	—
ATPase	—	0.5	—	—	—	—	—	—	—	—	—	—	1	—	—	—
Acetylcholinesterase	—	—	0.12	0.2	—	—	—	—	—	—	—	—	—	—	—	—
Phospholipase A	—	—	—	0.5	0.5	—	0.67	—	—	—	—	—	—	—	—	—

\*The enzyme activity found in crude venom is taken as 1

Table 5 Enzyme activities of fractions obtained from column run (figure 2)

Peak No.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Enzyme Activity*											
Protease	—	0.32	0.2	—	—	—	—	—	—	—	—
5' - Nucleotidase	0.2	—	—	—	—	0.2	0.84	1.4	1.6	—	0.4
Deoxyribonuclease I	—	—	—	—	—	—	—	—	—	—	—
Deoxyribonuclease II	2.0	0.8	—	—	—	—	—	—	—	—	—
Alkaline phosphomonoesterase	—	—	—	—	—	—	—	—	—	—	—
Phosphodiesterase	—	—	—	—	—	—	0.3	—	—	—	—
Ribonuclease	—	0.3	—	—	—	—	—	—	—	—	—
Inorganic pyrophosphatase	—	—	—	—	—	—	2.6	5.0	10	14	—
ATPase	—	—	—	—	—	—	—	1.0	—	—	—
Acetylcholinesterase	—	0.16	—	—	—	—	—	—	—	—	—
Phospholipase A	—	1.50	2.2	—	—	—	—	—	—	—	—

\*The enzyme activity found in crude venom is taken as 1

Similarly SDS-electrophoresis of both fraction V-A (figure 1) and fraction V (figure 2) revealed only a single cathodic band at pH 4.2 and 6.0 (figures 4C & D). Only four protein fractions (I, II, III & VIII) from the Triton X-100 run (figure 2) exhibit slight heterogeneity (figure 4B) on disc gel electrophoresis.

It can be seen from the toxicity and LD<sub>50</sub> data (tables 1 & 2) that, while the lethality of fractions is not affected by the incorporation of a detergent like Triton X-100 in the eluents, the aggregates themselves show differences in lethal potency. This is reflected in the fact that while fractions V-A (figure 1) V (figure 2) and V-A<sub>2</sub> (figure 3) have identical LD<sub>50</sub> values, fraction V-A<sub>1</sub> (figure 3) has its potency greatly reduced to a value equalling that of V-B (table 2). It is possible that aggregation among identical molecules of the toxin leads to the aggregates acquiring different lethal potencies. Heterogeneity of fractions may in part arise due to the particular ion-exchange system (CM-Sephadex) employed. Co-elution of different proteins of venom observed by some earlier workers (Louw & Carlsson 1979) has been attributed to hydrophobic interactions of protein molecules with uncharged parts of dextran gels containing hydrophobic groups. Triton X-100 abolishes such aggregations leaving the toxic potency unimpaired.

Strong protein-protein interactions are known to occur among venom proteins and separation of venom into pure proteins is for that reason often very difficult to accomplish (Meldrum 1965 a, b, Kabara 1971, Henriques & Henriques 1971 a). The crotoxin case is the most striking example of a strong protein-protein interaction which could be broken only on exposure to such a damaging reagent as 1-fluoro, 2, 4 dinitroben-

zene (Fraenkel-Conrat & Singer 1956). Interaction between cardiotoxin and phospholipase A<sub>2</sub> has been demonstrated by Louw and Carlsson (1979). The toxic protein, NVC, from Bulgarian viper venom, that was electrophoretically homogeneous was shown to be a protein complex consisting of at least two components (Tchorbanov et al. 1977). During an investigation of venom of the forest cobra *Naja melanoleuca*, Shipolini et al. (1974) were confronted with a similar situation, where several venom fractions revealed multiple bands upon disc electrophoresis.

Examples of toxin-toxin and toxin-nontoxin associations have also come to light during studies on proteins of scorpion venom (Rochat et al. 1967). Symmetrical peaks of constant specific activity from equilibrium chromatography on Amberlite (Miranda et al. 1966) have been later shown to represent an association of neurotoxic with non-neurotoxic material. Inversely, non-symmetrical peaks showing increasing or decreasing specific activity from beginning to end of a peak may be formed by a single molecular species in monomeric or dimeric forms (Miranda et al. cited in Miranda et al. 1970 a). Some earlier workers (Polson et al. 1946, Meldrum 1965 a, b, Miranda et al. 1966, Fischer & Kabara 1967, Kabara 1971) have made highly pertinent comments on toxin-toxin and toxin-nontoxin associations occurring in cobra (*Naja*) venoms. Fischer and Kabara (1967) postulated that the highly basic small molecular weight neurotoxic proteins may latch on to the large acidic protein molecules in venoms and the later thus act as "carriers" of neurotoxins. Meldrum (1965 a, b) explained the inability of neurotoxins to pass through dialysis membrane as being due to binding of the neurotoxins to large molecular weight



protein carriers. Kabara (1971) has indeed used the term "aggregate" to describe such associations. Our observations on the *Naja naja* neurotoxin are reminiscent of these earlier findings on scorpion and snake neurotoxins. Our studies confirm and extend the scope of these preliminary observations on venom protein interactions.

Aggregation among various protein components of cobra venom is also supported by data on enzymatic activities of cobra venom fractions. Thus, where elution was carried out without incorporation of Triton X-100 in the eluents (figure 1) several fractions exhibit overlapping and "multi-enzyme" activities (table 4). This is due to later eluting peak materials aggregating with earlier eluting protein (enzyme) fractions. But when Triton X-100 is incorporated in the eluents (figure 2) such intermolecular aggregations are abolished, the proteins are more cleanly resolved from one another and consequently enzymatic activities of individual fractions are more discretely restricted to specific fractions (table 5). Thus, protease activity is found in four fractions (table 4) when no Triton X-100 was used in chromatography (figure 1) but this activity is restricted to only two fractions (table 5) obtained from columns that were eluted with 0.1% Triton X-100 containing buffers (figure 2). Even more striking is the occurrence of DNase II activity in nine fractions (table 4), in the absence of detergent and its being limited to only two fractions in the presence of detergent (table 5). Similarly phosphodiesterase, activity is found in four fractions (table 4) and only one fraction (table 5), respectively. One early eluting fraction II (table 4) shows pyrophosphatase activity, while with detergent none of the early eluting fractions (table 5) show this acti-

vity. Also, while only three fractions out of eleven exhibited several enzyme activities (three or more) and two (fractions IV and V) are devoid of any activity when Triton X-100 was incorporated in the eluents (figure 2, table 5), as many as seven fractions out of fourteen obtained in the absence of Triton X-100 (figure 1, table 4) exhibited several enzyme activities (three or more) and two (fractions V-A and VIII-A) are devoid of any activity. Fractions V-A of figure 1 and V of figure 2, comprising 3.3% and 8% of total venom proteins, respectively, are devoid of all enzymatic activities and represent the major neurotoxic peak.

Venoms of Elapidae (e.g. cobra) and Hydrophiidae (sea snakes) are reported to show only very weak or no protease activity when casein or haemoglobin is used as substrate (Tu 1977 b). Ghosh (1936) and Rao and Rao (1956) have earlier reported the presence of proteolytic activity in unfractionated cobra venom. The latter authors have postulated the presence of more than one proteolytic enzyme in cobra venom. However, the activity was very weak as evidenced by the fact that a 24 hr incubation period was needed to obtain detectable activity. Contrary to this, we have detected high caseinolytic activity in crude venom and its fractions. In fact, the specific activity values obtained by us for cobra venom and its fractions (not given here) are as high as those obtained from venoms of Crotalidae and Viperidae by Friederich and Tu (1971). It is these later venoms, especially those of crotalid snakes, that have long been recognised as being rich in proteolytic activity, in marked contrast to venoms from elapid and hydrophid snakes. It is of particular interest to note the occurrence of inorganic pyrophosphatase activity in cobra venom

among the later eluting basic fractions (tables 4 & 5). The only suggestion so far on the possible presence in cobra venom of weak inorganic pyrophosphatase was made by Johnson et al. (1953), during a study of the capability of venom to hydrolyze ATP. One milligram of venom was found to hydrolyze 1.5  $\mu$  moles of pyrophosphate in six days. Inorganic pyrophosphatase activity is appreciable in fractions VIII-B and X (table 4) and IX and X (table 5). Fractions X (table 4) and IX and X (table 5) would correspond to cardiotoxins and cobramines (Condrea 1974). Differences in composition of venom of even the same species collected from different geographical regions (Miranda et al. 1970 a, b, Glenn & Straight 1978) are, however, known to exist.

The specific activity of phospholipase A from Triton X-100 runs (table 5) is higher than for samples obtained in the absence of Triton X-100 (table 4). Perhaps, the non-ionic detergent has a protective effect on the enzyme. Recently a detergent-resistant phospholipase A

was observed in *E. coli* K-12 cells. Triton X-100 effectively protected the enzyme against thermal inactivation. It was suggested that the binding of hydrophobic compounds like Triton X-100 stabilizes the enzyme (Tamori et al. 1979). In contrast, Triton X-100 appears to destabilise the protease. On the other hand, enzymes such as phosphodiesterase, ribonuclease, acetylcholinesterase and ATPase suffer diminution or lack of enhancement in specific activity on chromatography whether in presence or absence of Triton X-100. This may be related to instability under the conditions of chromatography, desalting or lyophilization. Similar effects have been documented for many enzymes and toxins, whether arising from denaturation, autodigestion, partial proteolysis or lyophilization (Bjork & Boman 1959, Henriques & Henriques 1971b, Prescott et al. 1976, Willemsse & Hattingh 1979).

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