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ACTIONS OF WHOLE AND FRACTIONATED INDIAN COBRA (*NAJA NAJA*) VENOM ON SKELETAL MUSCLE

BY

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Muscular weakness is a prominent feature in man of poisoning by the bite of the Indian cobra (*Naja naja*). The principal cause of death in experimental animals is asphyxia resulting from paralysis (Arthus, 1910; Cushny & Yagi, 1918; Kellaway, Cherry & Williams, 1932). The earliest studies (Brunton & Fayrer, 1873, 1874) showed that cobra venom has an action on the direct excitability of skeletal muscle as well as a " curare-like " action on the neuromuscular junction. In frog isolated skeletal muscle it is not possible to demonstrate a complete neuromuscular block with cobra venom in the absence of any effect on the direct excitability of the muscle (Kellaway & Holden, 1932).

Largely on the basis of comparison of different venoms, Houssay (1930) concluded that effects of venoms on muscle cell membrane permeability are due to an enzyme hydrolysing lecithin (phospholipase A) and that the neuromuscular block produced by cobra and other paralytic venoms is due to a separate " neurotoxin." Tobias (1955), from experiments with cobra venom that had been heated at low *p*H, claimed that phospholipase A depolarizes lobster giant axons and frog skeletal muscle. The present work reports an attempt to separate and study the factors acting directly on the muscle cell membrane and those acting at the neuromuscular junction. A preliminary account of some of this work has been published (Meldrum, 1963, 1964).

METHODS

Starch-gel electrophoresis. Weighed samples (10 to 30 mg) of lyophilized *Naja noja* venom from Ross Allen's Reptile Institute, Florida, U.S.A., were absorbed as a 10% solution on filter paper strips. These were inserted in the centre of horizontal starch-gels made with 300 ml. of tris-citrate buffer, *p*H 8.9, and 36 g of hydrolysed starch (from Connaught Laboratories, Toronto, Canada). The tris-citrate buffer consisted of 0.076 M-tris[2-amino-2-(hydroxymethyl) propane-1,3-diol hydrochloride] and 0.005 M-citric acid. Borate buffer (0.3 M-boric acid and 0.06 M-sodium hydroxide), *p*H 8.4, was used in the electrode troughs (Poulik, 1957). Current was passed at a constant voltage (usually 18 V/cm) for 3 to 5 hr until the buffer boundary had travelled the 12 cm from the cathodal to the anodal connecting papers. The 8-mm-deep gel was cut in the horizontal plane and the upper section (3 mm thick) was stained with Amido Black or azocarmine. The lower section (5 mm) was cut into blocks corresponding to the stained bands in the upper section. These were eluted in 10 or 20 ml. of saline at 4° C for 24 or 48 hr. The eluates were used for toxicity tests in mice and for the further procedures described below.

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Phospholipase A activity. Phospholipase A activity was assessed by measuring the rate of release of fatty acid from an emulsion of chromatographically purified ovolecithin (Gallai-Hatchard & Thompson, 1965). Ovolecithin (1 mg) was emulsified in 0.9 ml. of glycylglycine buffer (*p*H 8.5, final molarity 0.05 M) with 2 mg of sodium deoxycholate. Enzyme solution (0.1 ml.) was added to the emulsion whilst shaking it in a 37° C water-bath. The reaction was stopped after appropriate intervals by adding 5 ml. of a mixture of isopropanol (40 vols), heptane (10 vols) and N-sulphuric acid (1 vol). The solution was allowed to stand for 5 to 10 min. 3 ml. of heptane was added and the tube shaken. The mixture was centrifuged and 3 ml. of the upper layer was pipetted off into 1 ml. of Cresol Red (5% saturated aqueous Cresol Red in 95% w/w ethanol). The solution was titrated against 0.005 N-sodium hydroxide (in 90% w/w aqueous methanol) in a Conway microburette. All reactions were performed in duplicate. From the mean values were subtracted blank values obtained by adding the isopropanol-heptane-sulphuric acid mixture before or immediately after the enzyme. Palmitic acid dissolved in heptane was used as a standard.

External recordings from frog sartorius muscle. Sartorius muscles from *Rana temporaria* were isolated with their attached pelvic bone and mounted vertically in frog-Ringer solution at room temperature. The system of Fatt (1950) was used to record the potential difference between the tibial tendon at the top and the surface of the muscle at the level of the fluid meniscus. To make a recording of the surface potential along the length of the muscle the fluid was allowed to run out at a uniform rate in such a way that the meniscus scanned the muscle in 5 sec and the oscilloscope beam was triggered to give a 5-sec sweep concurrently.

Motor end-plate recordings from rat diaphragm. The diaphragm with its rib-cage attachments was dissected from rats weighing between 125 and 195 g during ether anaesthesia. The central segment from the left side was mounted in a bath perfused with oxygenated Krebs-Henseleit-Ringer solution at room temperature. Glass microelectrodes filled with 3 M-potassium chloride solution (resistances 10 to 40 MΩ) were inserted with a micromanipulator (Huxley, 1961) into the muscle fibres as close to the fine nerve terminals as possible. Recording was via silver-silver chloride electrodes, a cathode follower and a DC amplifier.

RESULTS

Starch-gel electrophoresis

After starch-gel electrophoresis of whole venom, staining with Amido Black or azocarmine revealed eight or nine separate bands on the anodal side of the origin and five bands on the cathodal side. These were called A1–9 and C1–5 respectively in order of decreasing mobility; A9 was not always seen. C1 stained purple rather than blue with Amido Black and it sometimes resisted staining with azocarmine. The following band, C2, was diffuse and its staining properties were a variable combination of those of C1 and of the fine protein bands, C3–5. C3 stained with Amido Black as an extremely fine line. The bands C4 and C5 had the appearance of typical protein lines. Some stainable material commonly remained at the origin.

When whole venom was dissolved in acetate buffer at *p*H 4.5 and heated to 100° C for 10 min and then submitted to electrophoresis, bands A4, A5, C1 and C2 were but little altered. The other bands appeared very faint or were completely absent.

Eluates from specific zones of the gel when freeze-dried and resubmitted to electrophoresis produced bands closely resembling those from which they were derived. Eluates of C1 tended to show slight staining in the C2 region. Eluates of C2 were apparently not contaminated with C1.

Toxicity tests in mice

Mice were injected intraperitoneally with whole venom or eluates from starch-gels to determine the location of the toxic elements in the gels and the percentage recovery of the

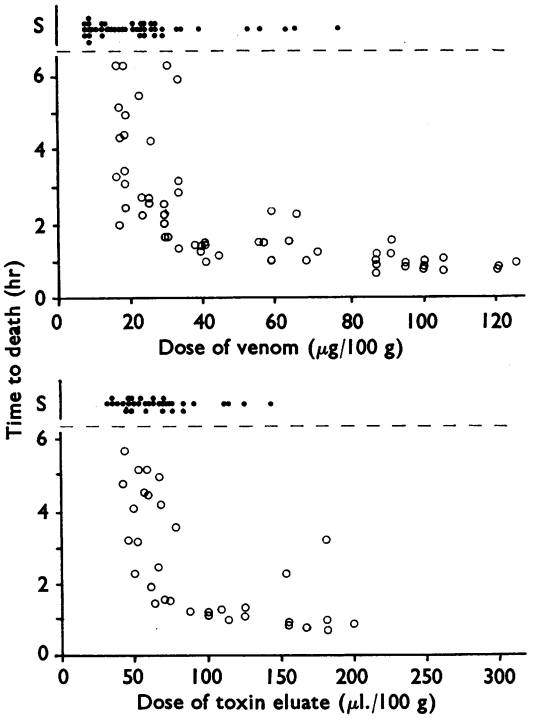

Fig. 1. In the top graph, survival (S) or time to death is plotted against the dose of venom injected intraperitoneally in a total of 103 mice. In the bottom graph, survival or time to death is plotted against the dose of C1 eluate injected intraperitoneally in a total of 67 mice. 10 ml. of saline were used for eluting the gel (in the C1 zone) which had on it 11 mg of venom.

toxicity. The time to death was recorded for mice dying within 6 to 8 hr of injection; survival was assessed at 22 hr. Fig. 1 shows the relationship between time to death and dose for whole venom and for an eluate from the C1 region of a starch gel.

Mice injected with whole venom or with eluates of C1 or C2 showed the same *ante mortem* syndrome of muscular weakness, respiratory distress and cyanosis with terminal convulsive movements. Eluates from the C3–5 region also proved fatal but were twenty- to forty-times less potent than eluates of C1. Mice given eluates from the anodal fractions always survived even when given volumes corresponding to 100-times a lethal volume of C1 eluate.

The relative potencies of eluates of C1 and C2 fractions compared to whole venom were estimated from graphically determined LD50s and from log-log plots of time to death against dose. (It was found to be valid to fit straight lines by regression methods to plots of log dose and log time to death for intervals of 1 to 3 hr. Relative potencies were then estimated from doses producing death at 2 hr.) In the example illustrated (Fig. 1) the LD50 values indicated a recovery of 30% of the venom toxicity in the C1 fraction (with 90% confidence limits of 17 to 54%) and the 2-hr mean time to death values indicated a recovery in this fraction of 44% of the venom toxicity (with 95% confidence limits of 37 to 51%). For the C2 fraction LD50 values indicated a recovery of 9% of the venom toxicity (90% confidence limits, 6 to 15%) and the 2-hr mean time to death values indicated a

recovery of 9% (95% confidence limits, 8 to 10%). From the C3–5 region less than 1% of the toxicity was recovered. If we take the average value of 37% for the toxicity recovered in the C1 fraction and we allow for the retention of 6 ml. of fluid by the starch when each 10 ml. of eluting fluid was separated, we can account for approximately 75% of the venom toxicity in the cathodal fractions.

Phospholipase A from eluates

Eluates from five separate gels showed no measurable phospholipase A activity in the

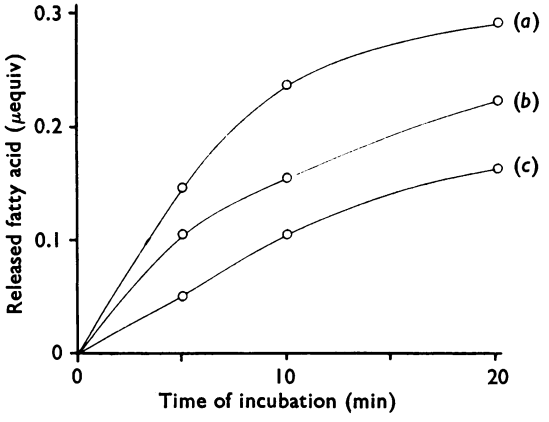

Fig. 2. The phospholipase A activity of venom and of a gel eluate is compared by plotting the rate of fatty acid release from emulsified ovolecithin. (*a*) 0.02 ml. of combined anodal eluates (total volume after dialysis 39 ml., representing 24 mg of venom); (*b*) 4 μg of whole venom; (*c*) 2 μg of whole venom.

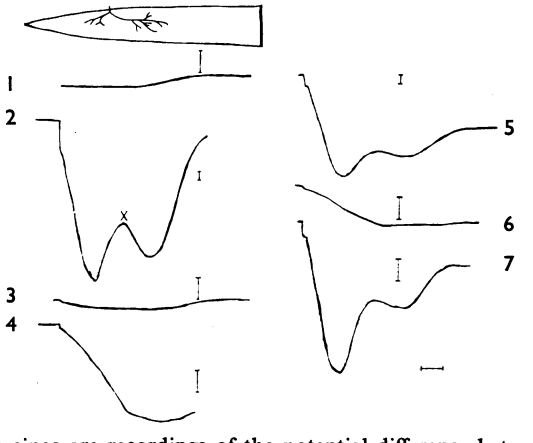

Fig. 3. The numbered tracings are recordings of the potential difference between the tibial tendon and the external surface of the frog sartorius along its length as indicated in the diagram at the top. 1, Resting trace ; 2, trace 1 min after a 45-sec exposure to 10^{-5} (w/v) carbachol; the cross marks the level to which the eluates were brought in 3 and 4; 3, after the lower half of the muscle had been immersed for 3 min in 24 ml. of Ringer solution plus 4 ml. of eluate from a gel in the region beyond C1, on the cathode side; 4, after the lower half of the muscle had been immersed for 3 min in 24 ml. of Ringer solution plus 3 ml. of a C1 eluate (equivalent to 1 mg of venom if recovery is 100%); 5, 1 min after a 60-sec exposure to 10^{-5} carbachol; 6, trace taken after overnight storage at 4° C; 7, 1 min after a 30-sec exposure to 10^{-5} carbachol. Vertical calibrations, 2 mV; horizontal calibrations, 3 mm.

C1 fraction whether they were tested directly or dialysed and freeze-dried before assay. The C3–5 fraction likewise had no phospholipase A activity. On one occasion the C2 region appeared to have just detectable activity. The combined anode fractions always had a high phospholipase A activity. In the experiment illustrated (Fig. 2), determination of initial velocities by graphical extrapolation shows that approximately 50% of the enzyme activity put on to the gel was recovered in the 39 ml. of eluate from the anode fraction.

Depolarization of frog sartorius muscle

Frog sartorius muscles after immersion for 60 sec in 10^{-5} w/v carbachol showed a characteristic pattern of depolarization (Fig. 3, trace 2).

The two peaks of depolarization corresponded to the two zones of maximum end-plate density; the pelvic end has no end-plates and was not depolarized. When the lower (pelvic) half of the muscle was exposed to Ringer solution containing 3×10^{-5} cobra venom a sustained depolarization involving the end-plate free zone of the muscle was produced

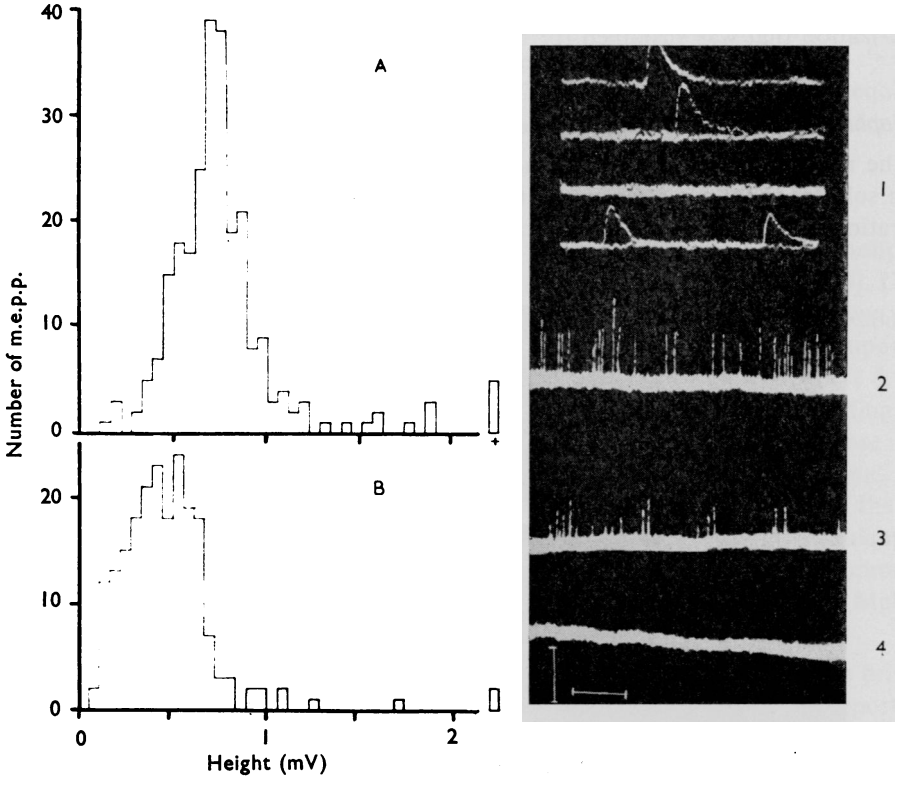

Fig. 4. Spontaneous miniature end-plate potentials in rat diaphragm. Records 1 and 2 were at respectively fast (reading from left to right) and slow (reading from right to left) speeds before the addition of venom. Record 3 shows miniature end-plate potentials 11 min after exposure to 0.017 mg/ml. of acid-heated venom. Record 4 was taken after 14 min exposure to venom. Vertical calibration, 1 mV; horizontal calibrations, 10 msec for 1, 0.88 sec for 2, 3 and 4. Histogram A shows heights of miniature end-plate potentials (m.e.p.ps) for a 60-sec period before addition of venom (total number, 255; mean height, 0.82 mV); histogram B is for a 60-sec period 10 min after exposure to venom began (total number, 203; mean height, 0.56 mV).

within 30 sec. After the venom had been washed off the depolarization remained static for many hours.

When the pelvic half of a muscle was exposed to Ringer solution containing C1 or C2 eluate the effect was identical to that seen with whole venom (Fig. 3). Apparently 50 to 100% of the depolarizing activity of whole venom was recovered in the C1 and C2 eluates.

In another experiment, using a gel containing 24 mg of venom and eluting the C1 fraction with 15 ml. of saline, 0.5 ml. of eluate in 35 ml. of Ringer solution depolarized a sartorius by 10 mV with a 2-min exposure (equal to the effect of 2×10^{-5} cobra venom). The C2 eluate was about one-quarter as active as this. Thus an approximate overall recovery of 100% (divided in the ratio 8 : 2 between C1 and C2) was achieved.

Eluates from the anodal bands at high concentrations did not produce measurable depolarization with 5-min exposures at room temperature.

Intracellular recordings confirmed that anodal fractions were without significant effect on the resting membrane potential, but brief exposure to C1 or C2 eluates produced a rapid depolarization that was sustained for several hours.

Rat diaphragm miniature end-plate potentials

In the rat diaphragm a sustained neuromuscular block could be produced with dilute venom solutions without altering the twitch response to direct muscle stimulation. This preparation was therefore used for a study of the neuromuscular blocking action of venom.

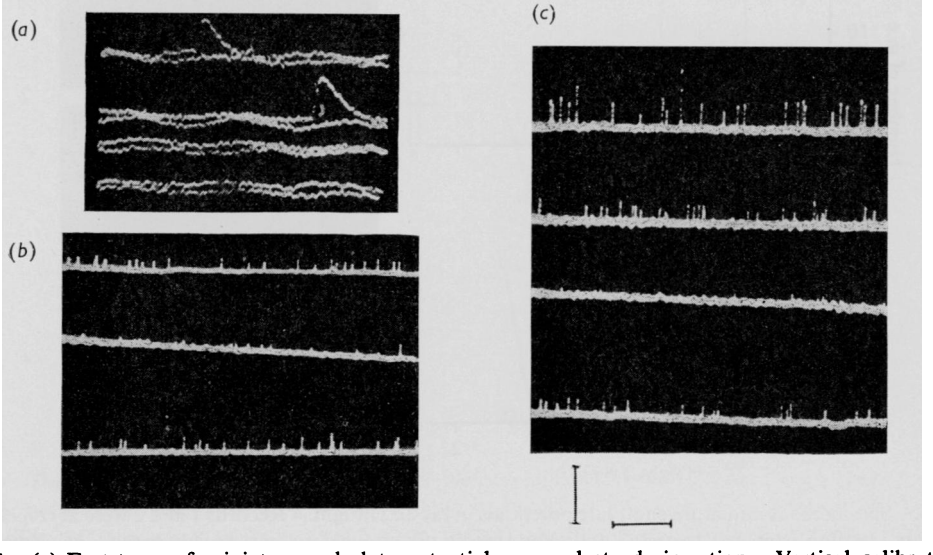

Fig. 5. (*a*) Fast trace of miniature end-plate potentials upon electrode insertion. Vertical calibration, 1 mV; horizontal calibration, 10 msec. (*b*) Upper trace: miniature end-plate potentials upon electrode insertion; middle trace: 12 min after washing out the carbachol. Vertical scale, 4 mV; horizontal scale, 0.88 sec. (*c*) First trace: 5 min exposure to C1 eluate corresponding to 0.006 mg/ml. venom; third trace: 30 to 40 sec after 1.3×10^{-5} carbachol; fourth trace: after washing out the carbachol. Vertical calibration, 2 mV; horizontal calibration, 0.88 sec.

Concentrations of whole venom in the range 5 to 10×10^{-6} or slightly higher heated at 100° C for 10 min at *p*H 5.0 (to destroy cholinesterase; Braganca & Quastel, 1953) produced a striking and consistent effect on the spontaneous miniature end-plate potentials.

Such an experiment is illustrated in Fig. 4. A progressive decline in the height of the miniature end-plate potentials leading to their complete disappearance was followed by a fall in the resting membrane potential. Usually when the miniature end-plate potentials diminished to zero in 10 to 15 min the resting potential fell by 2 to 5 mV over the same period. In the following 10 min the resting potential fell by 20 to 50 mV. Similar effects were produced by C1 eluates. Measurement of the depolarization produced by carbachol showed that this effect on the size of the miniature end-plate potentials was postsynaptic (Fig. 5). The maximal rate of depolarization during a 60-sec exposure to 1.3×10^{-5} carbachol as measured before the addition of a C1 eluate equivalent to 6×10^{-6} venom was 5.0 mV in 30 sec. After an exposure to the eluate sufficient to halve the height of the miniature end-plate potentials the maximal rate of depolarization by the same concentration of carbachol was 2.3 mV in 30 sec.

End-plate potentials recorded intracellularly during stimulation of the phrenic nerve (with neuromuscular transmission blocked with excess of magnesium ions) showed a progressive decline in amplitude after exposure to C1 eluates (at concentrations equivalent to 2×10^{-5} whole venom, assuming 100% recovery) comparable to the changes seen in the miniature end-plate potentials.

DISCUSSION

Separation of the toxic activity of cobra venom (*Naja naja*) from its phospholipase A activity by electrophoresis has been reported previously by Bussard & Côté (1954), Detrait, Izard & Boquet (1959) and Master & Rao (1961). Phospholipase A from *Naja naja* venom has an isoelectric point of *p*H 5.2 (Dawson, 1963), whereas that of the toxic component is above *p*H 9.0. The failure of some investigators (for example, Polson, Jourbert & Haig, 1946) to separate them by electrophoresis in free solution is probably due to binding of the basic toxin by acidic proteins or to a specific complexing of toxin and phospholipase A such as occurs in rattlesnake venom (Fraenkel-Conrat & Singer, 1956; Habermann, 1957). This presumed binding of toxins to proteins can also account for the failure of the cobra venom toxins to pass out of a cellophane dialysis membrane from a solution of snake venom at neutral or mildly alkaline *p*H and their ability to pass through such a membrane after they have been separated by electrophoresis on starch (Bussard & Côté, 1954; Meldrum, 1964).

That the depolarization of skeletal muscle produced by whole cobra venom is primarily attributable to a basic toxin and not to the phospholipase A (previously reported by Meldrum, 1963) is contrary to the conclusions of Tobias (1955). The experimental results of Tobias are not incompatible with this finding, but he interpreted the work of Braganca & Quastel (1953) as showing that venom which has been heated to 100° C for 10 or 15 min at *p*H 5 contains phospholipase A free from other activities, and subsequently other authors have also erroneously attributed effects of " acid-heated " venom to phospholipase A.

Habermann & Neumann (1954), employing electrophoresis on paper at *p*H 7.0, reported that the venom components acting on frog sartorius and rat diaphragm moved more rapidly towards the cathode than did phospholipase A. A venom element producing direct lysis

of washed human red blood cells could be similarly separated from phospholipase A. More recently, Condrea, de Vries & Mager (1964) and Condrea, Mammon, Aloof & de Vries (1964), who fractionated cobra venom by paper electrophoresis, have shown that cobra venom phospholipase A does not haemolyse washed human red blood cells or hydrolyse their phospholipids, but the most basic fraction from the venom does have a direct lytic action on red cells. If the direct lytic fraction and the phospholipase A are added together rapid haemolysis with hydrolysis of the red cell phospholipids occurs.

Cobra venom produces a progressive diminution in the height of miniature end-plate potentials in the rat diaphragm leading ultimately to their total disappearance. This effect is produced by the basic toxin or toxins in the venom and precedes the depolarization of the muscle cell membrane that these elements produce. There is apparently an interference with the electromotive action of acetylcholine (and related active compounds) occurring either at the receptor site or in the membrane surrounding the receptor site. If combination with the acetylcholine receptor is the mechanism of the toxin's postsynaptic action it is unusual in being irreversible. It is possible that the toxin can also produce a presynaptic block of neuromuscular transmission, but it is difficult to assess this in the presence of a postsynaptic block of more rapid onset. The present experiments do not permit a decision between the following possibilities: that there are two toxins, one acting on the acetylcholine receptor and one of the muscle cell membrane; that there is one toxin which has both these actions; or that there is one toxin which has one action on the membrane, manifest at low doses only as a " postsynaptic " block of neuromuscular transmission and in higher doses as depolarization. Chang (1960) and Chang & Lee (1963) have studied these problems using krait venom (from *Bungarus Multicinctus*). In a rat diaphragm with total neuromuscular block due to venom the rate of release of acetylcholine in response to phrenic nerve stimulation was initially normal but later declined. By electrophoresis on starch they separated three fractions (called α-, β- and γ-bungarotoxins) which could block neuromuscular transmission. The first of them inhibits the response of frog rectus abdominis to acetylcholine; the other two decrease the acetylcholine output of the diaphragm but do not alter the response of the frog rectus abdominis or the chick biventer cervicis to acetylcholine.

SUMMARY

1. Starch gel electrophoresis at *p*H 8.9 of *Naja naja* venom reveals fourteen components staining with Amido Black.

2. Fractions eluted from starch-gel possessing high phospholipase A activity are free from any paralysing or toxic action in mice or any depolarizing action on skeletal muscle.

3. The paralysing, toxic and depolarizing activities of the venom are present in the two fast-moving bands found nearest to the cathode.

4. The depolarization of skeletal muscle by venom is irreversible; in the rat diaphragm, it is preceded by a reduction in size and final disappearance of spontaneous miniature end-plate potentials. End-plate potentials in the phrenic nerve-diaphragm preparation blocked with excess magnesium ions show a similar decline in amplitude after exposure to eluates of toxin.

5. It is concluded that basic toxins are responsible for the paralysing action of *Naja naja* venom. They act both at the neuromuscular junction (producing a block which is primarily postsynaptic) and on the muscle cell membrane.

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