

BIOCHEMICAL AND PHARMACOLOGICAL STUDIES
ON THE VENOM OF ENHYDRINA SCHISTOSA
(COMMON SEA SNAKE) FROM THE WESTERN COAST
OF INDIA

THESIS SUBMITTED
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
MASTER OF PHARMACY FACULTY OF TECHNOLOGY
IN
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SUBMITTED BY
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UNDER THE GUIDANCE OF
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A
THESIS



Submitted To
The University of Bombay

For The Degree Of
MASTER OF PHARMACY
FACULTY OF TECHNOLOGY
In Pharmacology

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P R E F A C E

This thesis is an account of work carried out in the Department of Pharmacology, Haffkine Institute for the degree of Master of Pharmacy in Pharmacology of the University of Bombay.

The Introduction is a brief outline of the problem, identification of species, aim and importance of the problem.

The work is divided into three main parts. The first part is concerned with enzyme studies of the venom. It includes introduction, review of existing literature of sea snake enzymes, experimental part and discussion.

The second part deals with fractionation of the venom and purification of the principal toxic component. The physicochemical, immunological methods used are included in this part.

The third part is concerned with the pharmacological studies of the purified neurotoxic component from the venom. Each chapter has its introduction, review of literature and discussion. This is followed by common discussion. Finally, summary and conclusion of the whole work and bibliography have been given.

..... S. P. GAVADE.

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CHAPTER I

INTRODUCTION

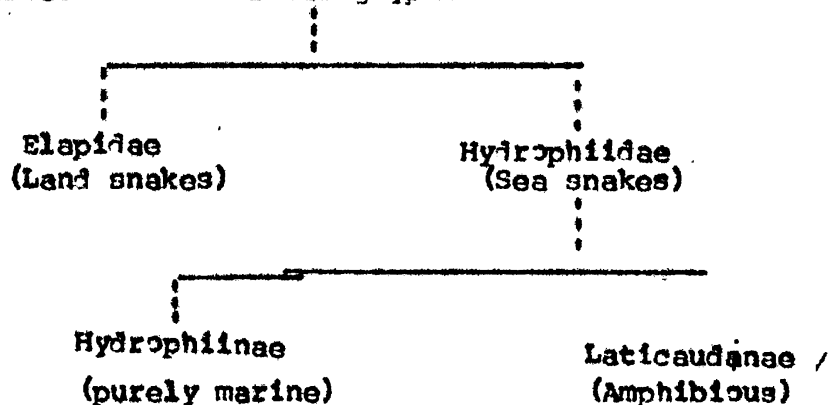
Sorting fish at the net,
 Someone has bitten,
 He never knew,
 he felt asleep,
 and died.

The snakes that are almost exclusively adapted to marine life are numerous. Many of them are venomous and dangerous to man. They are commonly known as sea snakes.

1.1 Classification :

According to Boulenger (1896), sea snakes are classified as below :

Kingdom	- Animalia
Phylum	- Chordata
Subphylum	- Vertebrata
Class	- Reptilia
Order	- Ophidia
Suborder	- Proteroglypha



The family Hydrophiidae is one of the most widely distributed families in the world. Very little is known about the evolution of Hydrophiidae, but their specialized form and the fact that all of them are venomous is the evidence of their late arrival among the serpents (Smith, 1926). They may be derived from cobras and their allies of the family Elapidae (Smith, 1926). Family Hydrophiidae is further divided into two sub-groups - Hydrophiinae and Laticaudinae. Laticaudinae snakes are amphibious and oviparous in contrast to Hydrophiinae snakes which are exclusively of marine origin and oviviviparous. The distinction being based mainly on the differences in the breadth of the ventral scales and the position of the maxillary teeth in relation to palatine. In Hydrophiinae internasal is absent and nostrils are situated at the upper part of the snout while in case of Laticaudinae internasal is present and nostrils are situated laterally.

1.2 Geographical distribution :

Sea snakes are widely distributed in tropical and subtropical regions of the Indian and Pacific Oceans. They are also found along the Asian coast ranging from the Persian Gulf to South Japan, through the Indo-Australian seas. They are also found among the islands of Oceania as far east as the Samoan islands. One species

namely Pelamis platurus is found beyond this range.

1.3 General characteristics :

The tail of sea snakes is rudder-like and distinctly flattened. Body is transversely, uniformly marked with dark bands. Ventral scales are very much reduced and body scales are less imbricated. The head and eyes are small. Nostrils are provided with a small internal valve to prevent the entry of water.

Sea snakes in their native habitat are sluggish. They frequently get entangled in the nets and are caught along with the fishes. Almost every cast of the net results in the capture of several sea snakes. The fisherman hold them in a great contempt picking them up by catching their tail and tossing them back in the sea. Eventhough sea snakes are sluggish and bite only under provocation (Reid, 1956), their bites are quite common and deaths are not unusual among the fishermen frequently exposed to the sea. Frequent occurrence of sea snakes in the nets and their occasional biting constitute a real occupational hazard in the fishing folk.

1.4 Incidence of Sea snake bite :

Majority of the victims of sea snake bites are fishermen, who are bitten while working in the sea.

Bathers are rarely bitten by sea snakes. Reid and Lim (1957) while surveying sea snakes bite cases in Malaya, varified the great frequency of poisoning accidents among fishing folk. However, as such type of survey has not been done in India, it is difficult to estimate the exact incidence on Indian coast. However, at the Ratnagiri sea coast, it is given to understand by the native fishermen that, there are frequent sea snake bite cases among the fishermen and death cases are also observed due to sea snake bite poisoning. Barme (1968) reported 2 to 3 sea snake bites every day at Vietnam. Bite occurs usually when the fishermen handle their nets in the sea and sort fishes with which snakes are mixed.

1.5 Sea snake bite - Mystery :

The chief reason of failure to realise the frequency of bites may be superstitions and myth among the fishing folk. Superstitions forbid fishermen to relate casualties they have witnessed. In Vietnam, the king of snakes and Genie of the sea are believed to rule sea snakes. Those who talk, are liable to reprisal, in the form of bites from one of the kings subjects (Barme, 1968).

Fishermen readily proclaim that sea snakes are numerous in their country and that a sea snake bite may

be fatal. But when one is asked whether fatalities occur among the inhabitants of his tribe or village, he usually will answer that there are none, but further will add that in the next tribe or village there are many. Reid (1956) recorded common superstitions generally present in Malayan and Chinese fishermen as follows :-

1. Talk about sea snake bite offends the sea snakes and may change their timidity to aggressiveness.
2. If a victim talks about his bite, he will be bitten again.
3. If a pregnant woman hears of a sea snake bite, the victim will die.
4. Presence of Bomor (medicine man) in village attracts sea snakes and cause more bites.

1.6 Mortality :

It is difficult to estimate accurately the death rate among the victims of sea snakes, for many of these casualties are not reported. Recent available data regarding the mortality due to sea snake bites in Thailand, Malaya and Phillipines during the period of 1956 to 1969 is 4.5 % (Sawai et al., 1972). Reid (1973) reported mortality rate about 5.0 %. However, this type

of data is not available on the Indian coast. However, two such historical records are reported on the Indian coast. In Madras river-mouth (1820), M'kenzie noted an "epidemic" of sea snake bites. He had described 31 cases of sea snake bites. Peal (1903) had reported that he was told about 3 to 4 fishermen being bitten every year in a fishing village on the Orissa coast and 25 % of the victims died. Since then no report of sea snake bites have been recorded on the Indian coast.

1.7 Symptomatology of sea snake envenomation :

We could not see any sea snake bite case throughout the course of our study. However, the clinical study of casualties from sea snake bites has been appreciably advanced by Reid's personal observations in Malayan fishermen (1957-1962).

The sea snake bite is always painless after the initial prick, which may be the chief reason why many of the bite cases often go unnoticed. There is no local swelling, ecchymosis or abnormal bleeding. Sea snake poisoning follows characteristic generalised myalgia starting usually half to one hour after the bite (Reid and Lim, 1957). Ptosis of eyelids, aches, pains and stiffness occur on moving arm, thigh, neck and trunk

muscles. Myoglobinuria becomes evident on inspection three to six hours after the bite. Early deaths (within 8 hours of ~~of~~ the bite) are due to a combination of respiratory-muscle damage, inhalation of vomit, and retention of secretions. Most deaths occur 12 to 24 hours after the bite and they are due to respiratory failure and hyperkalaemia; later deaths upto 12 days after the bite, are mainly due to renal failure (Reid, 1961).

1.8 Treatment :

1.8.1 Traditional methods :

Traditional methods mainly based on superstition and are more frequently employed. In Vietnam, fishermen used to lay the patient on a bed of water-bindweed and make loud noises in order to arouse the patient from sleep (Barne, 1968). Contrary to this, in India fishermen keep patient undisturbed and used to give him as much wine he could drink (personal communication). The fact that sea snake bites do not always induce death is probably the best explanation of the success of these methods.

1.8.2 Modern methods :

1.8.2.1 Nonspecific therapy : Application of tourniquet, incision and suction are the initial first aid measures.

Tracheotomy together with artificial respiration can be used as a supportive treatment since the death is due to respiratory paralysis. To encourage excretion of venom and to minimise renal damage, the patient is allowed to drink a litre of water and is given rapid intravenous infusion of litre of the mixture of 25% glucose, 10% calcium gluconate and 10% sodium lactate. Treatment of haemodialysis might be very much effective to cure the patient.

1.8.2.2 Specific therapy : Intravenous drip of specific sea snake antivenin (manufactured by Serum Research Laboratories, Melbourne, Australia) appears to be an adequate therapy.

1.9 Sea snake species :

The catalogue of snakes in the British Museum (Boulenger, 1893-96) enumerates 18 species of sea snakes distributed in the Indian and Pacific Ocean. Smith (1926) recorded 16 genera including 52 species of sea snakes. Species found along the Western coast of India so far are as follows :-

1. Enhydrina schistosa (Naudin)
2. Astrotia stokesi (Gray)

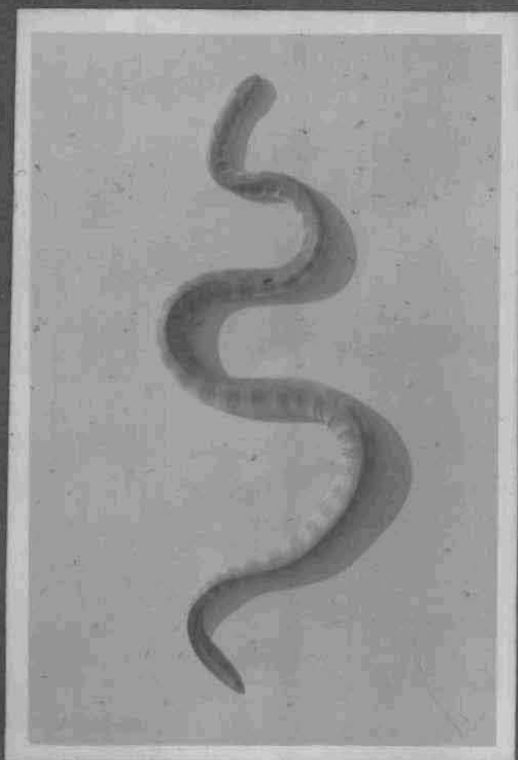
3. Hydrophis coeruleus (Shaw)
4. Hydrophis cyanocinctus (Daudin)
5. Hydrophis lapemoides (Gray)
6. Hydrophis mamillaris (Daudin)
7. Hydrophis ornatus (Gray)
8. Hydrophis spiralis (Shaw)
9. Lapemis curtus (Shaw)
10. Microcephalophis gracilis (Shaw)
11. Microcephalophis cantoris (Gunther)
12. Pelamis platurus (Linne)
13. Thalassophina viperina (Schmidt).

Sea snake Enhydrina schistosa being the most commonly available and the most dangerous (Reid, 1975) and poisonous species from the Western coast of India.

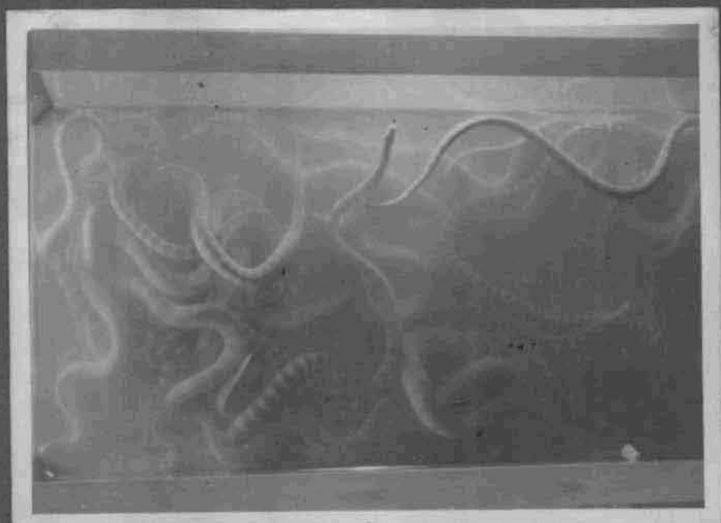
1.10 Identification of Enhydrina schistosa :

Species was identified using the keys for the identification by Smith (1926) and Wall (1921). Smith (1926) has given following characteristics for the identification of E. schistosa.

*Eyes moderate, rostral higher than broad, prefrontals very much narrowed anteriorly; frontal small, shorter than its distance from the end of the



Enhydrina schistosa (Common Sea Snake)



Snakes collected in Tank from Bombay
Sea Coast

snout; 1 pre and 2 post oculars; 7 to 8 supralabials, the 3rd and 4th touching the eye, the last 2 to 3 very small; usually a single anterior temporal; mental narrow, elongate, partly hidden in a groove in the symphysis, 40 to 52 scale-rows on the neck in males, 42 to 55 in females, 49 to 60 on the body in males, 51 to 66 in females, the scales imbricate with a short central keel; ventrals 239 to 322, distinct throughout, a little broader than the adjacent scales; preanals feebly enlarged." The young ones are grey or bluish-grey above and whitish below in colour with black annuli broadest dorsally; these markings usually disappear in the adult. Average total length of adult is 1400 mm, tail 180 mm.

1.11 Importance of sea snake venom research :

Sea snake venom is a heterogeneous mixture of proteins, many of which possess enzymatic activities, non-enzymatic polypeptides, non-protein contents, inorganic ions and many other unknown substances secreted through a venom gland which is a modified salivary gland lying immediately behind the eyes.

Sea snake venoms (studied so far) contain potent neurotoxins which are remarkably similar to that of cobra neurotoxins and α -bungarotoxin. The extremely specific action of snake venom neurotoxins on different stages in

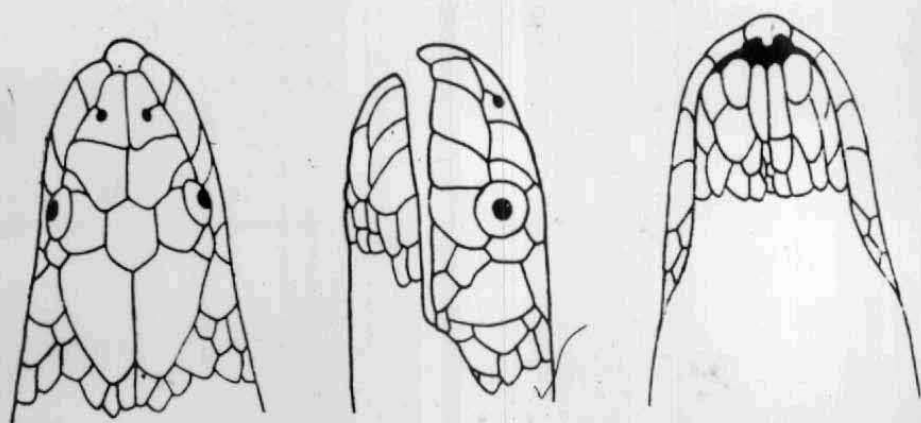


Fig. 145.—*Enhydrina schistosa*. (After Wall, Monogr. 1909.)

Identification of species : Head Shields

the complex mechanisms of signal conductance between nerve cells and the target organs have made them a valuable tool in the hands of neurobiologist. Neurotoxins from all species of sea snakes have not been investigated. Continued investigations on sea snake neurotoxins may clear the evolutionary aspects of sea snakes. Neurotoxins may also be useful in the investigation of certain neuropathological conditions.

Besides neurotoxins, sea snake venom also contains several enzymes. Snake venom enzymes have very much important application in the research. Exonucleolytic phosphodiesterase and endonucleolytic deoxyribonuclease were showed to be very much useful in determining the nucleotide sequence in oligonucleotides (Razzell and Khorana, 1958). Phosphomonoesterase have been used for quantitative analysis of oligonucleotides (Knorred et al., 1973). Phospholipase A have been used to study the biochemistry of subcellular particles (Habermann, 1968) and also in studying the transport mechanism across the cell membrane (Larson and Wolff, 1968). The venom protease have been used for amino acid sequence determination in proteins (Mella et al., 1967).

Sea snake bite cases are very less as compared to land snakes, but they do exist and death are also possible.

Therefore, to wipe out the universal fear of sea snakes and for precautionary measures it is necessary to prepare sea snake antivenin in our country. Continued studies on sea snake venom may lead to useful pharmacologically active new moieties. Some specific enzymes may also prove useful in basic studies on cellular metabolism and pharmacological mechanisms and could ultimately lead to valuable discoveries.

1.12 Aims and objects :

Two separate groups reported different results of the fractionation of E.schistosa venom from the same geographical region i.e., Strait of Malacca (Tu and Toom, 1971; Karlsson et al., 1972). Miranda et al. (1970) reported the difference in the toxins of the same species and Zeller (1948) reported the considerable variations in the venom of the same species collected from different geographical origin.

The work on sea snake venom is very much scanty in India. It is, therefore, very interesting to study the same venom from the Indian coast.

Sea snake antiserum is the only specific therapy for sea snake bite poisoning. At present it is only

available from Serum Research Laboratories, Melbourne, Australia. It is, therefore, the present need of our country to prepare indigenous sea snake antivenin.

In pursuance of the above objectives pharmacological, biochemical and immunological studies were carried out on the venom of Enhyrina schistosa (common sea snake). Being the pioneering work, lot of difficulties were faced right from the collection of sea snakes, their handling, identification and extraction of venom. An experimental sea snake antivenin (monovalent) is prepared by immunizing rabbits. Attempts were made to study enzymatic activities of the crude venom, to fractionate the venom by using mild physicochemical methods and to isolate and purify pharmacologically active component. The active component was further studied for its pharmacological action.

CHAPTER II

ENZYME STUDIES

2.1 Introduction :

Enzymes are protein catalysts for specific chemical reactions in biologic system. Enzymes are of universal occurrence in biological materials and life itself depends on a complex network of chemical reactions brought about by specific enzymes. The functions of this biologic catalyst is to influence the rate of chemical reaction but is not itself used up during the process and can in ideal cases be recovered at the end of the reaction. They often serve as the focal point for the regulation of body function.

2.2 Enzymes in sea snake venom :

The presence of large variety of enzymes, mostly hydrolases is outstanding characteristic of the chemical composition of snake venoms. The enzymes of snake venoms and their biological significance have been reviewed by Zeller (1948, 1951), Meldrum (1965a), Boquet (1964, 1966), Sarkar and Devi (1968) and Tu (1974).

In comparison with the terrestrial snake venoms, sea snakes are much simpler in composition containing fewer enzymes as evidenced from isoelectric focusing distribution patterns (Toom et al., 1969). Following enzymes have been reported to be present in sea snake

venoms, namely, phospholipase A (EC 3.1.1.4), phosphodiesterase (exonuclease) (EC 3.1.4.7), 5'Nucleotidase (EC 3.1.3.5), phosphomonoesterase (EC 3.1.3.2) acetylcholinesterase (EC 3.1.1.7), deoxyribonuclease (EC 3.1.4.6), adenine triphosphatase (EC 3.6.1.8), hyaluronidase (EC 3.2.1.36).

It has been reported that venom of Enhyridina schistosa lacks proteolytic activity and arginine ester hydrolysing activity (Toom et al., 1969). E.schistosa venom have been reported to have following enzyme activities, namely, fibrinogen clotting activity, hyaluronidase, alkaline phosphatase, phosphodiesterase, deoxyribonuclease, acetylcholinesterase and leucine amino peptidase (Tu and Toom, 1971).

2.3 Literature review :

2.3.1 Phospholipase A (EC 3.1.1.4) : Condrea and DeVries (1965), Meldrum (1965a) have reviewed the snake venom phospholipase A. The presence of phospholipase A in the venom of E.schistosa was first recognised by Carey and Wright (1960). This enzyme was isolated in purified form from the venom of Laticauda semifaciata (Tu and Passey, 1971; Tu et al., 1970; Uwatoko-Setoguchi and Obo, 1969; Uwatoko-Setoguchi, 1968).

The phospholipase A exhibits positional and steric specificity in catalysing the hydrolysis of the fatty acyl ester bond at the β -position of phosphatide molecules with the formation of lysophosphatides and release of free fatty acids, mostly unsaturated.

Ibrahim (1970) made an attempt to study the mode of action of phospholipase A in heated E.schistosoma venom on purified phospholipids, plasma phospholipids and intact muscle cells. He showed that deoxycholate is required for the activation of phospholipase A to achieve maximal hydrolysis of purified lecithin. The choline, serine and ethanolamine phosphatides of the intact muscle cells were also hydrolysed by this enzyme.

Phospholipase A from L.semifaciata was isolated and purified by CM-cellulose column chromatography followed by DEAE cellulose chromatography (Tu and Passey, 1971). The molecular weight was found to be 11,000 by the combination of sedimentation coefficient and diffusion constant, 10,700 by gel filtration method. Selectively L- α -lecithin was hydrolysed by this enzyme. This enzyme exhibited haemolytic activity which was greatly intensified by the addition of lecithin and relatively little activity on mouse embryo cells and very weak myolytic activity (Tu and Passey, 1971).

Phospholipase A activity of snake venoms is considered to be responsible for many of the biological actions, such as haemolytic action (Condrea et al., 1964), the release of histamine, adrenaline, acetylcholine and bradykinin from tissue cells (Lewis and Mustad, 1965), coagulant effect (Kirschmann et al., 1965), neurotoxic effect (Weil, 1930; Braganca and Questel, 1953), direct action on muscle cells (Houssay et al., 1922) and anti-bacterial activity (Dorey and North, 1960).

2.3.2 Acetylcholinesterase (EC 3.1.1.8) : Hydrolysis of acetylcholine by snake venom was first demonstrated by Iyengar et al., (1938). The enzyme capable of hydrolysing variety of acetic esters was observed in variety of snake venoms (Zeller et al., 1949; Boquet, 1964). Tu and Toom (1971) detected acetylcholinesterase activity in E. schistosa venom, but this activity was not observed in L. semifaciata venom (Uwatoko et al., 1966a, 1966b). Zeller (1948) did not find acetylcholinesterase activity in E. schistosa venom which was 50 years old and impure. Because of the role of acetylcholine in the transmission of nerve impulses, the presence of acetylcholinesterase in elapid and few hydrophid venoms, stimulated the interest of many investigators to search the evidence of possible action of this enzyme in the transmission of nerve impulses.

2.3.3 Phosphatases : Various types of phosphatases have been reported in snake venoms (Sarkar and Devi, 1968). Phosphatases are the group of enzymes capable of hydrolysing phosphate esters or anhydride bonds in mono or polynucleotides with a variable degree of specificity (Yang et al., 1958; Meldrum, 1965). Among phosphatases present in snake venoms, 5'nucleotidase, exonuclease (phosphodiesterase), deoxyribonuclease, nonspecific phosphomonoesterase were reported to be present in E. schistosa venom (Tu and Toom, 1971). Phosphodiesterase and phosphomonoesterase were also reported in L. semi-faciata venom (Uwatoko-Setoguchi, 1970).

2.3.3.1 5'Nucleotidase (EC 3.1.3.5) : 5'Nucleotidase (AMPase) is known to occur in all snake venoms so far tested (Gulland and Jackson, 1938b; Zeller, 1950; Sulkowski et al., 1963). 5'Nucleotidase hydrolyses the terminal phosphate from adenylic acid. It is the most active enzyme among all phosphatases. This enzyme along with phosphodiesterase have been used in the determination of nucleic acids.

2.3.3.2 Exonuclease (Phosphodiesterase) (EC 3.1.4.1) :

It was Uzawa (1937) who first time recognised this enzyme in the snake venom. Phosphodiesterase performs stepwise exonucleolytic degradation of polynucleotide

molecule, starting at the mononucleotide that has a free 3'-hydroxyl group (Felix et al., 1960; Williams et al., 1960; Bjork, 1961). This enzyme has been widely employed to determine the nucleotide sequence and to study structure of RNA and DNA (Razzell and Khorana, 1959a; 1959b). It has been suggested that this enzyme may be responsible for the immediate fall in blood pressure following intravenous injection of venom (Meldrum, 1965a).

2.3.3.3 Nonspecific phosphomonoesterase (EC 3.1.3.1) :

This enzyme was detected in variety of snake venoms (Gulland and Jackson, 1938a). This enzyme hydrolyses monophenylphosphate and phenol is liberated. This can be distinguished from phosphodiesterase in its pH optimum and by releasing 3'-nucleotides. Nothing is known about the pharmacological properties of the venom-phosphomonoesterase.

2.3.3.4 Deoxyribonuclease :

It is a heat stable endonuclease capable of hydrolysing deoxyribonuclease (DNA) or ribonuclease (RNA) yielding tri or higher 3'-phosphate terminated oligonucleotides (Georgatsos and Laskowski, 1962).

2.3.4 Hyaluronidase (EC 3.2.1.36) :

This enzyme have been detected in all snake venoms so far examined (Zeller, 1948; Russell, 1967; Barne, 1968). The enzyme specifically depolymerizes hyaluronic acid, a mucopolysaccharide which appears to bind water in the interstitial spaces and to hold cells together in a gel. Hyaluronic acid, while allowing metabolites to pass through, it has the important function of offering resistance to penetration by foreign matter including agents of infectious diseases. This inhibition was broken by the hyaluronidase. This break down allow other venom fractions to penetrate the involved tissues. This enzyme has been related to the edema and swelling caused by the venom (Russell, 1971).

2.3.5 Proteases (Endopeptidases and Exopeptidases) :

In 1881, Lacerda first demonstrated the presence of proteolytic activity in snake venom. Hydrophid venoms either have no or very little proteolytic activity (Sarkar and Devi, 1968; Robertson et al., 1969). Venoms that are rich in protease produce marked tissue changes and destruction. The anticoagulant effect of several snake venoms may be attributable in part to the proteolytic disintegration of the fibrinogen. Proteolytic activity was not found in L.semifaciata (Uwatoko et al., 1966a; 1966b). Tu et al. (1966b) studied the proteolytic

activity in hydropheid venom and concluded that hydro-
pheid venom did not hydrolyse either TAME (p-Toluene
sulfonyl-L-arginine methyl ester) or BAEE (Benzoyl arginine
ethyl ester) nor BTEE (N-benzoyl-L-tyrosine ethyl ester)
or ATEE (N-acetyl-L-tyrosine ethyl ester).

2.3.6 L-Amino acid oxidase : (EC 1.4.3.2) :

The L-amino acid oxidase was first reported to
be present in the venom by Zeller (1944), and Zeller
and Maritz (1944). The enzyme attacks the L-isomers of
most amino acids by oxidative deamination resulting in
the formation of corresponding L-keto acids. The enzyme
of snake venom is a flavoprotein and the yellow colour
of the venom is attributed to riboflavin content of this
enzyme. However, this enzyme has not been detected so far
in hydropheid venom. Zeller (1951) reported that the
enzyme is probably integrated with the digestive function
of the venom.

2.3.7 Coagulase (Blood clotting activity) :

Some snake venoms contain substances that
accelerates the coagulation process. Denson et al. (1972)
have characterized the coagulant activity of some snake
venoms. Venom of Enhydrina schistosa did not find any
effect on blood clotting (Reid, 1956b) while Tu et al.

(1966b) reported slight direct coagulant action on fibrinogen in the venom of L.colubrina. Fibrinogen clotting activity was also reported to be present in E.schistosa venom (Tu and Toom, 1971).

2.3.8 Anticoagulase :

The factor that delays the coagulation was reported in some snake venoms (Krause and Dam, 1950). Barne (1968) reported slight anticoagulant activity in the venom of sea snake Lapemis hardwickii. However, this action has not been reported yet in E.schistosa venom.

2.4 Materials :

2.4.1 Enhydrina schistosa :

Snakes were collected from Bombay sea coast from the local fishermen in the month of March/April 1975. Species were identified using keys for the identification described by Smith (1926) and Wall (1921).

2.4.2 Crude dried venom :

Snakes were anaesthetised with pentobarbitone sodium 15 mg/kg (I.P.). Venom glands were dissected out, homogenised with hand homogeniser at 4°C in distilled water. The suspension was centrifuged at 4000 r.p.m. for 15 minutes and the supernatant liquid was lyophilized. The lyophilized powder was used as crude dried venom.

The venom preparation was kept in the desiccator over calcium chloride until use. About 2 g of venom was obtained from about 170 snakes.

2.4.3 Enzyme assays :

For enzyme assays following substrates were used. Casein (BDH), Calcium di-p-nitrophenyl phosphate (Fluka), Sodium di-p-nitrophenyl phosphate (E.Merck), Adenosine monophosphate (E.Merck), Acetylcholine chloride (Koch Light), 2:6-Dichlorophenol indophenol (DCIP, E.Merck), L-Leucine (E.Merck), Benzoyl-arginine ethyl ester (BAEE, Koch Light). All other chemicals and reagents used were of analytical and reagent grade. All solutions were made in distilled water.

2.5 M e t h o d s :

2.5.1 Assay of phospholipase A activity :

Phospholipase A activity was determined quantitatively by turbidimetric method (Marinetti, 1965). The enzyme phospholipase A breaks down the phospholipids (lecithin) present in the egg yolk resulting into clearing of egg yolk suspension. Intensity of the clearing depends upon degree of the concentration of enzyme. A stock egg yolk suspension was prepared in 0.9 % sodium chloride. Working solution was prepared from this stock by diluting

with 0.9 % sodium chloride in such a way that the absorbance of this working suspension at 925 nm was around 1.2 . Different concentrations of the venom (0.1 ml) was incubated with 2.0 ml of the working solution at 37°C for 20 minutes. The blank contained 2.0 ml of working solution only. The reaction was stopped by adding 3.0 ml of chilled 0.9 % sodium chloride and optical density was measured at the same wave length i.e. 925 nm against 0.9 % sodium chloride. One unit of the activity is defined as the amount of enzyme which produces a decrease in 1 m unit of absorbance at 925 nm per minute.

2.5.2 Assay of proteolytic activity :

The proteolytic activity was determined according to the method of Murata et al. (1963) using casein and denatured haemoglobin as a substrate. The enzyme protease hydrolyses casein and denatured haemoglobin to liberate free amino acids, the concentration of which can be detected by modified Lowry's method. Colour intensity being varies with the degree of enzyme concentration.

2.5.2.1 Casein digestion : The casein solution was prepared by suspending 2.0 g of casein in 100 ml of 0.4M Tris-HCl buffer at pH 8.5 and heating for 15 minutes in a boiling water-bath to form a complete solution. 0.5 ml of the venom solution of varying concentrations and 1.0 ml

of casein solution were incubated at 37°C for 15 minutes. The reaction was stopped by adding 1.0 ml of 0.44 M trichloroacetic acid (TCA) and after standing for 30 minutes the mixture was filtered. To 0.5 ml of the filtrate, 2.5 ml of 0.4M Na_2CO_3 and 0.5 ml of Folin-Ciocalteu reagent (Centron) was added and the optical density was measured at 660 nm. One unit of protease is defined as the amount of enzyme which yield a colour equivalent to 1 μg of tyrosine per minute under the above conditions.

2.5.2.2 Denatured haemoglobin digestion : Haemoglobin solution was prepared by dissolving 1.0 μg of haemoglobin in 40 ml of water and was denatured by heating for 30 minutes at 80°C . pH of the solution was adjusted to 8.5 with 0.1N NaOH. The same assay procedure was followed as described above in casein digestion replacing casein with haemoglobin.

2.5.3 Assay of phosphodiesterase (exonuclease) activity :

The phosphodiesterase activity was determined according to the method of Sulkowski *et al.* (1963) using calcium di-p-nitrophenyl phosphate (Fluka) as a substrate. The enzyme hydrolyse the substrate to liberate p-nitrophenol which has yellow colour, which can be measured colorimetrically. Intensity of the colour depends upon the degree of concentration of the enzyme.

The reaction mixture contained 1.0 ml of 0.1M Tris-HCl buffer at pH 8.9, 1.2 ml of 0.001M calcium di-p-nitrophenyl phosphate, 0.2 ml of venom solution (of varying concentration) and distilled water to make 3.0 ml. The reaction mixture was incubated at 37°C and the reaction was stopped by adding 3.0 ml of 0.05N NaOH. The absorbance was measured at 400 nm against the blank that contained all the reagents but the venom solution. One unit of phosphodiesterase activity was defined as the amount of enzyme which liberated 1 μ mole of p-nitrophenol per minute under the above conditions.

2.5.4 Assay of phosphomonoesterase activity :

The phosphomonoesterase activity was estimated according to the method of Sulkowski et al. (1963) using sodium-di-p-nitrophenyl phosphate as a substrate. Principle of this method is the same as in phosphodiesterase. Activity was determined using changes in the absorption of p-nitrophenol liberated at 400 nm during 15 minutes of incubation at 37°C. The reaction mixture contained 1.0 ml of 0.1 M glycine NaOH buffer pH 9.5, 1.2 ml of 0.1M $MgCl_2$, 0.1 ml of venom solution (of varying concentration) and water to make 3.0 ml. The reaction was stopped by adding 3.0 ml of 0.05N NaOH. The absorbance was read at 400 nm against the blank that contained all the reagents except venom solution. One unit of activity was defined as the

amount of enzyme which liberated 1 u mole of p-nitrophenol per minute under the above conditions.

2.5.5 Assay of 5'-Nucleotidase activity :

The 5'-Nucleotidase activity was determined by the method of Sinsheimer and Koerner (1932) as modified by Sulkowski et al. (1963) using adenosine monophosphate (AMP) as a substrate. The 5'-nucleotidase hydrolyses the substrate adenosine monophosphate to liberate phosphate. The liberated phosphate was determined by the method of Fiske and Subba Row (1925). Colour intensity being depending upon the degree of enzyme concentration. The reaction mixture contained 0.1 ml of 1M glycine buffer pH 9.0, 0.1 ml of 0.1M $MgCl_2$, 0.3 ml of 0.01M adenosine monophosphate (AMP), 0.1 ml of the venom solution (of varying concentration) and water to make 1 ml. The reaction mixture was incubated for 15 minutes at 37°C. Determination of phosphorus depends entirely upon the reduction of phosphomolybdic acid to a blue pigment, which has a absorption range around 700-725 nm. One unit of 5'-nucleotidase activity was defined as the amount of enzyme liberated 1 u mole of inorganic phosphate per minute under the above conditions.

2.5.6 Assay of cholinesterase activity :

Cholinesterase activity was determined by colorimetric method of Huerfaga et al. (1952) using acetylcholine

chloride as substrate. The unhydrolysed acetylcholine is converted with hydroxylamine to acetyl hydroxamic acid, which can be determined as a brown ferric complex. The reaction mixture contained 2.0 ml of acetylcholine buffer salt mixture (containing 8 volume of veronal buffer, 0.1M, pH 8.2; 1 volume of 0.5M acetylcholine chloride; 1 volume of mixture of 0.44 M $MgCl_2$ and 0.03M KCl) and 0.2 ml of venom solution (of varying concentration). After incubation for one hour at $37^{\circ}C$, 2.0 ml of alkaline hydroxylamine solution (prepared just before use by mixing equal volumes of 14% w/v hydroxylamine (Sigma) and 14% w/v NaOH) was added. After one minute, 6.0 ml of 0.5N HCl was added by shaking thoroughly. Acetylhydroxamic acid is formed from the unhydrolysed acetylcholine. From the sample and control tubes 0.5 ml hydroxamic acid was pipetted into centrifuge tubes containing 10 ml of ferric chloride (1 % w/v) solution and was centrifuged for 5 minutes at 2000 r.p.m. and the supernatant was measured colorimetrically at 540 nm.

2.5.7 Assay of L-amino acid oxidase activity:

The L-amino acid oxidase activity was determined by measuring the rate of reduction of 2,6-dichlorophenol indophenol (DCIP) at 600 nm according to the method of Shaham and Bdolah (1973). The reaction mixture contained 7.5 mM L-leucine, 0.1 mM DCIP, 0.05M Tris-HCl buffer

pH 7.4 and 0.5 M KCl in a final volume of 1.0 ml.

The reaction mixture was preincubated for 2 to 3 minutes before the addition of leucine in order to check endogenous activity.

2.5.8 Assay of amino acid esterase activity :

Amino acid esterase activity was determined by using the method of Toom and Chan (1972) with slight modifications to comply with our laboratory conditions. The enzyme esterase hydrolyses synthetic amino acid esters to liberate free amino acids which can be titrated with 0.05N NaOH using phenol red as an indicator. 15 μ mole of BAGE in 4 ml of distilled water was incubated with 1.0 ml of venom solution (1 mg/ml) at 37°C in constant temperature bath. The acid liberated was titrated using microburette with 0.05N NaOH at pH 8.5.

2.6 R e s u l t s :

The activities of various enzymes in the crude venom were determined according to the methods given in Section (2.5). Standard curves for acetylcholine chloride, p-nitrophenol and L-tyrosine are given in the figures 1 to 3 respectively.

The plots, concentration of venom against absorbance for the enzymes phospholipase A, phosphodi-

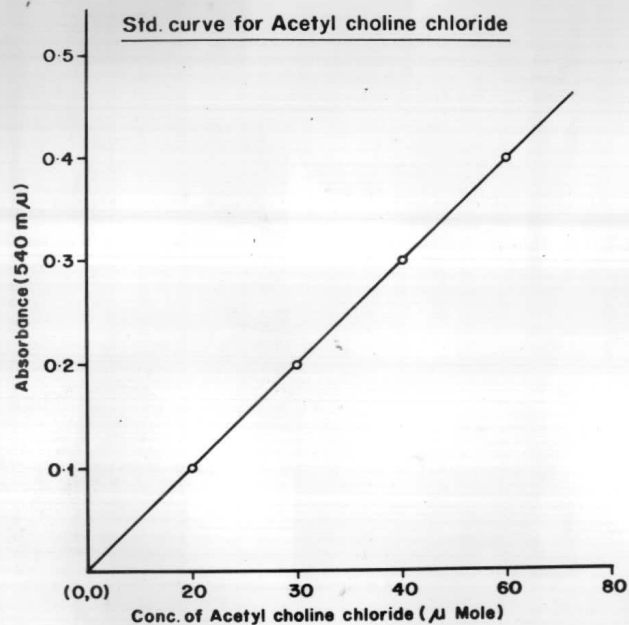


Figure - 1

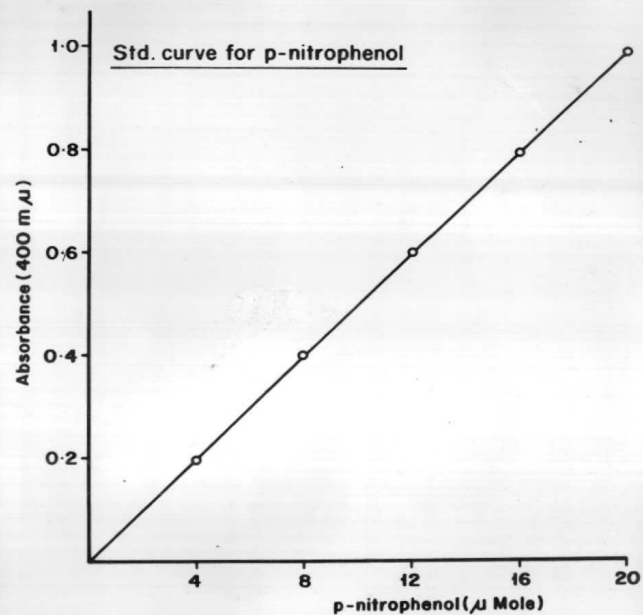


Figure - 2

esterase, phosphomonoesterase and 5'-nucleotidase are given in the Figure 4.

Specific activities were expressed in Units/mg of lyophilized venom. The results are listed in Table 1.

TABLE - 1
Enzymatic activities of E.schistosa venom.

Enzyme	Specific activity (Units/mg)
1. Phospholipase A (EC 3.1.1.4)	78.50
2. 5'-Nucleotidase (EC 3.1.3.5)	0.15
3. Phosphodiesterase (exonuclease) (EC 3.1.4.5)	0.05
4. Phosphomonoesterase (EC 3.1.3.2)	0.15
5. Acetylcholinesterase (EC 3.1.1.7)	7.60
6. L-Amino acid oxidase (EC 1.4.3.2)	Nil
7. Protease	12.00
8. Amino acid esterase	Nil

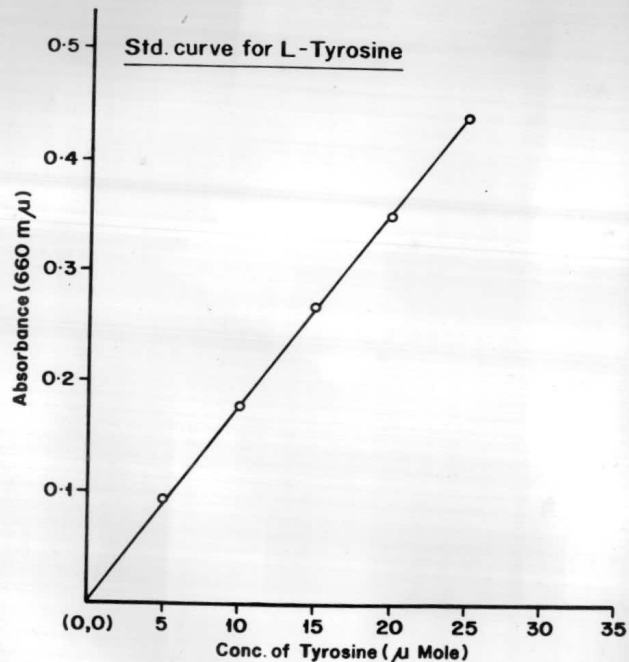


Figure - 3

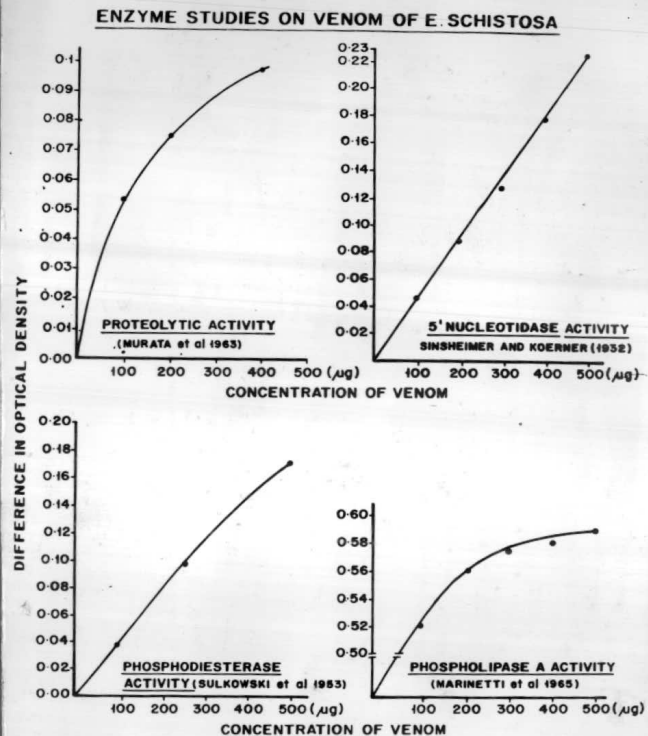


Figure - 4

2.7 D i s c u s s i o n

Sea snake venom is a heterogeneous mixture of proteins, many of which possess enzyme activities. Enzymes are important and essential component of biological system. Their function being to catalyse the chemical reactions that are essential to life. Mainly hydrolytic enzymes are present in snake venoms. Since the venom enzymes have very specific substrate requirements, they found very important tool as a reagent in the elucidation of the complex structures of natural products and in the determination of finer points in the structure of nucleic acid (Razzell and Khorana, (1958).

Several earlier investigators have tried to correlate the toxicity of snake venom with their enzyme activities (Iyengar et al., 1938; Bovet and Bovet, 1943; Taborda et al., 1952b; Braganca and Questel, 1952a; Slotta and Fraenkel-Conrat, 1938; Eagle, 1937; Deutsch and Diniz, 1955). However, with the progress in new techniques of protein chemistry, snake venoms were fractionated and their lethal actions and enzyme activities were separated (Yang et al., 1959; Ghosh and Sarkar, 1956).

Zeller (1948) showed the considerable variation in venom composition of the same species from different

geographical areas. The studies on sea snake venom from the Indian coast are very much scanty in India. Our objectives was to study the enzymatic composition of the venom of E.schistosa from the Western coast of India.

Tu and Toom (1971) tested enzyme activities of the venom of E.schistosa obtained from Malaya and detected following enzymes namely, hyaluronidase, clotting activity, alkaline phosphatase, phosphodiesterase, deoxyribonuclease, acetylcholinesterase and leucine amino peptidase. Our studies showed that same venom obtained from the Western coast of India, in addition to phosphodiesterase (exonuclease), phosphomonoesterase, acetylcholinesterase and phospholipase A contain 5'-nucleotidase and proteolytic activity.

Proteolytic activity was studied by using casein and denaturated haemoglobin as substrates. This activity was reported to be absent in the venom of same species from Malaya (Tu and Toom, 1971). This difference may be due to the difference in the method of extraction of venom or this may be due to the variation in the composition of venom obtained from different geographical regions.

Formerly it was believed that the hydrolysing activity with synthetic substrates, BAEE and ATEE were

present together with protease activity. But Murata et al. (1963) and, Soh and Chan (1974) showed that the enzyme responsible for esterolytic activity is different from protease enzyme. We found that in E.schistosa venom amino acid esterase activity with BAEE as a substrate is absent while the protease activity with casein and denatured haemoglobin as substrates was present.

Presence of phospholipase A in E.schistosa venom was showed by Carey and Wright (1961), Tu and Toom (1971). We estimated this activity using egg yolk clearing assay. This activity was found to be very rich in this venom. Bee venom and cobra venom (Condrea and DeVries, 1965) and krait venom (Chhatwal, 1974) are among the richest sources of this enzyme. Our studies indicate that Enhydrina schistosa venom may be another rich source of phospholipase A.

C H A P T E R I I I

PURIFICATION OF BIOLOGICALLY
ACTIVE COMPONENT

3.1 Introduction :

Pharmacologically, actions of snake venoms are very complex, as it is clearly indicated by the knowledge of symptoms and signs of envenomation. The complexity of venom action has been demonstrated to be mainly due to combined effects of different components contained in the same venom. The pharmacological events taking place in a victim of snake bite will be fully understood only when the pharmacological activity of each individual constituent of the venom is well known. It is well known that the neurotoxins, if not alone, but the main and the most lethal factor present in the sea snake venoms.

3.2 Sea snake neurotoxins :

Sea snake neurotoxins are among the best chemically and pharmacologically characterized snake venom constituents. They are remarkably similar in their chemical structures and identical to their mode of action with that of cobra neurotoxins and α -bungarotoxin and therefore, sometimes recognised as "Isotoxins" (Lee, 1972). Sea snake neurotoxins could be regarded as one of the most toxic substances in the world (Tu, 1974). It is remarkable that the venoms of two types of snakes, hydrophids and elapids possess similar structure and neurotoxic action, although the former spend their

entire life in the sea while the later live on the land (Tu, 1974).

3.2.1 Literature review :

Toxins from eight varieties of sea snakes have been isolated so far, four hydrophiinae and four laticaudinae by different investigators from different laboratories. In some cases, one venom has been simultaneously studied at several different laboratories. Therefore, it is not unusual to find different names assigned for the same toxin. For instance, the laticauda toxin III and laticaudotoxin IV isolated from Laticauda semifasciata by Uwatoko et al. (1966) is identical with erabutoxin a and erabutoxin b isolated by Tamiya and Arai (1966).

Carey and Wright (1960a) showed that the toxic component of E. schistosa venom was dialysable. The neurotoxic component present in the dialysate was further purified by CM-cellulose column chromatography, which was four times more potent than the crude venom.

Tu and Tsem (1971) isolated major toxin from the same species by CM-cellulose column chromatography from Strait of Malacca. The toxic component was shown to be homogeneous by zonal electrophoresis, ultracentri-

fugation, isoelectric focusing and gel filtration. Amino acid composition of the toxin revealed 62 amino acid residues with the molecular weight, 6981. Further, Tu and Toom (1971) demonstrated that the compound N-bromosuccinimide completely destroyed the toxicity of the preparation indicating that tryptophan is necessary for the toxic action of this venom.

Karlsson et al. (1972) reported the isolation of two principal neurotoxins schistosa 4 and schistosa 5 from E.schistosa venom from the same geographical origin i.e., Strait of Malacca by Sephadex-gel filtration followed by gradient chromatography on Bio-Rex 70.

Fryklund et al. (1972) elucidated complete amino acid sequences of schistosa 4 and schistosa 5. Both the toxins consisted of single peptide chain of 60 amino acid residues cross linked by four disulfide bridges and contain a "masked" -SH group which can be alkylated with iodoacetate in 8 M urea without inactivation. The gradient pattern provided chromatographic evidence indicating the presence of 14 or more different toxins in addition to schistosa 4 and schistosa 5 (Karlsson et al., 1972).

Uwatoko et al. (1966b) isolated two major toxins and three minor toxins from the venom of Laticauda semifasciata. Further, two major toxins were isolated in crystalline form designated as Laticotoxin III and Laticotoxin IV.

Tamiya and Arai (1966) isolated four toxins from the same venom by CM-cellulose chromatography. Two of them were further characterized and called erabutoxins a and erabutoxin b. Direct comparison of chromatograms obtained from the two laboratories indicates that crystal laticotoxin III and laticotoxin IV similar to erabutoxin a and erabutoxin b. Each of these toxins consist of 62 amino acid residues.

A third component erabutoxin c was also isolated from the same Laticauda semifasciata venom in the crystalline form (Uwatoko-Setoguchi, 1970; Tamiya and Abe, 1972). Toxins of identical amino acid composition and pharmacological action were isolated in the crystalline form from Laticauda laticaudata and Laticauda colubrina were therefore given common name "Laticotoxin" (Sato et al., 1969). Laticotoxins like erabutoxin a and erabutoxin b contain 62 amino acid residues.

Tu et al. (1971) reported the isolation of two toxins 'a' and 'b' from Laticauda semifasciata from the

Philippines by sephadex gel filtration followed by CM-cellulose column chromatography. Toxins a and b contain 62 and 61 amino acid residues respectively. There are slight differences in amino acid compositions from the toxins of the same species of Japanese origin. This showed that the snake from Philippines may be a different subspecies (Tu ^{et al.} & Hong, 1971).

Tu & Hong (1971) isolated major toxin in purified form from the venom of Lapemis hardwickii by a four step purification procedure using sephadex gel filtration followed by CM-cellulose column chromatography.

Liu et al. (1973) fractionated Hydrophis cyanocinctus venom by CM-cellulose column chromatography. The most retarded fraction hydrophitoxin a was further studied for amino acid composition and showed that hydrophitoxin a was similar to other sea snake neurotoxins so far investigated.

A weak and reversibly acting neurotoxic component Ls III isolated from Laticauda semifasciata venom by CM-cellulose chromatography (Maeda and Tamiya, 1974). The component Ls III showed to contain 66 amino acid residues and five disulfide bridges. This is the only sea snake neurotoxin having five disulfide bridges so far isolated.

Yu et al. (1975) isolated major neurotoxin (toxin a) from Pelamis platurus from Philippines by Sephadex gel filtration and CM-cellulose column chromatography. The amino acid composition revealed 55 amino acid residues.

Liu et al. (1975) reported the isolation of major toxic component pelamitoxin a from the same venom of Pelamis platurus from the Formosa water by CM-cellulose chromatography with linear concentration gradient of ammonium acetate buffer pH 6.5. Its amino acid composition revealed that it is identical to that of hydrophiltxin b from Hydrophis cyanocinctus venom (Liu et al., 1974) and schistosa S from the venom of E.schistosa (Fryklund et al., 1972).

Recently three neurotoxic components a, b and c were isolated in purified form from Aipysurus laevis (Maeda and Tamiya, 1976). Fractionation was performed by CM-cellulose chromatography followed by Bio-Rex 70.

Yu et al. (1975) studied pure toxins isolated from E.schistosa and Lapemis hardwickii by laser Raman spectroscopy to elucidate further the structure-function relationship of neurotoxins.

3.2.2 Classification :

Neurotoxins, in general, are classified pharmacologically into two distinct groups (Lee, 1970; 1972). The first group produces a non-depolarizing N-M block by acting on the post-junctional membrane of the motor end plate similar to curare and different from curare in its slow development, in the imperfect antagonism by neostigmine and in the absence or slowness of reversibility (Jimenez-Porras, 1968), whereas the second group produces a N-M block by acting presynaptically on the motor nerve endings, leaving the sensitivity of the end plate to acetylcholine (Ach) unaffected. Sea snake neurotoxins (Tamiya and Arai, 1966; Sato *et al.*, 1969; Liu *et al.*, 1973; 1975; Tu *et al.*, 1976; Maeda and Tamiya, 1976), α -bungarotoxin (Lee, 1970) and most of the elapid neurotoxins belong to the first group (Lee, 1972).

Neurotoxins belonging to the first group can be further classified into three sub-groups according to their amino acid compositions, namely, short chain toxins composed of 60 to 62 amino acid residues of 15 to 16 amino acids in a single peptide chain cross-linked by four disulfide bridges, medium length chain toxin with 66 amino acid residues and five disulfide bridges (Maeda and Tamiya, 1974) and third, long chain

toxins consist of 71 to 74 amino acid residues of 17 to 18 amino acids with five disulfide bridges. All of the sea snake neurotoxins so far investigated belong to the first short chain toxin group (Tamiya and Arai, 1966; Sato et al., 1969; Tu and Hong, 1971; Tu and Toom, 1971; Tu et al., 1971; Liu et al., 1973; 1975; Tu et al., 1975; Maeda and Tamiya, 1976) and one medium length chain toxin group (Maeda and Tamiya, 1974).

3.2.3 Establishment of homogeneity :

Purity of the toxins isolated from venom is generally established by disc electrophoresis, ultracentrifugation, isoelectric focusing, immunoelectrophoresis, immuno gel diffusion and gel filtration. Three or more different methods were used for the determination of homogeneity.

3.2.4 Molecular weight :

The difference in the molecular sizes among the different snakes has a significant implication in sea snake poisoning effect (Tu et al., 1973). The onset of systemic symptoms due to envenomation of neurotoxins on sea snakes is extremely rapid. Rapid action may be due to their smaller size, which diffuse into vital target organs much faster causing the quicker toxic action (Tu, 1973). In general, molecular weight is determined

by three methods namely, amino acid composition, sedimentation equilibrium, sedimentation coefficient, and gel filtration. Molecular weights of the sea snake neurotoxins so far isolated are summarised in Table 2.

3.3 Immunochemistry :

Peal (1903) was the first to report the use of antivenin in sea snake bite. Carey and Wright (1960) prepared anti E.schistosa serum in goat. They showed by immunoelectrophoresis and gel diffusion that there is a distinct relationship between the venom of E.schistosa and other members of the Hydrophiinae namely, Kerilia jerdoni, Hydrophis cyanocinctus, Microcephalophis gracilis, Lapemis hardwickii, Hydrophis spiralis. Carey and Wright (1960) further reported that antivenin against cobra, krait, Russell's viper and Echis exhibited only negligible neutralization against E.schistosa venom .

Reid (1962) demonstrated the clinical efficacy of commercial E.schistosa antivenin, prepared by the Commonwealth Serum Laboratories, Melbourne, Australia against bites by the homologous species. He reported that before sea snake antivenin became available out of 11 cases of serious poisoning 6 died, subsequently 10 cases serious poisoning received specific sea snake antivenin, only two patients died and eight recovered dramatically.

TABLE - 2

Molecular weight of purified neurotoxin from the venoms of sea snakes
(Hydrophiidae)

Snake	Origin	Toxin	M e t h o d s				REFERENCE
			Amino acid composition	Sedimentation equilibrium	Gel filtration	S and D	
<u>HYDROPHIINAE</u>							
<u>Enhydrina schistosa</u>	Straits of Malacca	Toxin	6878	-	-	7300	Tu & Toom (1971)
		Schistosa-4	6689	-	-	-	Karlsson <u>et al.</u> (1972)
		Schistosa-5	6679	-	-	-	Karlsson <u>et al.</u> (1972)
<u>Hydrophis cyanocinctus</u>	Formosa	Hydrophitoxin-a	6635	-	-	-	Liu <u>et al.</u> (1972)
		Hydrophitoxin-b	-	-	-	-	Liu <u>et al.</u> (1972)
<u>Lapemis hardwickii</u>	Gulf of Thailand	Toxin	6774	6800	-	6800	Tu & Hong (1971)
<u>Pelamis platurus</u>	Formosa	Toxin	6223	-	6600	-	Tu <u>et al.</u> (1975)
		Pelamitoxin	7000 (approx.)	-	-	-	Liu <u>et al.</u> (1975)

Contd.....

TABLE - 2 (Contd...)

Snake	Origin	Toxin	M e t h o d s				REFERENCE
			Amino acid composition	Sedimentation equilibrium	Gel filtration	S and D	
<u>LATICAUDINAE</u>							
<u>Laticauda semifasciata</u>	Okinawa	Laticotoxin-III	-	-	7000	-	Uwatoke-Setoguchi (1970)
		Erabutoxin-a	6850	7430	-	-	Sato <u>et al.</u> (1969)
		Laticotoxin-IV	-	-	7000	-	Uwatoke-Setoguchi (1970)
		Erabutoxin-b	6870	7430	-	-	Sato <u>et al.</u> (1969)
		Erabutoxin-c	-	-	7000	-	Uwatoke-Setoguchi (1970)
	South China Sea	Toxin-a	6847	-	7000	-	Tamiya & Abe (1972)
		Toxin-b	6840	6800	6600	-	Hong (1970), Tu <u>et al.</u> (1971)
<u>Laticauda colubrina</u>	Okinawa	Toxin-b	6677	6500	6400	-	Hong (1970), Tu <u>et al.</u> (1971)
<u>Laticauda colubrina</u>	Okinawa	Laticotoxin-a	6970	-	-	-	Sato <u>et al.</u> (1969)
<u>Laticauda laticaudata</u>	Okinawa	Laticotoxin-a	6970	6520	-	-	Sato et al. (1969)
<u>Aipysurus laevis</u>	Australia	Toxin-a	-	-	-	-	Maeda and Tamiya (1976)
		Toxin-b	-	-	7000	-	Maeda and Tamiya (1976)
		Toxin-c	-	-	-	-	Maeda and Tamiya (1976)

Barne (1963) reported that antivenin against Lapemis hardwickii manufactured in Vietnam effectively neutralised the venoms of E.schistosa and Hy.cyanocinctus.

Minton (1967) reported that different cobra venoms are neutralised by E.schistosa antivenin. Tu and Ganthavorn (1969) demonstrated that venoms of Hydrophis cyanocinctus, Pelamis platurus and Lapemis hardwickii cross reacted immunologically with anti E.schistosa serum. Sato and Tamiya (1970) reported that eventhough erabutoxins a and b of L.semifasciata differ slightly, they formed fused precipitation line with anti erabutoxin b.

Tu et al. (1971) showed that the detoxified neurotoxins formed a precipitin line with antibody made from the original venom. Rankonkar et al. (1972) studied the efficacy of Haffkine Institute's polyvalent antivenin and showed that it is ineffective against Malayan E.schistosa venom.

Baxter and Gallichio (1974) showed cross neutralization of Notechis scutatus (Tiger snake) antivenin and E.schistosa antivenin against several sea snake venoms. Tu and Salafranca (1974) reported that venoms of Latigauda semifasciata and Latigauda laticaudata did not form

precipitin lines with anti E.schistosae serum.

Tu and Salafranca (1974) further reported that the commercial antivenin for E.schistosae venom manufactured at the Commonwealth Serum Laboratory, Melbourne, Australia is effective for the neutralising the venoms of sea snakes Pelamis platurus from Central America, L.semifasciata from Philippines and L.laticaudata from Japan.

3.4 Materials and Methods :

3.4.1 Protein estimation :

The following methods were used for the estimation of proteins.

3.4.1.1 Direct spectrophotometric determination :

Direct measurement of the absorbance at 280 nm was used for the rapid estimation of protein in the individual fractions. The total protein concentration of the crude venom and of individual proteins were determined by the method described by Kalckar (1947) using the formula :

$$\text{Protein mg/ml} = 1.45 E_{280} - 0.74 E_{260}$$

3.4.1.2 Protein determination by Folin-Lowry method :

This is the most sensitive method for the estimation of protein. The method proposed by Lowry

et al. (1951) using Folin-Ciocalteu (Centron) reagent was followed. The principle of this method is that a copper tartarate complex is allowed to react with the proteins in alkaline solution. The protein copper complex can then reduce phosphomolybdate to form a blue substance with a broad absorption peak around 750 nm.

3.4.2 Fractionation techniques :

Methods like dialysis, salt fractionation and solvent fractionation have been successfully employed by many workers for the separation of proteins (Michael and Jung, 1936; Carey and Wright, 1960a; ^{Gherli}Chaudhury, 1943; Muic and Piantanida, 1955; Doery, 1958). However, with the advent of protein chemistry, new techniques have been developed. Gel filtration and ion exchange chromatography have become the method of choice for the fractionation of venom proteins.

In the present investigation gel filtration and CM-cellulose chromatography were used.

3.4.2.1 Gel-filtration (Chromatography on cross linked dextran gels : Sephadex) :

Gel chromatography is the technique employed for the separation of substances according to their molecular size. Large molecules will emerge first from the bed, while smaller molecules are retarded. The term "Molecular

sieving" was introduced by McBain (1926), but the term "gel filtration" (Porath and Flodin, 1959) is often used.

Various types of gels are commercially available, namely, dextran gel, polyacrylamide gel (Bio gel), agar gel and agarose gel (Sevavac), cross linked dextrans i.e., Sephadex (Pharmacia) gels are particularly very useful for the separation of substances of biological origin with the molecular weight from several hundred to several hundred thousand. Different types of Sephadex grades are available commercially which differ in degree of cross linkage and so the swelling properties.

Molecules larger than the largest pores of the swollen Sephadex i.e., above the exclusion limit, cannot penetrate the gel particles and therefore they pass through bed in the liquid phase outside the particles and are eluted first. Smaller molecules, however, penetrate the gel particles to a varying extent depending on their size and shape and are eluted from a Sephadex bed in order of decreasing molecular size. The choice of the appropriate Sephadex type depends upon the molecular size and the chemical properties of the substances to be separated.

In the present work various types of Sephadex grades were used. The column dimensions and eluents were

also different depending upon the nature of the solute to be filtered. The details of the particular experiments were given in the experimental section.

3.4.2.1.1 General procedure :

Sephadex gels were allowed to swell in the buffer for the period of 3 hours to 3 days at room temperature depending upon the type of grade used. The gel and the supernatant were mixed gently to form a slurry of uniform consistency for packing the bed. Good separation depend upon the uniform packing. In our work columns were packed according to manufacturer's suggestion .

After completion of the packing, the column was equilibrated with the same buffer by allowing to flow the buffer over-night through the bed. Flow was regulated by gravity feed and by keeping the buffer reservoir at higher level. As the separation results are entirely dependant on the quality of column packing, bed was checked before use. For this purpose Blue dextran (Pharmacia) was used. All experiments were performed at 2-5°C. The sample was dissolved in the elution buffer containing sucrose (1%) and applied to the top of the column without disturbing the gel surface. Small amount of eluent was carefully filled and the eluent reservoir

was connected. Fractions of 3 ml each were collected at the rate of 18 ml per hour. The fractions obtained were measured at 280 nm on Carl-Zeiss spectrophotometer. The fractions under each protein peak were pooled, and their protein content was determined and used further for testing toxicity and pharmacological activity.

3.4.2.2 Chromatography on cellulose ion exchangers :

Peterson and Sober (1956) were the first to introduce the cellulose ion exchanger in separation of larger polypeptides such as proteins and nucleic acids. The separation of proteins on cellulose ion exchangers is believed to be depend mainly on the electrostatic interactions of the polyelectrolytic protein with the oppositely charged adsorbent. The relative affinity of a protein, therefore, depends on its charge density. This affinity can be modified a) by altering the charge of solute and/or that of the adsorbent and b) by increasing the concentration of the buffer salts where ions also have affinities for the adsorbents. Proteins exhibit an absorption equilibrium between the adsorbent and the eluent of a fixed buffer composition. For this reason, chromatography of proteins is often carried out under the conditions nearly approaching to that of all or none absorption by either a stepwise or a gradient increase of eluent.



3.4.2.2.1 Activation procedure :

CM-cellulose CM-II (Whatman), 0.7 m equiv/gm (carboxymethyl group content) was used (Te Palo King, 1968). 15 g of dry cellulose was suspended in 600 ml of 1.0N NaOH and 1.0N NaCl. The suspension was dispersed with the magnetic stirrer until free of trapped air bubbles. After setting for about 30 minutes the supernatant was decanted and the cellulose was resuspended in 600 ml of 1.0N NaCl and filtered by suction on a Buchner funnel and washed with about 2 litres of 1N NaCl. The moist cake was suspended in 600 ml of 1N HCl just for one minute and immediately filtered by suction and washed with water until the washed solution was near neutral. The ion exchanger was then brought to the desired pH by three cycles of resuspension and washing in 600 ml of concentrated solution of the starting buffer. After decantation of the excess of concentrated buffer, the cellulose was resuspended in 600 ml of the starting buffer and stored in the same buffer.

3.4.2.2.2 General procedure :

The column (1X 30 cm) was used for an ion exchange chromatography. Packing of the column is similar to that of gel filtration. Gradient increase in the concentration of buffer was achieved by using linear gradient mixer. The linear gradient increase and stepwise

increase in the concentration of buffer salt was used for elution. The fraction of 3 ml each were collected at the flow rate of 18 ml per hour and the absorbance of each fraction was measured at 280 nm on Carl-Zeiss spectrophotometer. The fractions belonging to the same peak were pooled and desalted. Protein content of the desalted fractions were determined and further used for toxicity and pharmacological studies.

3.4.3 Desalting of proteins :

Sephadex G-15 (Pharmacia) was used for the desalting of venom proteins. In this method the large molecules (i.e., proteins) move with the void volume and are separated from the low molecular weight salts. Sephadex G-15 was packed in the column (1 X 25 cm) according to the procedure given in Section (3.4.2.1.1). Distilled water being used as an eluent. Sample volumes upto 30% of the total bed volume was used for desalting.

3.4.4 Testing of homogeneity of purified toxic fraction :

The testing of homogeneity is very important step in the purification of proteins from the complex mixture. Various methods have been used for this purpose viz., disc electrophoresis, immunoelectrophoresis, gel diffusion and gel filtration. Out of these disc electro-

phoresis and immunoelectrophoresis are widely used for testing the homogeneity.

3.4.4.1 Polyacrylamide disc gel electrophoresis :

This method has been pioneered by Ornstein and Davis (1964) and is the method of choice for high resolution in the separation of components of the protein mixtures and for testing their homogeneity.

Principle : This is achieved by taking advantage of the Kohlraush "regulatory function " (1897). If one layer of solution of a slower moving ion over a solution of a faster moving ion of the same charge, the boundary between the two ions will be maintained under the conditions of Kohlrausch's regulatory function. If a solution of a third ion of the same charge but of intermediate mobility is placed in the solution, it will assume a position at the boundary between the first two ions and will be "sandwiched" into a narrow zone.

The cationic system of Resfield et al. (1962) using 15% running gel which stacks at pH 5.0 and runs at pH 3.8 was followed. All the reagents used in this experiment were purchased from Eastman Chemical Co..

The perfectly cleaned and dried tubes were held in a vertical plane by placing them in a rubber stopper. The running gel solution which was composed of a sufficient concentration of polyacrylamide to effectively sieve the molecules, was prepared and carefully layered by water, which was drained off by tilting the tubes when the running gel was completely polymerized. Spacer gel solution then placed over the running gel. The tubes were placed directly under the daylight fluorescent light to photopolymerize the spacer gel. The sample (50 to 200 ug) was mixed with about 0.15 to 0.2 ml of the sample gel working solution and carefully placed over the photopolymerized spacer gel. Sample gel was then allowed to photopolymerize. Rubber stoppers were then removed carefully without disturbing the gel column. The tubes were then placed in the apparatus. Reservoirs were filled with the buffers, 0.1 % methyl green was used as reference marker stirrer into the upper reservoir buffer.

The electrophoresis was carried at 4°C. The current of 4 mA per tube was passed for 90 minutes. The gels were removed from the tubes and were stained with 1% Amido black 10 B in 7% acetic acid. The excess dye was removed by washing with 7% acetic acid. The gels were stored in 7% acetic acid.

3.4.4.2 Immunogel diffusion :

Gel diffusion reactions are dependant on the higher water content (99%) of the gelatinous supporting media. This permits the antigens and antibodies to migrate through the gel by diffusion following the Fick's law -

$$dx/dt = Dd^2C/dt^2$$

where C represents the concentration of diffusing agent, t the diffusion time, x the distance of diffusion and D the coefficient of diffusion.

This method was introduced by Oudin (1946). This is a simple and widely used method for the examination of antigenic compositions of unknown samples as well as for the evaluation of the antigenic relationship between different protein antigens. When antigen and antibody are allowed to diffuse in opposite direction through agar gel, precipitin line appears at the point of equivalence where the proportion of two substances is optimal for flocculation. Each precipitation line represents the presence of one antigen and its specific antibody.

Experiment was performed according to Ouchterlony's technique (1948). 1.0% w/v agar (Difco) was

prepared by heating the agar in 0.9% (w/v) sodium chloride to which 0.1% sodium azide was added as a preservative. The agar was filtered hot through moist cotton and 20 ml of this agar solution was poured into a petri dish (10 cm in diameter) in which cups (5 to 7 mm diameter) has arranged at equal distances of 1 cm from central cup. The agar was allowed to set. Cups were then removed and bottoms of the wells were sealed with 1 or 2 drops of melted agar. Central well was filled with the antiserum while the surrounding wells were filled with the venom and fractions.

For micro gel diffusion clear microscopic slides (2.5 X 7.5 cm) were used. 3 ml of 1% melted agar solution (prepared as above) was poured over the slide and allowed to solidify. Wells of 2 mm diameter were made into the agar at equal distance from the central well. 1 to 2 drops of appropriate sample of antigen and antibody were carefully placed in the well by Pasteur pipette.

3.4.4.2.1 Washing and Staining :

After complete development of precipitation lines (48-72 hours for macro and 24 hours for micro) the agar was washed for 5 to 6 days with normal saline. The final washing was done with distilled water and gel was

dried. Staining of the gel was carried out according to the method of Graber (1959) with 0.5 % Amido black 10B (E.Merck) in methanol-acetic acid-water (40:10:50). Petri dish or slide was kept in the stain for 10 minutes. The excess stain was removed by washing with 2% glacial acetic acid solution. The final washing was done with 15 % glycerol solution which prevents the agar from cracking and the slide can be preserved indefinitely.

3.4.4.3 Immunoelectrophoresis :

This method was introduced by Graber and Williams (1955) and Scheidegger (1955). It consists of a combination of electrophoresis and immunogel diffusion. The complex antigenic mixtures may be separated because of the additional resolving power obtained through the electrophoretic step.

Agar (Difco) was used as a medium in the experiment. One percent agar solution was prepared in Veronal acetate buffer, 0.025M, pH 8.6. Melted agar (3.0 ml) was poured on a suitably levelled microslide previously washed and dried. When the agar solidified, a hole (2 mm dia.) was made in the centre. The slide was then transferred to the electrophoretic chamber and kept in a perfectly levelled conditions. The chambers were then filled with buffer (0.05M Veronal buffer, pH 8.6) The test sample was poured in the well and the slide was

subjected to electrophoresis for 3 hours at 3.5 mA current per slide. At the end of the electrophoresis, 2 mm wide trench was cut on either sides of the point of application of the sample. The trenches were made lengthwise and kept at equal distance from the sample on either side. The antiserum was then carefully placed in these trenches. The slides were kept under moist condition (by keeping water soaked cotton around the slide in the petri dish) for the development of precipitation lines. The lines were formed within 48 hours. The washing and staining were carried out as given in Section (3.4.4.2.1).

3.4.5 Preparation of monovalent antisera :

Monovalent antiserum against the venom of Enhydrina schistosa (common sea snake) was prepared by immunising rabbits. Albino rabbits of 2 to 2.5 kg were selected. A stock solution of 1.0% (w/v) venom in glycerol was prepared and sterilised by millipore filtration. Primary immunization was carried out by administration of two subcutaneous injections, 1st and 8th day of 1.0 μ g of the venom solution. Then at weekly intervals, supplemental subcutaneous injections of increasing doses of venom emulsified with equal volumes of Freund's incomplete adjuvant (Liquid paraffin 82% + Lanolin 15% + Aluminium monostearate 3%) were given.

Freund's complete adjuvant (Difco) was used for last three doses (1000 µg to 1500 µg). Rabbits were bled 7th day after 9th, 12th, 14th, 16th and last injections. Their serum was collected and freeze-dried until use.

TABLE - 3

Immunizing schedule of the rabbits with the *Enhyalina*
schistosa venom.

No.	Day	Dose of venom (µg)	Volume (ml)	Route of Injection	Adjuvant
1	1st	1.0	1	Subcutaneous	-
2	8th	1.0	1	-do-	-
3	15th	2.0	1	-do-	-
4	22nd	4.0	1	-do-	-
5	29th	10.0	1	-do-	-
6	36th	20.0	1	-do-	Freund's incomplete
7	43rd	40.0	1	-do-	-do-
8	50th	40.0	1	-do-	-do-
9*	57th	80.0	1	-do-	-do-
10	67th	80.0	1	-do-	-do-
11	74th	150.0	1	-do-	-do-
12*	81st	150.0	1	-do-	-do-
13	91st	300.0	1	-do-	-do-
14*	98th	500.0	1	-do-	-do-
15	108th	1000.0	1	-do-	Freund's Complete
16*	115th	1000.0	1	-do-	-do-
17*	125th	1500.0	1	-do-	-do-

* Rabbits bled after these doses.

3.4.6 Molecular weight determination :

The molecular weight of the purified toxic fraction separated from E.schistosoma venom was determined by the method described by Andrews (1964) using gel filtration.

Previously washed and equilibrated Sephadex G-75 (Pharmacia) was suspended in 0.05M Tris-HCl buffer (pH 7.8). The column (2.5 X 50 cm) was packed according to the procedure given in Section (3.4.2.1.1). After packing, the column was equilibrated with the same buffer overnight. Experiments were carried out at 5-8°C. Blue dextran 2000 (Pharmacia) was used to determine void volume.

The proteins were dissolved in the elution buffer (2.0 ml) containing 5 mg sucrose and applied to the column. The column was then eluted with the same buffer. Fractions of 3 ml each were collected at the rate of 18 ml per hour. The column was calibrated against the proteins of known molecular weights. Standard proteins used for the calibration were Insulin (Boots Pure Drugs), Cytochrome C (Sigma), Trypsin (Nutrition Biochem.CO.) and Pepsin (Met. Drugs). The elution volume (V_e) for each of the standard proteins were determined and plotted against the log molecular weight.

3.5 Experimental and Results

3.5.1 Protein estimation of the venom :

The protein concentration of the lyophilized E.schistosa venom gland extract was determined by direct spectrophotometric method and by Polin-Lowry's method as described in the Section (3.4.1). The results of these experiments are given in Table 4.

TABLE - 4
Protein content of E.schistosa venom

Method	Protein (%)
1. Direct Spectrophotometric Method	90.0
2. Lowry's Method	92.6

3.5.2 Protein components of E.schistosa venom :

Lyophilized venom gland extract was subjected to electrophoresis on polyacrylamide gel according to the procedure described in Section (3.4.4.1). Figure 5 shows the protein pattern of the venom. The E.schistosa venom showed as many as 8 to 9 visible bands.

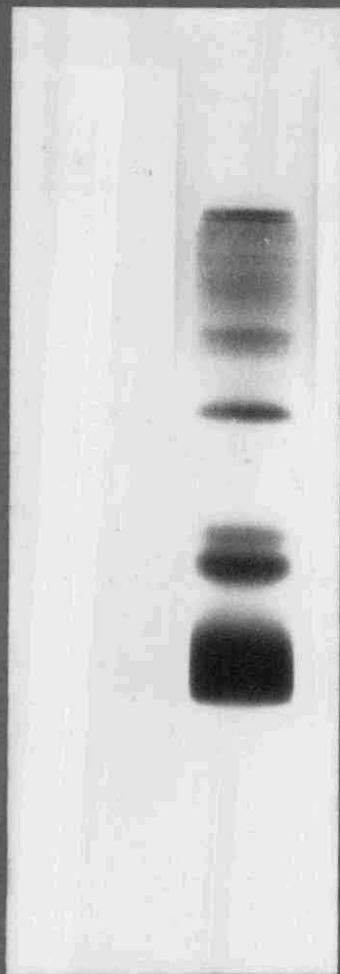


Fig. 5 : Disc Electrophoresis of the crude venom

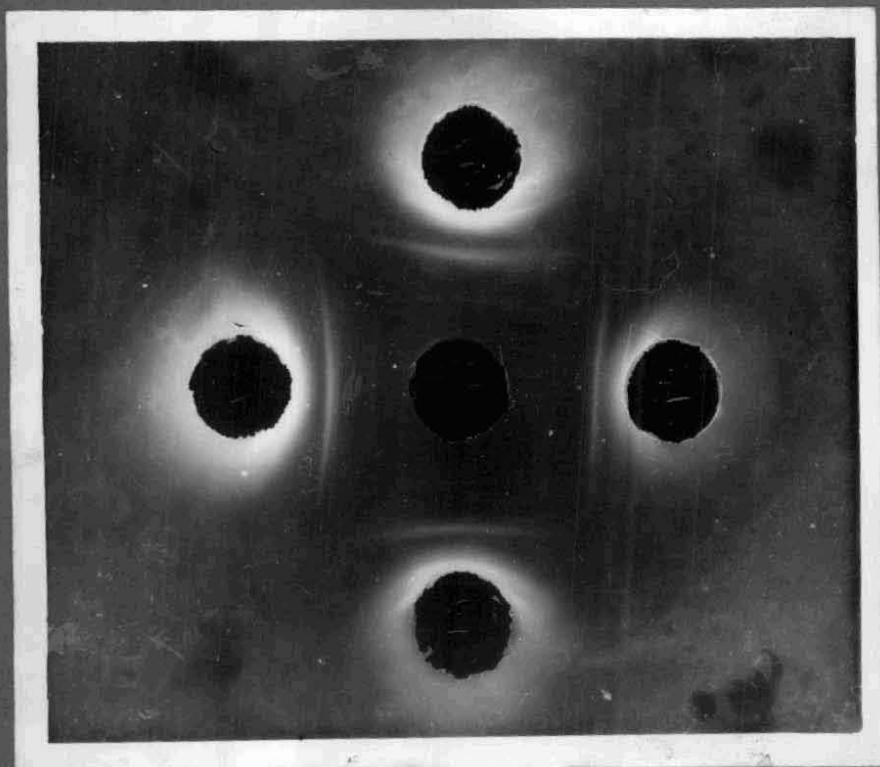


Fig. 6 : Gel diffusion pattern of crude venom against
Anti E.schistosa serum.

3.5.3 Antigenic composition of the venom :

Antigenic composition of the E.Schistosa venom was determined by gel diffusion and immunoelectrophoresis. Figure 6 and Figure 7 show the gel diffusion and immunoelectrophoretic patterns of E.schistosa venom against experimental monovalent E.schistosa antiserum prepared in rabbits.

3.5.4 Purification of biologically active component from E.schistosa venom :

The venom was fractionated by CM-cellulose chromatography followed by gel filtration on Sephadex G-50.

3.5.4.1 CM-cellulose chromatography :

Previously washed and activated CM-cellulose was equilibrated with the initial buffer 0.005M ammonium acetate (pH 5.0). The equilibrated CM-cellulose was packed in a glass column (1 X 30 cm). The flow rate was adjusted to 18 ml per hour. The column was loaded with 50 mg of the venom dissolved in 2.0 ml of starting buffer (0.005M ammonium acetate, pH 5.0). Fractions of 3 ml each were collected. All operations were carried out at cold room temperature (4 to 8°C). The absorbance of each tube was measured at 280 nm on Karl-Zeiss spectrophotometer. The fractions from each protein peaks were pooled, desalted on Sephadex G-15 and lyophilized, the

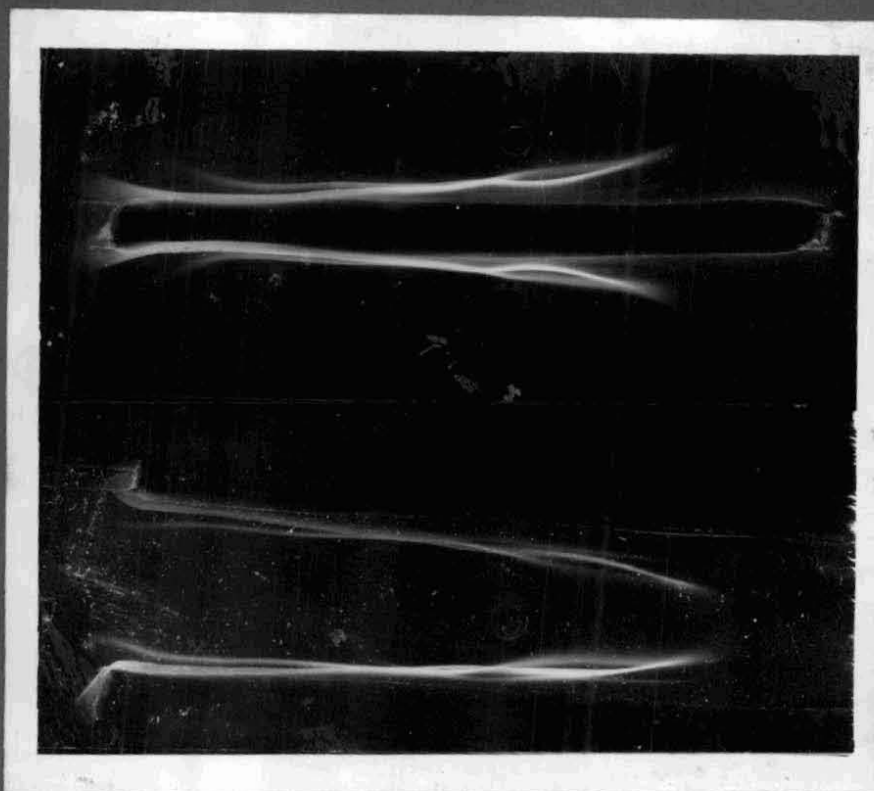


Fig. 7 : Immunoelectrophoretic pattern of the crude
venom against Anti E. schistosa serum.

toxicity of each fraction was determined. Both the methods, gradient increase and step-wise increase in the concentration of the buffer, were used. Advantage of step-wise method over gradient increase is that fractions are obtained in the concentrated form. Venom was separated into four major peaks as shown in the Figure 8 and Figure 9. The fraction CM-IV was the most toxic and had LD_{50} (i.m.) in mice .07 mg/kg of body weight. Fractions CM-I, CM-II and CM-III were nontoxic to mice even in the doses of 100 µg/mouse. The fraction CM-IV was the only toxic and contained about 70 % of the recovered protein. Fraction was further purified by gel filtration on Sephadex G-50.

3.5.4.2 Gel chromatography of fraction CM-IV on Sephadex G-50 :

Sephadex G-50, previously swelled in distilled water and washed with buffer (0.05M Tris-hydrochloride, pH 8.0) was packed in a glass column (1.8 X 51 cm) and equilibrated for overnight with the same buffer. Lyophilized fraction CM-IV (26.9 mg) was dissolved in 1.0 ml of Tris-HCl buffer (0.05M, pH 8.0) and applied to the column. Flow rate was adjusted to 18 ml per hour and fractions of 3 ml each were collected. All operations were carried out at 4 to 8°C. Absorbancy was measured at 280 nm on Karl-Zeiss spectrophotometer.

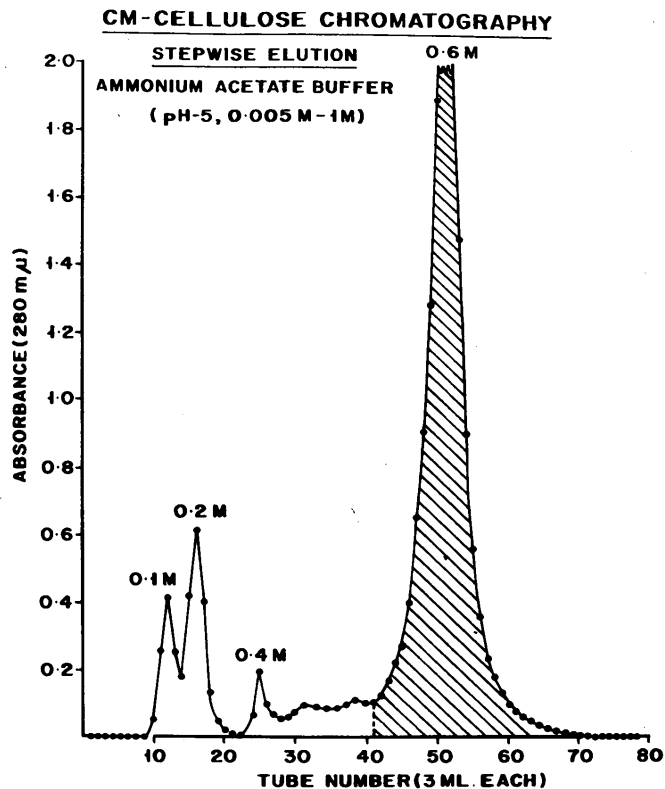
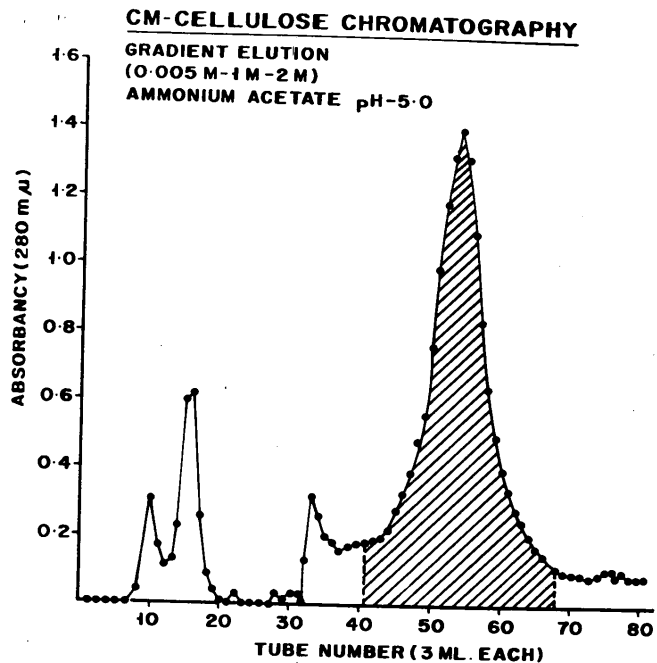


Figure - 8

Fraction CM-IV was separated into two fractions CM-IV-Sa and CM-IV-Sb. The elution profile is shown in figure 10. The fraction CM-IV-Sb was non-toxic to mice even at the doses upto 100 µg/mouse, while fraction CM-IV-Sa was found to be three times as toxic as the original venom. Toxicity of the purified fraction CM-IV-Sa is given in Table 5.

TABLE - 5

Toxicity of purified fraction CM-IV-Sa from *E. schistosoma* venom by CM-cellulose chromatography and Sephadex gel filtration.

Fraction	Protein (mg)	Recovery of protein (%)	Toxicity LD ₅₀ (i.m.) (mg/kg)	Apparent purifica- tion.
Venom	50	100	0.210	1.00
CM-I	1.6	3.2	Non-toxic*	0.00
CM-II	5.2	10.4	Non-toxic*	0.00
CM-III	1.2	2.4	Non-toxic*	0.00
CM-IV	26.9	53.8	0.075	2.80
Recovery	34.9	69.8		
CM-IV-Sa	19.0	38.0	0.070	3.00
CM-IV-Sb	1.5	3.0	Non-toxic*	0.00

* All the mice survived by intravenous injections of 100 µg of fraction per mouse.

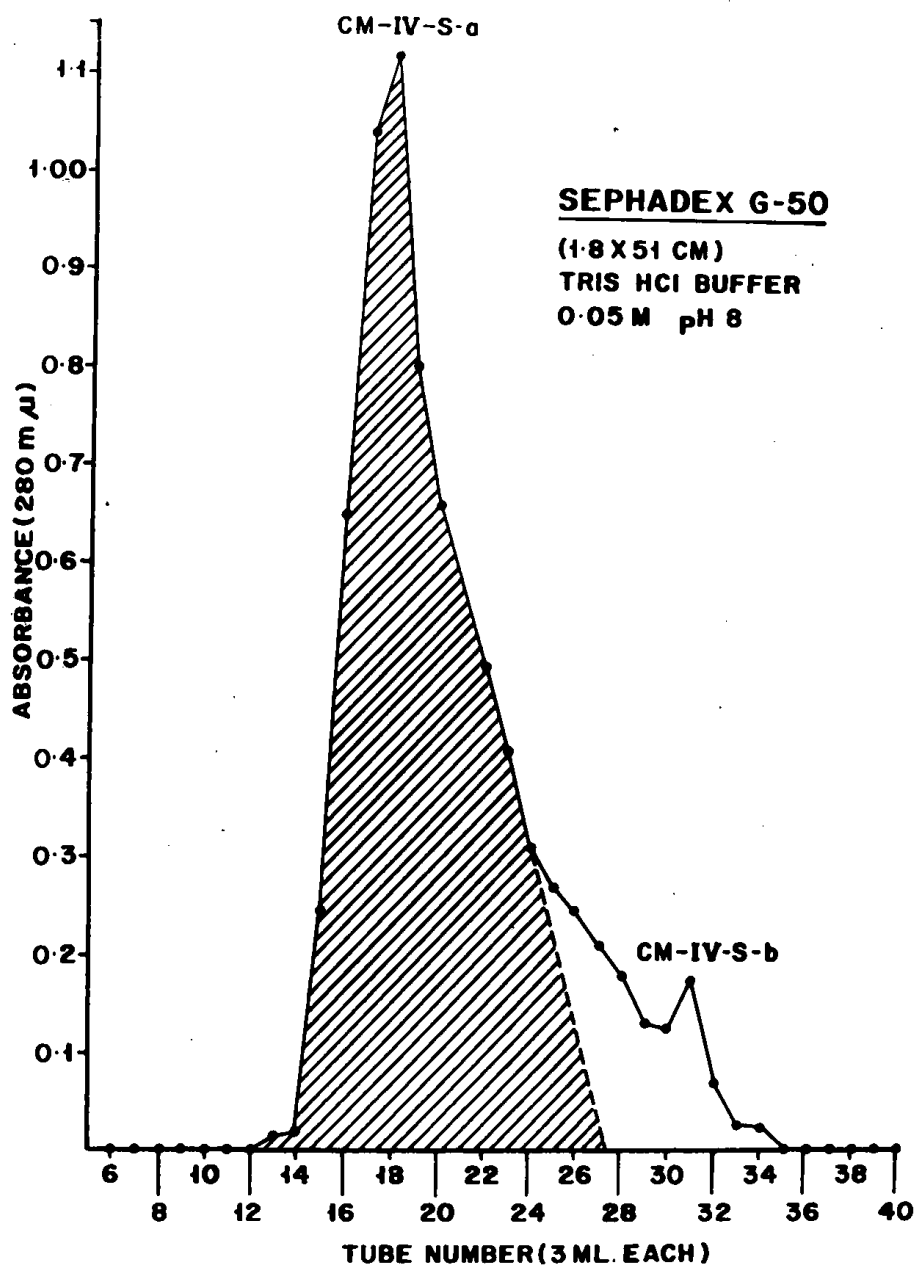


Fig. 10: Gel Chromatography of the fraction CM-IV on Sephadex G-50.

3.5.4.3 Homogeneity of the toxic fraction CM-IV-Sa :

The homogeneity of the toxic fraction was determined by the following methods.

3.5.4.3.1 Polyacrylamide disc electrophoresis :

Figure 11a and Figure 11b show disc electrophoretic pattern of crude venom and the purified toxic component CM-IV-Sa (25 µg) at pH 4.4 on 15 % polyacrylamide gel. Crude venom showed 8 to 9 bands while the toxic component revealed a single band indicating that the toxic component is reasonably homogeneous.

3.5.4.3.2 Immunoelectrophoresis :

Figures 12 and 13 show the immunoelectrophoretic patterns for crude E.schistosoma venom and the purified toxic component against experimental monovalent antiserum. Only one precipitin line was obtained with the toxic component while venom showed 3 to 4 precipitin lines.

3.5.4.3.3 Immunogel diffusion :

Figures 6 and 14 show gel diffusion pattern for crude venom and the toxic component against experimental monovalent antiserum.

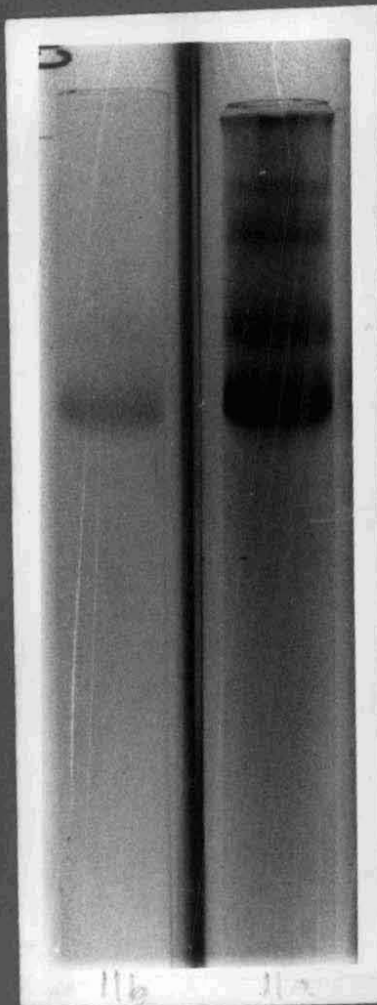


Figure - 11a

11b

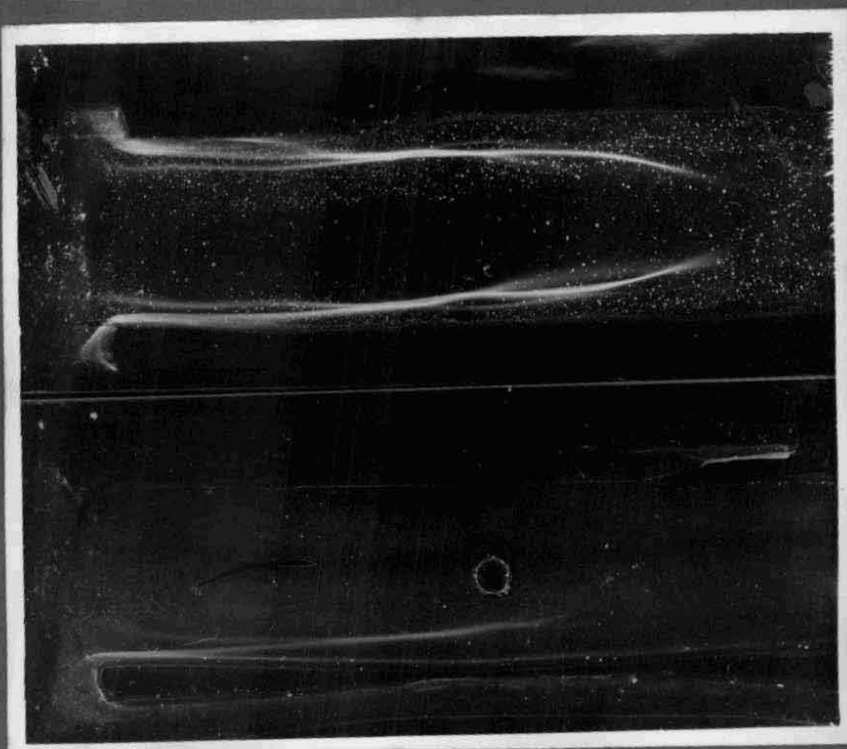


Fig. 12

Fig. 13

Figure-12 : Immunoelectrophoresis of Crude Venom against Anti E.schistosa Venom.

Figure-13 : Immunoelectrophoresis of the Toxin CM-IV-Sa against Anti E.schistosa Venom.

3.5.4.3.4 Gel filtration :

Gel filtration was used as an additional criterion for the homogeneity of the toxic component. As shown in the Figure 15, the toxic component was eluted as a single peak on Sephadex G-50 using Tris-HCl buffer at pH 8.0.

3.5.5 Molecular weight of the purified toxic component:

Molecular weight of the toxic component CM-IV-8a was determined by gel filtration according to the procedure described in Section (3.4.6). The molecular weight was calculated from the standard graph of log molecular weight against elution volume (V_e) as shown in Figure 16. It was found to be 7000 (approximately).

3.6 D i s c u s s i o n

The literature reviewed in Section (3.2.1) clearly showed that neurotoxins are the main and most powerful lethal factors present in the sea snake venoms studied so far, inspite of the presence of large number of enzymes which are shown to be non-toxic.

Our objective was to fractionate the venom and to isolate and characterize biologically active component from the venom of E.schistosa from the Western coast of India.

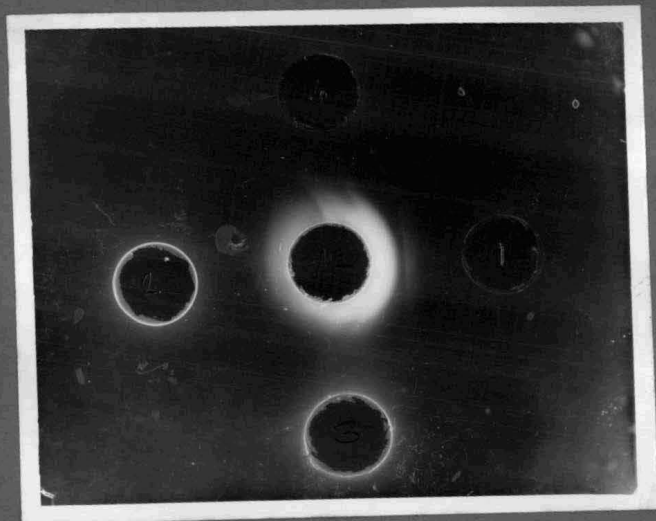


Fig. 14 : Gel diffusion of (1) Venom, (2) Toxin,
(3) Normal saline, against Anti *E.schistosa*
Venom.

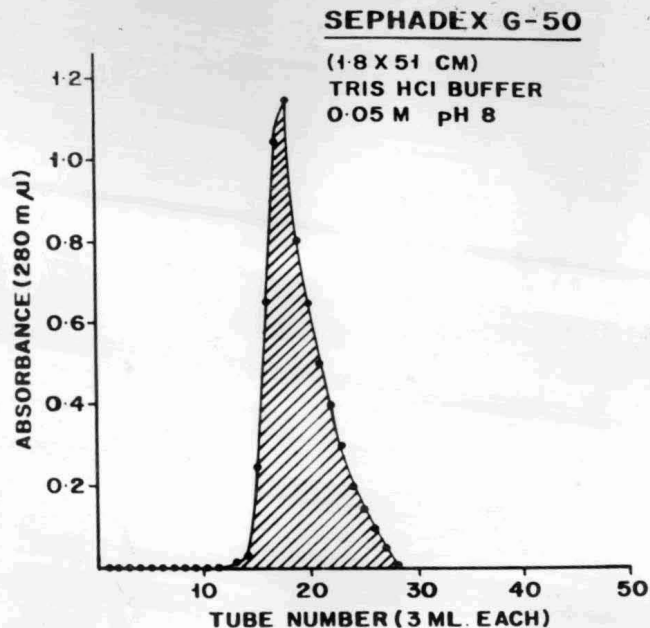


Fig. 15: Gel filtration of the toxin CM-IV-Sa
on Sephadex G-50.

Tu and Toom (1971) isolated the toxic component from the venom of Enhyrina schistosa from the Strait of Malacca by two-step purification procedure. 500 mg of venom was applied to CM-cellulose column and fractionation was carried out by stepwise increase in salt concentration in 0.01M sodium phosphate buffer, pH 7.5. The venom was separated into eight protein peaks four of which were highly toxic. The most toxic fraction (fraction V) was further purified by re-chromatography on second CM-cellulose column previously equilibrated with 0.01M glycine buffer, pH 9.7. Fractions were eluted by increasing salt concentrations. The most toxic fraction was shown to be homogeneous by zonal electrophoresis, ultracentrifugation, isoelectric focusing and gel filtration. The toxin accounted for about 30% of the total venom proteins. The molecular weight of the toxin was 7300 by sedimentation velocity and diffusion experiments. The amino acid analysis of the toxin showed that the toxin consists of 62 amino acids with a molecular weight 6981.

Karlsson et al. (1972) fractionated the same venom obtained from the same region i.e., Strait of Malacca by gel filtration followed by gradient chromatography on Bio-Rex 70 in ammonium acetate buffers. The venom was separated into seven protein fractions by gel

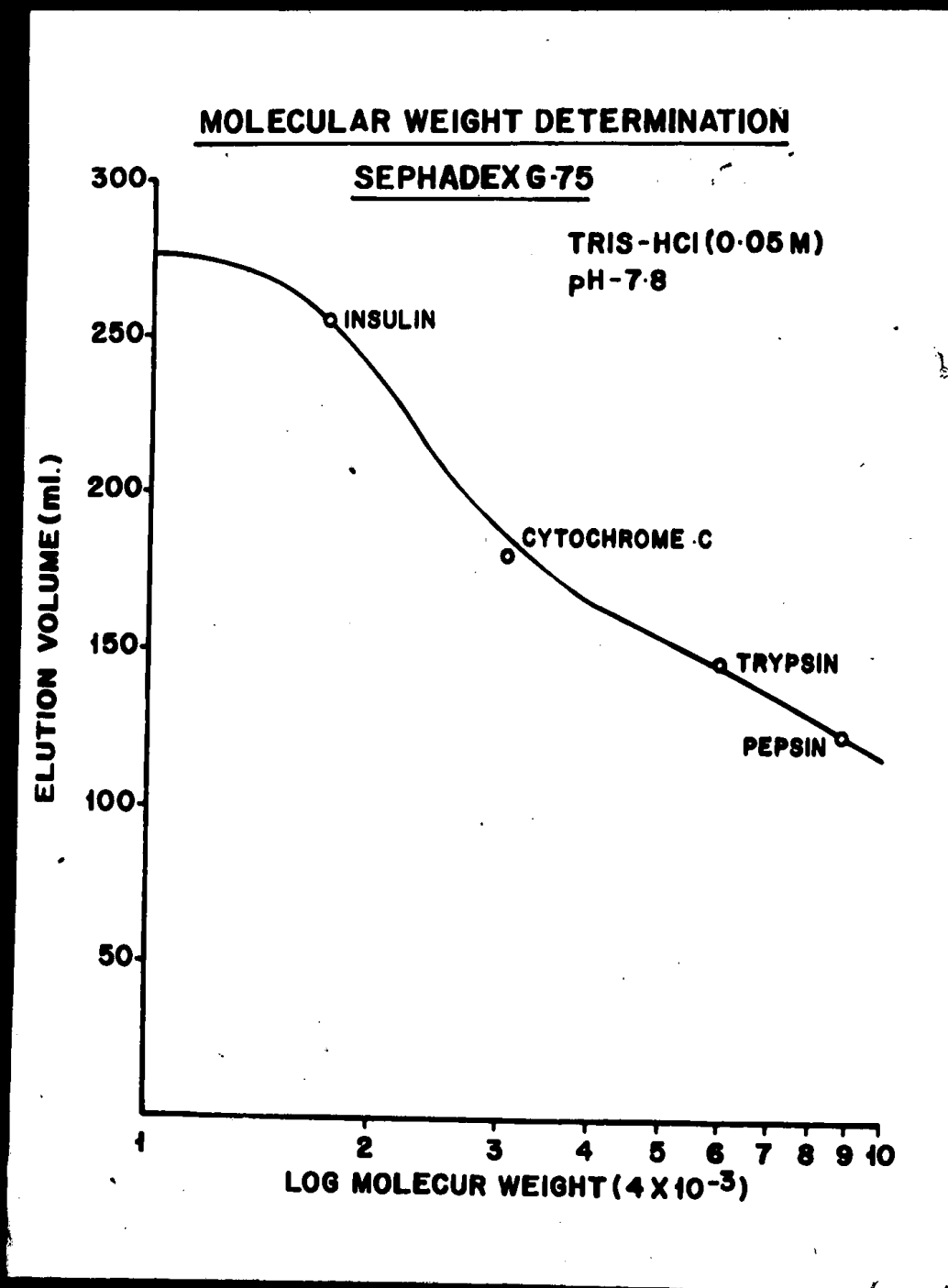


Fig. 16: Standard graph of log molecular weight against
elution volume (V_e).

filtration on Sephadex G-75 using ammonium acetate buffer (0.2M, pH 7.3). The toxic fraction was further separated by gradient chromatography on Bio-Rex 70 in ammonium acetate buffer (0.06M vs 1.4M, pH 7.3) showed that the venom protein might contain 16 or more different neurotoxins. The two principal neurotoxins schistosa 4 and schistosa 5 together accounted for 39% (14% and 25% respectively) of the soluble venom proteins. The amino acid analysis of the toxins revealed that toxins 4 and 5 consist of a single peptide chain of 60 amino acids cross linked by four disulfide bridges with the molecular weights 6689 and 6679 respectively. The toxins schistosa 4 and 5 had LD₅₀ towards mice 85 µg/kg of body weight.

We isolated the toxic component from E.schistosa venom from the Western coast of India by two-step purification procedure; CM-cellulose chromatography followed by gel filtration on Sephadex G-50. The best separation into its component fractions was achieved by CM-cellulose chromatography using gradient increase in the concentration of ammonium acetate buffer (0.005M to 1.0M) at pH 5.0. The venom was separated into four major fractions. The most retarded and the most toxic fraction (CM-IV) was accounted for about 70 % of the

recovered proteins. The toxic fraction was further purified by gel filtration on Sephadex G-50 using Tris-HCl buffer, pH 8.0. The preparation was shown to be homogeneous by disc electrophoresis, immunoelectrophoresis, gel diffusion and gel filtration. The molecular weight of the toxin CM-IV-Sa was determined by gel filtration and was found to be approximately 7000 and had LD₅₀ towards mice 70 µg/kg of body weight. The toxin CM-IV-Sa was accounted for about 38 % of the total venom proteins.

Molecular weight & the toxicity of the toxin CM-IV-Sa is compared with the toxins isolated from the same species from Strait of Malacca (Table 6).

TABLE - 6

Comparison of the molecular weight and the toxicity of the toxins from the *E. schistosa* venom.

Origin	Name	LD ₅₀ (mg/kg)	Molecular weight		Authors
			Struct- ure	Other method	
1. Strait of Malacca	Toxin	0.044	6978	7300	Tu & Toom (1971)
2. Strait of Malacca	Schistosa-4	0.085	6689	-	Karlsson et al (1972)
	Schistosa-5	0.085	6679	-	
3. Western coast of India	CM-IV-Sa	0.070	-	7000	Gawade & Bhife (1976)

On comparison, the toxicity and the molecular weight, the toxin CM-IV-3a appears to be slightly different from the toxin (Tu and Toom, 1971) and two neurotoxins schistosa 4 and schistosa 5 (Karlsson et al., 1972) isolated from the same venom from the Strait of Malacca. However, this difference may be due to different laboratory conditions or variations in the animals used or the species of E.schistosa available along the Western coast of India may be from different subspecies. The variation in toxins of the given species from different geographical areas has already been demonstrated by Miranda et al. (1970). However, it is too early to conclude anything at this stage. Further work regarding amino acid analysis, I.R., N.M.R. and mass spectroscopy may reveal the exact nature of the toxin.

CHAPTER IV

PHARMACOLOGICAL STUDIES

4.1 Introduction :

Studies of the pharmacology of the neuromuscular junction and progress in the physiology of the neuromuscular junction are closely linked. The first physiological demonstration of the neuromuscular junction dates from the studies on curare by Claude Bernard (1856), who showed that this agent blocked the transmission of impulses from nerve to muscle without apparent effects on either the nerve trunk or the muscle fibre themselves. Later, King (1935) isolated (+) tubocurarine from tubocurare and stimulated greater interest in the mode of action of neuromuscular blocking agents.

The important application of neuromuscular blockers i.e., curare is in the modern practice of anaesthesia to obtain the necessary muscle relaxation (Griffith and Johnson, 1942; Cullen, 1943). It has also been used as a muscle relaxant, as adjuvant to general anaesthesia in abdominal surgery so the general anaesthesia required is less by orthopaedic surgeons (Coch and Lundskog, 1950) in the treatment of poisonings and diseases connected with increased motor activity (Strychnine, Tetanus). A further application of curare is in psychiatric electroshock therapy.

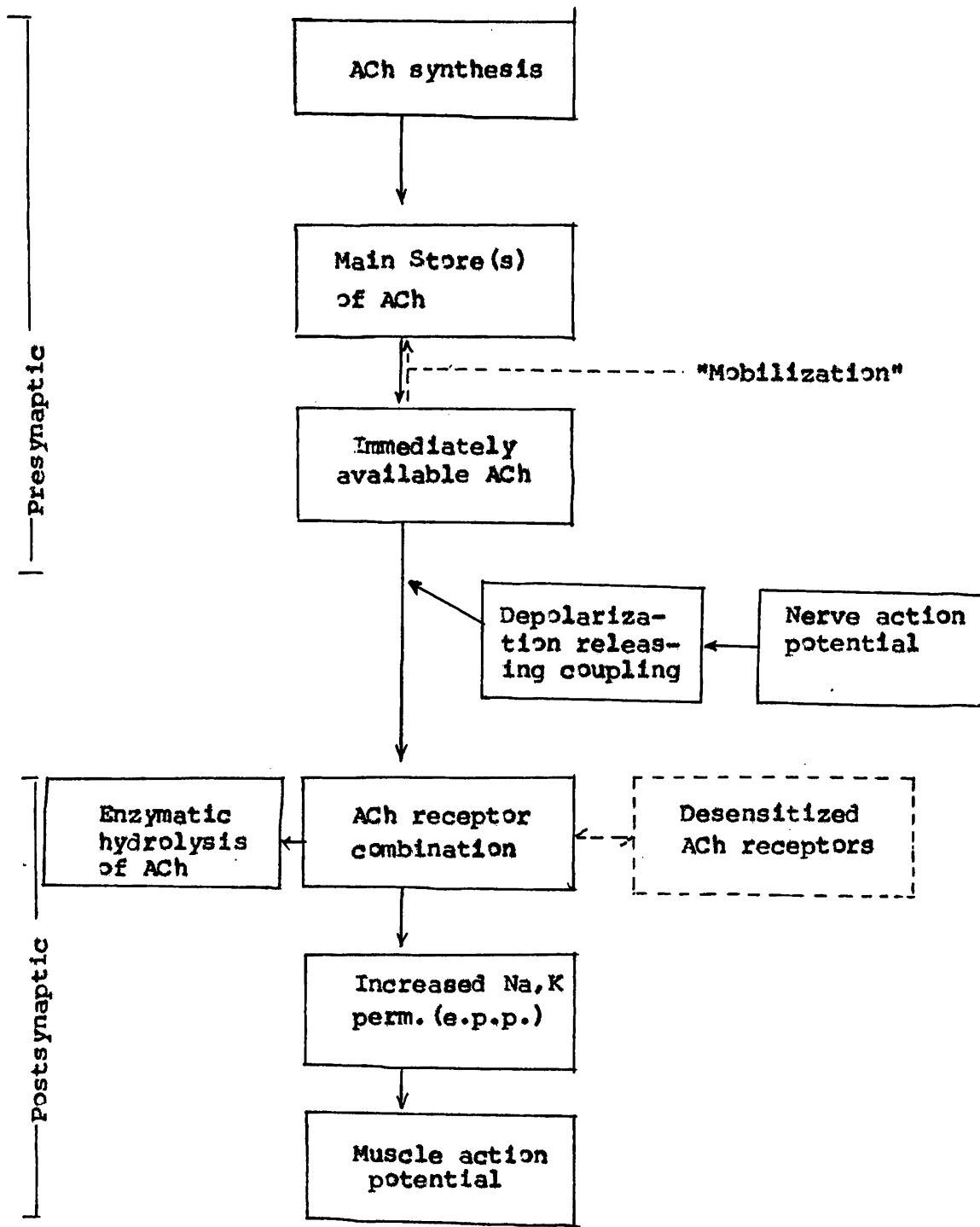
The difficulty in obtaining plant source of tubocurarine led investigators to search for another compound either of synthetic or of natural origin with similar type of action.

4.2 Physiology of neuromuscular transmission :

The sequence of events from liberation of acetylcholine (Ach) by nerve action potential (AP) to contraction of the muscle fibres are illustrated in Figure 17.

At rest, interior of the typical mammalian axon is approximately 70 mV negative to the exterior. The resting potential is based mainly on the 30 to 50-fold higher concentration of potassium ion in the axoplasm as compared with the extracellular fluid, and the relatively high permeability of the resting axonal membrane to potassium ions. Sodium and chloride ions are present in higher concentrations in the extracellular fluid than in the axoplasm but their concentration gradients across the membrane are lower than that of potassium and the axonal membrane at rest is considerably less permeable to these ions; hence their contribution to the resting potential is relatively minor. These ionic gradients are maintained by an energy-dependent

**Fig. 17 : DIAGRAMMATIC REPRESENTATION OF THE NEURO-
MUSCULAR TRANSMISSION**



(Thesleff and Quastel, 1965)

active transport or pump mechanism, involving an adenosine triphosphatase (ATPase) activated by sodium at the inner and by potassium at the outer surface of the membrane (Albers, 1967; Thomas, 1972). At some sites, an electrogenic sodium pump may also contribute to the net resting potential (Casteels et al., 1971).

In response to a stimulus above the threshold level, a nerve action potential (AP) or nerve impulse is initiated at a local region of the membrane. This is detectable first by a rapid deflection of the internal resting potential from its negative value toward zero, and continuing uninterruptedly to a positive overshoot. This local reversal of the membrane potential is due to a sudden, selective increase in the permeability of the membrane to sodium ions, which flow rapidly inward, in the direction of their concentration gradient. Repolarization of the membrane follows immediately and results from the rapid replacement of this change by one of increased permeability to potassium. The transmembrane ionic currents produce local circuit currents around the axon which activates adjacent inactive regions. This brings about the propagation of the AP.

The arrival of the AP at the axonal terminals initiates series of events as follows :

Acetylcholine synthesis : As shown in the figure 18, acetylcholine (ACh) is manufactured in motor nerve terminal cytoplasm from acetyl coenzyme A (acetyl Co A) of mitochondrial origin, the choline derived from extracellular fluid by an active membrane mechanism. A cytoplasmic enzyme choline-3-acetyltransferase (ChAc) accelerates synthesis and unknown mechanisms load the ACh into synaptic vesicles. The vesicles, like the mitochondria and ChAc molecules, are derived from the cell body by transport along the motor nerve. The vesicles fuse with the terminal membrane and empty their contents into the synaptic cleft. The vesicle membrane is then incorporated into the terminal membrane and later recovered as a complex vesicle, which in turn sheds its coat to reveal a synaptic vesicle, which is refilled with ACh and recycled.

Even in the absence of nerve impulses there is a spontaneous release of acetylcholine from the nerve endings giving rise to minute voltage fluctuations in the region of motor end-plate (Del Castillo and Katz, 1954; 1956; Fatt and Katz, 1952). Each of these miniature end-plate potentials is the result of the discharge on the motor end-plates of an amount of acetylcholine much too small to excite a propagated muscle excitation wave and this is released into the synaptic gap when the

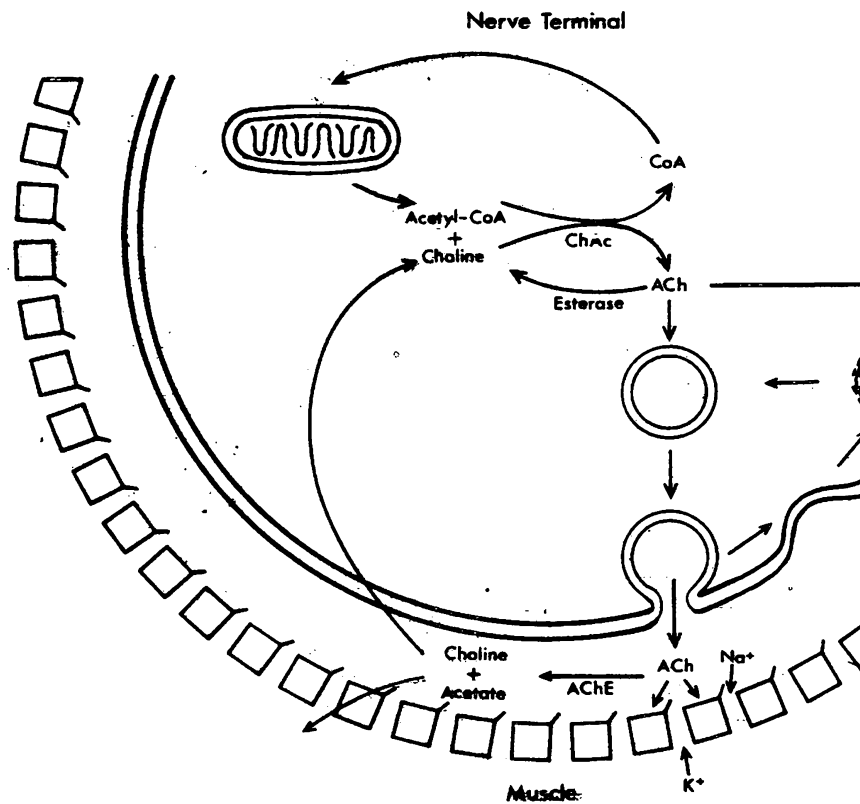


Figure - 18 : Neuromuscular transmission at the Hubbard and Quastel, (1973).

vesicle collides with or penetrates the pre-synaptic terminal membrane. The effective end-plate potential, or the response which triggers the muscle excitation wave, is the sum of large number of these minute effects occurring simultaneously, immediately after the nerve impulse arrives at the nerve terminal (Del Castillo and Katz, 1954; 1956). It is believed that the mechanism of release involves the passage of calcium ions into the pre-synaptic axoplasm (Hodkin and Keynes, 1957) and the amount of acetylcholine released varies over a wide range with the calcium ion concentration (Hutter and Kostial, 1954; Del Castillo and Katz, 1954).

The acetylcholine diffuses across the synaptic or junctional cleft, a distance of 100 to 500^oA and combines with specialized macromolecular receptors on the post-junctional membrane, which results in a localized, non-propagated increase in the ionic permeability, or conductance of the membrane. Two types of permeability changes occur - (i) a generalized increase in permeability to all types of ions, resulting in a localized depolarization of the membrane i.e., an excitatory post-synaptic potential (EPSP) or (ii) a selective increase in permeability to only the smaller ions (e.g., potassium, chloride), resulting in stabilization of actual hyperpolarization of the membrane, which constitutes an inhibitory post-synaptic potential (IPSP).

4.3 Mechanism of neuromuscular blockade :

The transmission of impulse from the nerve to the muscle can be intercepted by the action of a drug either at presynaptic or postsynaptic membrane and lead to muscle relaxation.

Presynaptic action :

Drugs that depress acetylcholine output

- a) by inhibition of synthesis or storage,
- b) by inhibition of release.

Postsynaptic action :

Drugs that prevent the action of released acetylcholine

- a) by depolarizing the muscle end plate,
- b) by preventing the depolarizing action of acetylcholine,
- c) by a mixed action.

4.3.1 Drugs acting presynaptically :

4.3.1.1 Drugs that depress acetylcholine output :

Hemicholiniums and Triethylcholine : These compounds compete with choline for occupation of intracellular transport mechanism to the site of synthesis.

4.3.1.2 Drugs that inhibit the release :

- 1) Tetrodotoxin blocks the action potential (AP) by preventing the selective increase in permeability to

sodium ions associated with the rising phase of AP (Kao, 1966).

ii) Batrachotoxin increases the selective permeability which induces a persistent depolarization (Albuquerque et al., 1973).

iii) Botulinum toxin prevents the release of acetylcholine by obstructing the exists by which the transmitter leaves the nerve terminals after its release from the synaptic vesicles.

iv) Other toxins :

β -bungarotoxin from Bungarus multicinctus venom reduces the acetylcholine output from the nerve endings and leaving the sensitivity of the end plate to acetylcholine unaffected (Lee, 1970; Lee, 1972).

Diphtheria toxin inhibits the conduction of nerve impulses in the motor nerve and may also inhibits acetylcholine synthesis.

v) Local anaesthetics interfere with impulse transmission in nerve fibres. Fine nerve terminals are particularly sensitive to local anaesthetics. Some local anaesthetics reduce the sensitivity of the end plate to acetylcholine.

vi) Cardiac glycosides interfere with the sodium pump mechanism.

vii) Changes in ionic balance : Increase of magnesium ions and reduction in calcium ions produce a neuromuscular block.

viii) Antibiotics : Kanamycin, neomycin and streptomycin produce neuromuscular block by a mechanism similar to that caused by calcium lack. They are also capable of causing an antidepolarizing blockade of muscle.

4.3.2 Drugs acting postsynaptically :

4.3.2.1 Drugs that prevent the action of released acetylcholine :

These drugs are broadly classified into two classes according to their type of action.

- i) Antidepolarizing type,
- ii) Depolarizing type.

4.3.2.1.1 Antidepolarizing agents : These are the drugs that produce blockade without depolarization of end plate. These drugs compete with acetylcholine for the receptors at the end plate but do not themselves cause depolarization.

Tubocurarine : (+) Tubocurarine is the prototype of the series. The block is easily reversed by anticholinesterase compounds i.e., neostigmine, physostigmine. (+) Tubocurarine exhibits a parallel shift of the dose response curve to ACh without reduction in the maximum response, indicating simple competition.

Other antidepolarizing agents are dimethyl-tubocurarine, gallamine triethiodide, benzoquinonium, dihydro- β -erythroidine, laudexium, methylsulphate and pancuronium bromide.

4.3.2.1.2 Depolarizing blocking agents : These agents are not broken down by cholinesterase. By administration of these agents the end plate is held in its depolarized state and the muscle will no longer respond to motor nerve stimulation. Substances that lead to muscle paralysis in this way, are called depolarizing blocking agents. Following are the drugs belonging to this class namely, Decamethonium, Succinylcholine, Suxethonium and carboclonium.

4.3.2.1.3 Dual action : Decamethonium produces block by depolarization in man, while in many mammalian species e.g., dog, monkey, guinea pig and rabbit, decamethonium

always produces a dual type of block. When the drug is injected, it produces block by depolarization but when the block is fully established it can be antagonized by an anticholinesterase or by tetanus and prolonged by d-tubocurarine which shows the presence of an anti-depolarizing blockade (Zaimis, 1953).

4.3.2.1.4 Effect of anticholinesterases : Anticholinesterases e.g., neostigmine, physostigmine bring about a rapid accumulation of acetylcholine at the motor end plate and reverse the neuromuscular blockade produced by agents that compete with acetylcholine. Depolarizing blocking agents have the same effect. Potassium ions, adrenaline and ephedrine, all potentiate the action of acetylcholine at the neuromuscular junction and, used alone or in conjunction with an anticholinesterase, they antagonized the effect of antidepolarizing drugs.

4.4 Mechanism of action of sea snake neurotoxins :

The venom of snakes belonging to the family Hydrophiidae is extremely toxic and produces flaccid paralysis and respiratory failure in animals (Lee, 1972). These effects are attributed to the so called neurotoxins present in these venoms.

Neurotoxins in snake venoms fall into two groups according to the mechanism by which they block the neuromuscular transmission - 1) presynaptic and 2) postsynaptic. All sea snake neurotoxins studied so far act by postsynaptic mechanism (Tamiya and Arai, 1966; Sato et al., 1969, 1970; Tamiya and Abe, 1972; Cheymol et al., 1972; Takamizawa, 1970).

4.4.1 Presynaptic neurotoxins :

These toxins act exclusively on the presynaptic site e.g., β -bungarotoxin (Bungarus multicinctus) which blocks the neuromuscular junction by reducing the Ach output from the presynaptic terminals. Electron microscopic study has shown that β -bungarotoxin induces increased profiles of opened synaptic vesicles at the axolemma, accompanied with a decreased number of synaptic vesicles and subsequently, almost complete depletion of the vesicles in the axon terminals (Chen and Lee, 1970).

4.4.2 Postsynaptic neurotoxins :

It is well exemplified that cobra neurotoxins, α -bungarotoxin and sea snake neurotoxins, all produce an antidepolarizing neuromuscular block by acting on the postfunctional membrane of the motor end plate (Meldrum, 1965; Jimenez-Porras, 1968; Lee, 1970; 1971). These

neurotoxins produce competitive inhibition of Ach response in the frog rectus abdominus muscle without affecting KCl response (Tamiya and Arai, 1966; Chang & Lee., 1963; Su et al., 1967). The Ach response of the isolated chick biventer cervicis (Chang & Lee., 1963) and cat gastrocnemius muscle in vivo (Lee, 1974) was abolished, before complete neuromuscular block by these neurotoxins. The response to micro-iontophoretically applied Ach or carbachol in the isolated frog sartorius is also blocked by these neurotoxins (Miledi, et al., 1971; Lester, 1970). These observations indicated that the mode of neuromuscular blocking action of these neurotoxins is identical with that of d-tubocurarine (Lee, 1972). Direct comparison of several neurotoxins from different species revealed that α -bungarotoxin has the highest degree of irreversibility followed by the neurotoxins from N.naja naja venom, while both erabu-toxin b and cobrotoxin are slowly reversible, suggesting that the richness in hydrophobic amino acids in their molecules may account for the irreversibility (Lee, 1972).

Toxins have been isolated from eight varieties of sea snakes, four from Hydrophiinae and four from Laticaudinae. All of them have been studied for their major pharmacological action. Toxicity of the purified toxins is listed in Table-7.

TABLE - 7
Toxicity of the purified toxin

Species	Origin	Toxin	LD ₅₀ (mg/kg)	REFERENCE
<u>HYDROPHIINAE</u>				
<u>Enhyalira schistosa</u>	Strait of Malacca	Toxin	0.044	Tu and Toom (1971)
		Schistosa-4	0.085	Karlsson <u>et al.</u> (1972)
		Schistosa-5	0.085	Karlsson <u>et al.</u> (1972)
	Western coast of India	CM-IV-Sa	0.070	Gawale and Bhide (1976)
<u>Lapemis hardwickii</u>	Gulf of Thailand	Toxin	0.060	Tu and Hong (1971)
<u>Pelamis platurus</u>	Formosa	Pelamitoxin-a	0.070	Liu <u>et al.</u> (1975)
	Costa rica	Toxin	0.044	Tu <u>et al.</u> (1975)

Contd...

TABLE - 7 (Contd...)

Species	Origin	Toxin	LD ₅₀ (mg/kg)	REFERENCE
<u>LATICAUDINAE</u>				
<u>Laticauda semifasciata</u>	Japan	Laticotoxin-III	0.015	Uwatoko <u>et al.</u> (1966a)
		Laticotoxin-IV	0.021	Uwatoko <u>et al.</u> (1966a)
	East China Sea, Okinawa	Erabutoxin-a	0.15	Tamiya and Arai (1966)
		Erabutoxin-b	0.15	Tamiya and Arai (1966)
		Erabutoxin-c	0.13	Uwatoko-Setoquchi (1970) Tamiya and Abe (1972)
	South China Sea, Philippines	Toxin-a	0.07	Tu <u>et al.</u> (1971)
		Toxin-b	0.05	Tu <u>et al.</u> (1971)
	Japan	Laticotoxin-a	0.13	Sato <u>et al.</u> (1969)
<u>Laticauda laticaudata</u>	Japan	Laticotoxin-a	0.13	Sato <u>et al.</u> (1969)
<u>Laticauda colubrina</u>	Japan	Laticotoxin-a	0.13	Sato <u>et al.</u> (1969)
<u>Aipysurus laevis</u>	Australia	Toxin-a	0.076	Maeda and Tamiya (1976)
		Toxin-b	0.076	Maeda and Tamiya (1976)
		Toxin-c	0.076	Maeda and Tamiya (1976)

4.5 Review of Pharmacological studies on
E.schistosa venom :

It was Rogers (1903-1904) who studied first the effect of Enhyrina schistosa venom on variety of vertebrates. He reported primary paralysis of respiratory centre in rabbits and cats. In more protracted cases primary failure of respiration is accompanied by a marked rise in blood pressure due to increase in the viscosity of blood. Blood pressure suddenly falls in the absence of artificial respiration, while with artificial respiration, gradual diminution and violent convulsions repeatedly noted. Rogers further concluded that the paralysis of respiratory centre is not the only cause of asphyxiation, but that paralysis of the end plates of the phrenic nerves. Direct stimulation of the diaphragm gave a marked contraction. It is interesting to note that the end plates of sciatic nerves remained excitable much longer than those of the phrenic nerve. Rogers further reported that even 1% of the solution of venom acting for five minutes had no effect on the nerve trunk. The venom in 1:100 solution showed no any appreciable effect when applied directly to frog's heart, while few drops of 1:1000 solution were quickly fatal when given intravenously. Finally, Rogers concluded that E.schistosa venom acts both on neuromuscular junction as well as on central nervous system.

Fraser and Elliot (1904, 1905) demonstrated that E.schistosa venom did not block conduction of the mammalian nerve trunks in vivo even with the supra-lethal doses. Further dry venom was applied to the floor of the fourth ventricle of the anaesthetized animal which caused death by rapid failure of respiration without any curarization of the diaphragm. Fraser and Elliot thus concluded that E.schistosa venom has got central action.

Kellaway et al. (1932) found that, when injected subcutaneously or intravenously into rabbits the hydropheid venom able to induce respiratory paralysis in the injected animal. Kellaway et al. (1932) concluded that this effect resulted from a peripheral action, because, when a lethal dose of the venom was injected into the fourth ventricle, very little effect was observed.

Barme (1958) studied the toxicity of E.schistosa venom and investigated its electrophoretic pattern on the paper. Carey and Wright (1960) reported LD₅₀ studies of E.schistosa venom on various laboratory animals.

Carey and Wright (1961) studied pharmacological effects of the fractions separated by dialysis and

CM-cellulose column chromatography. Injection into the medulla of anaesthetized rabbits, crude venom and lecithinase (Phospholipase A) fraction caused stimulation followed by depression of respiration. The toxic fraction did not show this effect. However, Carey and Wright (1961) did not find central activity to any fraction tested by intra-arterial injection.

Tamiya and Arai (1966) analysed the action of erabutoxins a and b from Laticauda semifasciata on frog sciatic nerve-sartorius muscle preparation and found that both the erabutoxins when added to the bath at the concentration of 0.12 µg of nitrogen per ml of Ringer solution cause a gradual amplitude decrease of the muscular contractions produced by repeated electrical stimulation of the muscle. Erabutoxin a caused a gradual decrease of respiration, amplitude and blood pressure. When injected in anaesthetized mice, both erabutoxins caused dyspnea and paralysis of the hind legs.

Tamiya and Arai (1966) also found that both erabutoxins blocked the contraction induced by acetylcholine on the frog rectus abdominis muscle. In contrast, rectus abdominis muscle contraction caused by

increasing concentration of potassium (20 to 25 mM) was not affected by the presence of erabutoxin a.

Chan and Gen (1967) reported antagonism of intra-arterial acetylcholine induced contractions of skeletal muscle by sea snake Enhyrinia schistosa venom and postulated that acetylcholine may not be the actual substance affecting the subneural membrane of the motor end plate as the transmitter substance which was the traditional concept of acetylcholine.

Cheymol (1972) demonstrated paralysing action of the Laticauda semifasciata venom and its neurotoxins (erabutoxin a and erabutoxin b) on cat in situ and rat neuromuscular preparation. Cheymol (1972) found that toxins have more potent paralysing activity and further concluded that the paralysis is peripheral and blocks end plate receptors without affecting the muscle fibres or the acetylcholine output at the nerve endings and concluded that the venom and erabutoxins a and b do not act by simple mechanism competition with acetylcholine as does d-tubocurarine.

Geh and Chan (1973) reported prejunctional site of action of E.schistosa venom. The venom of E.schistosa abolished the contractile response caused by

intra-arterial acetylcholine in the cat anterior tibialis muscle, without affecting the contractile response to indirect stimulation. Results obtained with facilitatory drugs and the effects observed with chronic denervation suggested that the venom probably produced this phenomenon by blocking acetylcholine action at a prejunctional site of the motor end plate.

In contrast to the view of neurotoxicity, Reid (1961) in consequence of clinical analysis of patients bitten by E.schistosa found reason to believe that the changes of muscular contraction caused by the venoms of sea snakes were by muscular necrosis and urged that the absence of skeletal muscle reflexes could also be due to direct muscle damage.

Marsden and Reid (1961) reported that the principal signs of sea snake envenomation in man were myalgia and myoglobinuria. They observed myonecrosis in dogs injected with E.schistosa venom.

Sitprija et al. (1971) reported that the myotoxic effect of sea snake venom might sensitize the muscles to the paralytic effects of hyperkalaemia and uremia. Removal of potassium and uremic factors by haemodialysis resulted in rapid movement of muscular activity.

Yeoh and Walker (1974) demonstrated the effect of E. schistosa venom on the ganglionic nicotinic actions of acetylcholine. The crude venom reduced nictitating membrane contraction responses to pre-ganglionic nerve stimulation. In the atropinised cat the venom depressed heart rate and pressor responses to intravenous acetylcholine but not the responses to adrenaline. It was concluded that the venom antagonised the actions of acetylcholine at autonomic ganglia and the adrenal medulla.

Walker and Yeoh (1974) reported in vitro neuromuscular properties of E. schistosa venom, in variety of skeletal muscle preparations and compared with those of δ -tubocurarine and diallyl bis nortoxiferrine. They used seven different muscles ranging from the monkey extracellular to the leech dorsal wall. Walker and Yeoh (1974) concluded that the blocking properties of the venom according to their observation presumably due to the presence of neurotoxins, were markedly different from those of δ -tubocurarine and diallyl-bis-nortoxiferrine, which were very similar to each other.

Recently, Schmidt et al. (1976) studied nephrotoxic action of sea snake venom and concluded that the cytotoxic action of sea snake venom is minor while the principal action is neurotoxic.

4.6 Materials and Methods :

4.6.1 Venom :

Venom was freshly collected by dissecting out venom glands according to the method described in Section (2.4.2) and stored in a desiccator over calcium chloride until use.

4.6.2 Toxicity studies :

Toxicity of the venom and each fraction was assayed in mice and MLD, LD₅₀ and ED₅₀ were determined.

4.6.2.1 MLD (Minimum lethal dose) : MLD was determined when the amount of fraction was not sufficient to carry out LD₅₀. White albino mice of Kasauli strain weighing 18-20 g bred and reared at Haffkine Institute were injected (i.v.) with various doses of fractions in normal saline (0.5 ml total) in 6 groups of mice. MLD is defined as the minimum amount of sample (expressed in μ g of protein) which killed all the mice in about 24 hours. Two mice were used for each dose.

4.6.2.2 LD₅₀ value : White albino mice of Kasauli strain (18 to 22 g) were injected intramuscularly and intravenously with varying concentrations of venom (1 μ g to 5 μ g per mouse) and the toxin CM-IV-Sa (0.5 to 2.5 μ g per mouse) in six dosage levels. Six mice being used for

each dose level. LD₅₀ (50% lethal dose) was determined using the method described by Litchfield and Wilcoxon (1949).

4.6.2.3 ED₅₀ value : white leghorn chicks 5 to 10 days old weighing between 25 and 30 g were injected into the jugular vein with toxin CM-IV-Sa (2 µg to 5 µg total). Six dosage levels of six chicks for each dose were used. ED₅₀, the effective dose in 50% animals was determined according to the Litchfield and Wilcoxon (1949). The animals were observed for complete head-drop.

Pharmacological action of the toxin CM-IV-Sa was studied on different biological preparations in vivo as well as in vitro.

4.6.3 Study of neuromuscular blocking action :

4.6.3.1 In vivo studies :

4.6.3.1.1 Chicks (i.v. injections) : white leghorn chicks 5 to 10 days old weighing between 25 and 30 g were injected intravenously with various doses of toxin (CM-IV-Sa).

4.6.3.1.2 The gastrocnemius sciatic muscle nerve preparation of cat : The method described by Brown et al. (1930) and modified by Koppányi and Kerczmar (1958) was followed. A cat weighing 2.5-3.0 kg was anaesthetized with chloralose

40 mg/kg intraperitoneally. Animal was placed on its back on a table. The fur on the dorsal aspect of the thigh and lower leg was removed. The trachea and femoral vein were cannulated according to the procedure given in Section (4.5.4.1.1). A vertical incision was made from the knob of bone on the inside of the foot along the front of the leg nearly to the knee, to expose subcutaneous fat and muscle. All the vessels were carefully ligated and the gap between the two major groups of muscles was opened up gently to expose the sciatic nerve. The nerve supplying the gastrocnemius muscle was carefully isolated and a loose ligature placed under it. A steel pin was driven through the lower end of the femur to fix the knee joint. The muscle was freed of its surrounding structures and tendon of insertion located on the anterior aspect of the ankle. The tendon was ligated and the suture led over a pulley to be attached to the bar of a palmer isometric lever. For close intra-arterial injection of drugs, the femoral artery was cannulated according to the procedure given in Section (4.5.4.1.1). The cannula was tied securely in place. Bipolar electrode was placed on the nerve to gastrocnemius muscle stimulated at the rate of six shocks per minute at 15 volts by square wave pulses of about 0.5 m-sec. duration.

4.6.3.2 In vitro studies :

4.6.3.2.1 Rat phrenic nerve diaphragm preparation : The method described by Bulbring (1946) was followed. A rat weighing 150 to 200 g was killed by stunning and bleeding. The muscles were freed from chest. The ribs were cut through along side the base of the sternum. The phrenic nerve was seen attached to the membrane. The ribs were then cut through, round towards the animal's flank. The upper part of the thorax was removed completely. An incision was made in the abdominal wall just below the diaphragm and two cuts were made through the diaphragm and ribs left attached to it, towards the spine, one from the front and one from the animal's flank to give a triangular shape with apex of tendinous material. The phrenic nerve was cut just below the thymus and the whole preparation was transferred to a petri dish containing Kreb's solution.

A thread was attached to the tendinous part of diaphragm. A second thread was attached to the cut end of the nerve. The preparation was then tied on electrode by threading at three places. The strip of ribs was tied onto the horizontal bar of the electrode while the nerve was gently placed on the electrodes. The preparation was mounted on electrode, was then set up

in an organ bath containing Kreb's solution, maintained at 37°C and aerated with a mixture of oxygen (95%) and carbon dioxide (5%). The thread tied to the tendinous apex and was connected to light loaded lever with a sideways writing point. The nerve was stimulated at the rate of 12 shocks per minute by rectangular wave pulses of about 0.5 m-sec duration and at 4 to 6 voltage. Drug was added to the bath fluid and its action on the muscle twitches were recorded.

4.6.3.2.2 Chick biventer cervicis preparation : The method described by Ginsborg and Warriner (1960) was followed. A chick 10 to 15 days old, weighing 25 to 30 g was anaesthetized with sodium pentobarbitone (injected intraperitoneally). Feathers were plucked from the back of the neck and a cut was made along the midline from the skull to below the base of the neck exposing two biventer cervicis muscles on either side of the midline, just below the skin. A thread was tied around the upper end of one muscle which was then cut free from its attachments and lifted gently. A thread was tied around the lower end and the preparation was attached to the hooked portion of aeration tube. The thread from the other end was carefully passed through the electrode and the whole assembly was placed in the organ bath and the thread attached to a light spring lever. The electrode was

adjusted to make contact with the upper tendinous part of the preparation. The organ bath (30 ml) containing Krebs solution aerated with oxygen (95%) and carbon dioxide (5%) and was kept at 37°C. The preparation was stimulated at the rate of 6 per minute at 20 volts by rectangular wave pulses of 0.5 m-sec. duration.

4.6.3.2.3 Frog rectus abdominis muscle preparation : The frog was stunned and decapitated and the spinal cord was destroyed. The skin of the anterior abdominal wall was cut and the pair of muscles attached to it were dissected out and transferred to a dish containing frog Ringer solution at room temperature. The muscles were divided, the thread was tied to one of them to both the sides of the piece. One thread was attached to the aeration tube and the other to the frontal writing lever. The load 0.5 to 1.0 g was adjusted to just balance the lever and additional load by plasticin about 1 g was used to bring back the lever to base line. The organ bath of 10 ml capacity was used. The preparation was kept at room temperature in frog Ringer solution through which air was blown.

4.6.4 General pharmacology :

4.6.4.1 In vivo studies :

4.6.4.1.1 Cat blood pressure and respiration : Cats weighing 2.5 to 3 kg were used. They were anaesthetized by injecting pentobarbitone sodium 40 mg/kg intraperitoneally.

Blood pressure was recorded on a smoked kymograph by a glass cannula placed in the carotid artery and connected by polythene tubing to a mercury manometer. Drugs were injected intravenously (i.v.) through the cannulated femoral vein, in a volume not exceeding 0.2 ml followed by a wash of 0.3 ml distilled water.

Cannulation of the trachea :

When anaesthesia was induced, the front of the neck was shaved with electric clippers. The skin below the larynx was picked and cut open with scissors. The trachea was freed from connective tissue. Two strong ligatures were passed around the trachea but not tied. A cut was made in the trachea between the two ligatures big enough to allow the insertion of the cannula. The top ligature was tied and pulled towards the head. The second ligature was then tied around the cannula.

Cannulation of the artery :

Carotid artery was selected for cannulation. Two threads were passed around the cleaned length of artery, and half-tied. The thread furthest from the heart was then tied and when the artery was distended with blood, the bulldog clip was placed at the end nearest to the heart. The arterial cannula was prepared, and was connected to a mercury manometer through a three-way tap with a syringe full of heparinized saline attached. The connection between the three-way tap and manometer was closed and the air bubbled in the arterial cannula flushed out with heparinized saline and the tap set so as to close off the arterial cannula. A slanting cut was made in the artery between the tied ligature and the bulldog clip. The cannula was inserted and tied into place. The pressure in the manometer was raised by means of syringe to the expected blood pressure of the animal. The tap was set so that the syringe was closed off and the arterial cannula was connected to the recording device. As an added precaution to prevent clotting, the animal was given heparin (1000 units/kg, i.v.).

Cannulation of vein :

The femoral vein was selected and prepared free from connective tissue and branches were tied off. The

blood flow was occluded by placing a bulldog clip on the end nearest to the heart. Two threads were carefully passed around the vein and half-tied. Then vein was made to distend fully by using a finger to push blood towards the bulldog clip. The thread furthest from the heart was then tied firmly and held taut so as to stretch the vein slightly. A small cut was made at an angle and the cannula was inserted by lifting the small flap at the cut. The second thread was then tied around the cannula and the bulldog clip was removed. The first thread was then tied to keep the cannula correctly. The cannula and the tubing connected to it filled with saline containing heparin (50 units/ml).

4.6.4.2 In vitro studies :

4.6.4.2.1 Guinea pig ileum : Guinea pigs weighing 450 to 550 g of both the sexes and starved for 48 hours, were used. The animal was killed by a blow on a head and cutting the throat to bleed it to death. The abdomen was opened; first 10 cm portion from ileo-caecal junction was cut and placed in a dish containing warm Tyrode's solution. The lumen of ileum was gently rinsed out. Mesentery was trimmed away and pieces were cut into segments, about 4 cm length starting above Peyer's patch. The method described by Magnus (1904)

was followed for mounting the preparation. Sutures were taken through either end of the segment. The thread of the lower suture was tied to the hooked end of a hollow aeration tube and the other to the isotonic recording lever with frontal writing point balanced to provide a tension of about 0.5 g. The tissue was positioned in an organ bath (10 ml) in Tyrode's solution at 37°C aerated with oxygen. The tissue was stabilized for about 30 minutes before starting the experiment. The cycle used for this experiment is as follows :-

0 min Kymograph started
 1 min Drug added
 1½ min Kymograph stopped and the preparation washed
 3 min Kymograph started.

4.6.4.2.2 Isolated rat uterus : Young female rats weighing 120 to 150 g and in oestrus were used for this experiment. The animal was brought into oestrus by injecting stilboesterol 0.1 mg/kg (i.m.) in arachis oil 24 hours prior to the experiments. The animal was killed by stunning and the abdomen was opened. The two horns of the uterus were dissected out and transferred to a dish containing DeJalon's solution. The two horns were separated and freed from fat and each was cut open

longitudinally. One horn was then mounted in an isolated organ-bath in DeJalon's solution at 37°C in a similar way to guinea pig ileum. The preparation was bubbled with oxygen, it was allowed to stabilize. The drug was allowed to act for 30 seconds and three minutes of cycle was maintained.

4.6.4.2.3 Isolated rabbit heart (Langendorff) : A rabbit weighing 1.5 to 2.5 kg was killed by stunning and cutting its throat. The thorax was opened quickly and the heart was removed with at least 1 cm of aorta attached and placed in a dish containing warm Ringer-Locke solution through which oxygen was bubbled. The preparation was squeezed gently several times so as to remove as much blood as possible. The heart was then cleared of extraneous connective tissue and the aorta was tied onto the glass cannula. Thread was attached to the ventricle by a hook and was led over the system of pulleys to the bar of the starling lever. Sample was added to the preparation by injection through the rubber cap into the perfusion fluid. The perfusion fluid was oxygenated Ringer-Locke solution, which was applied at a constant pressure from the reservoir and warmed to 37°C by the water circulated from the thermostat. Contractions of the heart were recorded on a smoked paper and coronary flow was measured by collecting the perfusate per minute.

4.6.5 Physiological solutions :

Physiological solutions for different biological preparations were prepared by dissolving the ingredients in the following composition in one litre of distilled water.

4.6.5.1 Tyrodé solution (1910) :

Sodium chloride			8.0 g
Potassium chloride			0.2 g
Calcium chloride			0.2 g
Magnesium chloride			0.1 g
Sodium dihydrogen phosphate			0.05 g
Sodium bicarbonate			1.0 g
Dextrose			1.0 g.

4.6.5.2 Krebs solution (1950) :

Sodium chloride			5.5 g
Potassium chloride			0.35 g
Magnesium sulphate			0.11 g
Calcium chloride			0.28 g
Potassium dihydrogen phosphate			0.16 g
Sodium bicarbonate			2.1 g
Dextrose			2.0 g

4.6.5.3 DeJalon's solution (1945) :

Sodium chloride			9.0 g
Potassium chloride			0.42 g
Calcium chloride			0.06 g
Sodium bicarbonate			0.5 g
Dextrose			0.5 g

4.6.5.4 Frög-Ringer solution (Gaddum, 1953) :

Sodium chloride			6.5 g
Potassium chloride			0.14 g
Calcium chloride			0.12 g
Sodium dihydrogen phosphate			0.01 g
Sodium bicarbonate			0.2 g
Dextrose			2.0 g

4.6.5.5 Ringer-Locke solution (1901):

Sodium chloride			9.0 g
Potassium chloride			0.42 g
Calcium chloride			0.42 g
Sodium bicarbonate			0.15 g
Dextrose			1.0 g

4.7 Results :

4.7.1 Toxicity studies :

4.7.1.1 General observations :

Toxin CM-IV-3a was injected intramuscularly in group of 10 mice with the doses ranging from 0.1 µg to 5.0 µg per mouse. The animal became dull, quiet and drowsy, with unsteady movements. The following symptoms were observed, namely, ataxia, gasping, increase and irregular respiration, tachycardia, tachypnea, loss of righting reflex, severe gasping, heart fibrillation, muscle twitching, severe convulsions, respiratory failure, cyanosis and death. Heart beats persisted for 2 to 3 minutes after death.

In chicks (10 chicks, i.v., 1 µg to 10 µg total), the head gradually sinks lower and lower, to be raised again in one quick jerky movement when it almost touches the ground. This was repeated until the head could no longer be supported. Animal then loses power from leg and starts sitting and sleeping. At this stage the respiration was markedly laboured. Death often followed by severe convulsions, gasping, air hanger and respiratory failure.

4.7.1.2 LD₅₀ and ED₅₀ studies :

LD₅₀ of the crude venom and the toxin CM-IV-Sa and ED₅₀ of the toxin CM-IV-Sa are listed in Table 8.

TABLE - 8

Toxicity studies of *E.schistosoma* venom and toxin CM-IV-Sa

Sample	Species	Route of injection	Value	µg/kg
Crude venom	Mouse	i.v.	LD ₅₀	155 (144.8 to 165.9)
Crude venom	Mouse	i.m.	LD ₅₀	210 (196.2 to 224.7)
Toxin CM-IV-Sa	Mouse	i.m.	LD ₅₀	70 (65.4 to 74.9)
Toxin CM-IV-Sa	Chicken	i.v.	ED ₅₀	120 (111.1 to 129.6)

4.7.2 Study of neuromuscular blocking action :

4.7.2.1 In vivo studies :

4.7.2.1.1 Effect of injecting toxin CM-IV-Sa into chicken : Chicks were injected with 100 to 200 µg/kg (10 chicks) of toxin. The head drop was observed as shown in the Figure 17.

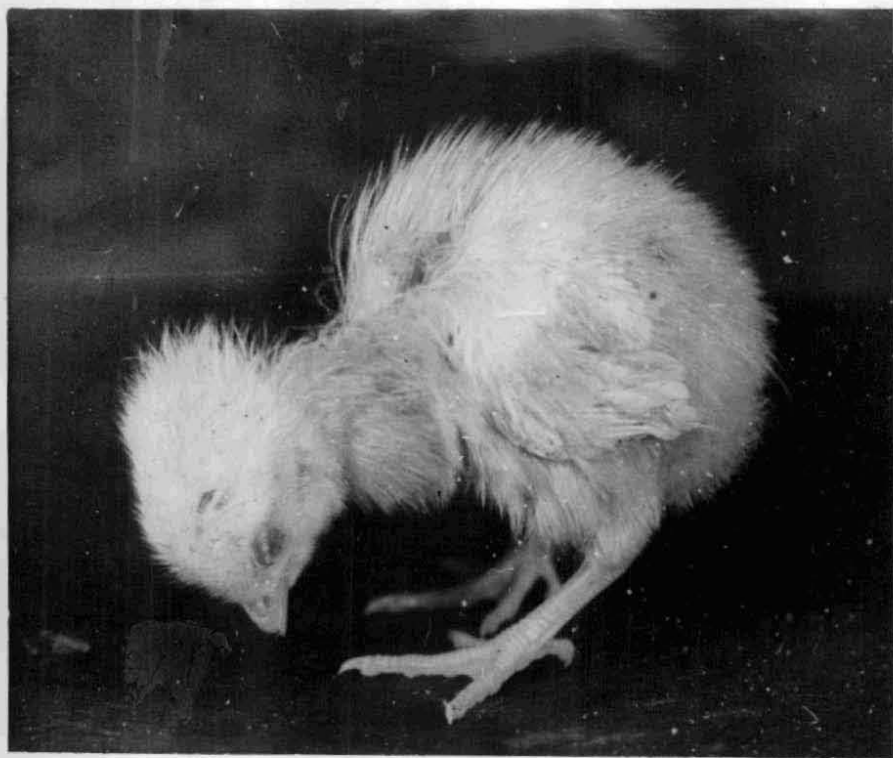


Fig. 19: Chicken injected with Toxin CM-IV-Sa showing
'head drop.'



Fig. 20: Chicken injected with Toxin CM-IV-Sa showing
'respiratory arrest'.

The action started after about 60 seconds and lasted for about 10 to 15 minutes, when chicken died due to respiratory failure as shown in the Figure 20. The type of paralysis observed was flaccid (Figure 21). At lower doses the effect was not seen for long time (2 hrs). Some animals looked depressed and partial head drop was observed.

4.7.2.1.2 Effect of toxin CM-IV-Sa on cat gastrocnemius sciatic nerve muscle preparation : When the toxin was injected in the doses ranging from 5 μ g to 10⁵⁰ μ g (Total, 5 experiments) by a close intra-arterial injection, a marked decline in the heights of the successive twitches was observed. The total block was observed in 10 minutes, when no muscle twitches were recorded, to indirect muscle stimulation.

The neostigmine 250 μ g (total) (5 experiments) was injected 5 minutes after the injection of toxin (5 μ g to 10⁵⁰ μ g, total), when the preparation was partially paralysed. Transient recovery of the paralysis was noted for only short time and the block progressed afterwards to total paralysis. The preparation was observed in this condition for 2 hours, but no recovery was observed. (Figure 22).



Fig. 21: Chicken injected with Toxin CM-IV-Sa showing flaccid paralysis.

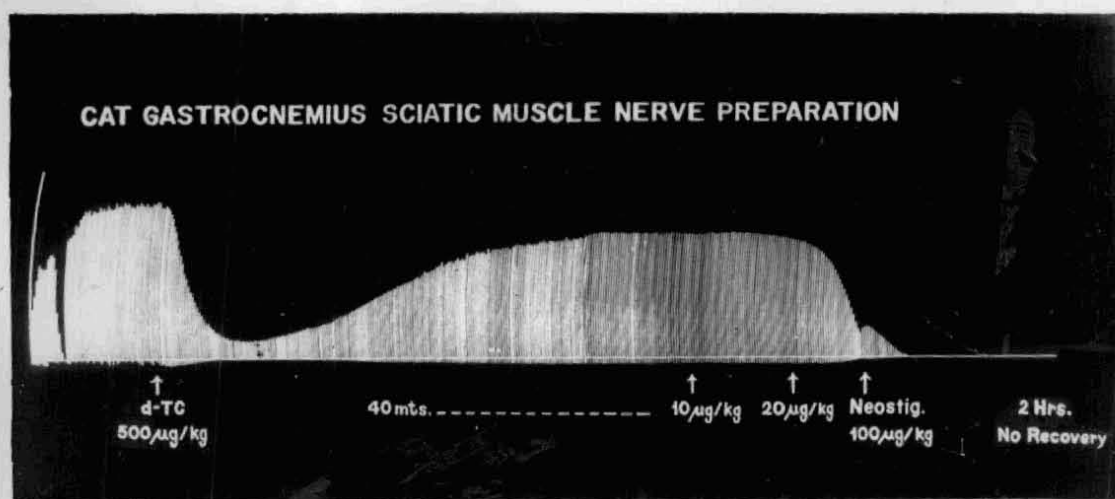


Figure-22

4.7.2.2 In vitro studies :

4.7.2.2.1 Effect of toxin CM-IV-Sa on rat phrenic nerve diaphragm preparation : When the preparation was exposed to toxin at the concentration 0.1 μg to 0.2 $\mu\text{g}/\text{ml}$ (five experiments), progressive decline in the heights of successive muscle twitches to indirect stimulation were observed. Within 60 minutes of the exposure of the toxin, total block was observed. The block did not recover after repeated washings for about 2 hours and even after addition of neostigmine (1 μg to 5 $\mu\text{g}/\text{ml}$; five experiments). The minimum concentration of the toxin in organ bath required to produce a complete blockade within 2 hours was 0.15 $\mu\text{g}/\text{ml}$ (five experiments).

After complete block to the responses of indirect stimulation, direct muscle stimulation was recorded at 100 volts with 5 m-sec duration and at a frequency of 0.2 Hz. The preparation was responded to direct muscle stimulation (Figure 23).

Effect of toxin on tetani and on post-tetanic potentiation : For recording tetani of the muscle, the preparation was stimulated at a rate of 100/sec at supra-maximal voltage of 6 to 8 volts and 0.5 m-sec duration for the period of 10 seconds.

A control untreated nerve muscle preparation at tetanic stimulation showed sustained contracture, which was maintained during tetanic stimulation. Its stimulation at normal frequency, immediately after tetanic stimulation showed improved amplitude as compared to normal responses. The phenomenon is called post-tetanic potentiation.

A partially curarized neuromuscular preparation at dose of 2 μ g to 4 μ g/ml α -tubocurarine at tetanic stimulation showed an initial tetanic contracture but with a falling tonus, which is termed as Wedensky inhibition. If the preparation immediately stimulated with normal frequency at this stage, posttetanic potentiation was observed.

A nerve muscle preparation was treated with the toxin CM-IV-Sa in a dose of 0.2 μ g/ml. The preparation was stimulated in a similar manner as in earlier experiments. After 10 minutes, when the neuromuscular block was incomplete, repetitive stimulations were interrupted by a brief tetanus by stimulating the preparation at a rate of 100/sec at supramaximal voltage of 6 to 8 volts and 0.5 m-sec duration for 10 seconds. No typical Wedensky inhibition like curarized muscle was observed. The contracture reduced steadily during tetani and no pronounced post-tetanic potentiation was observed.

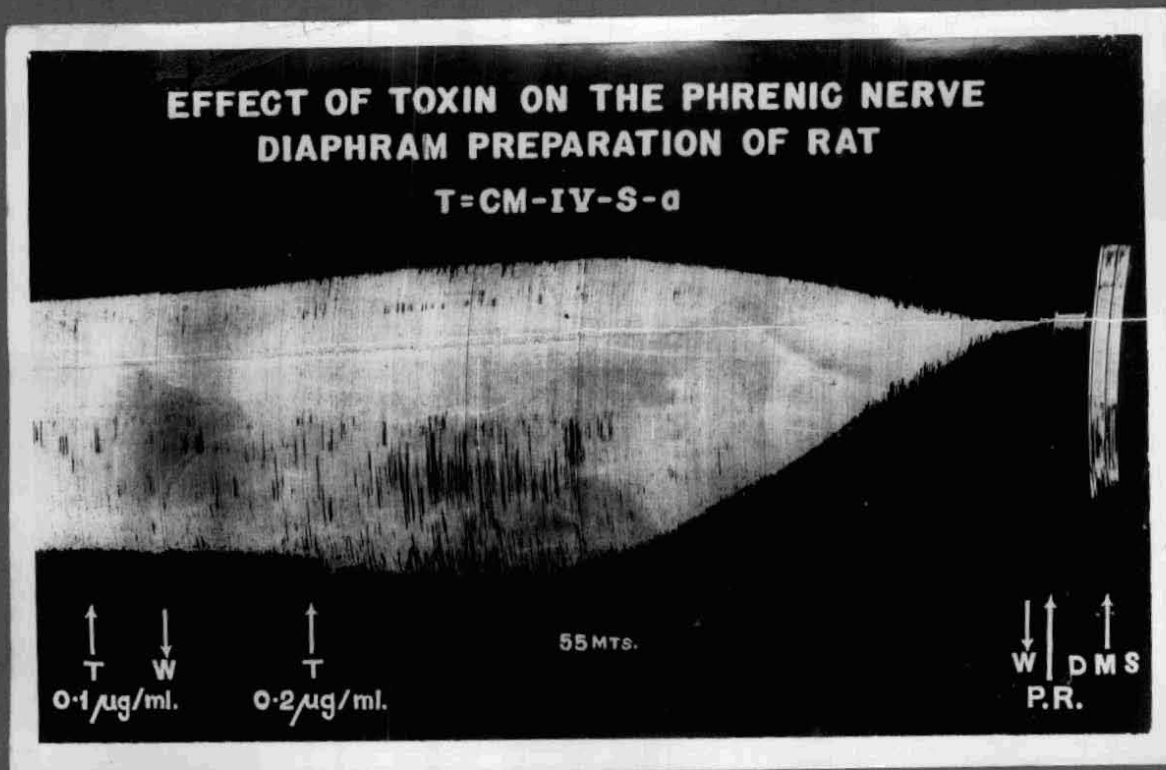


Figure-23

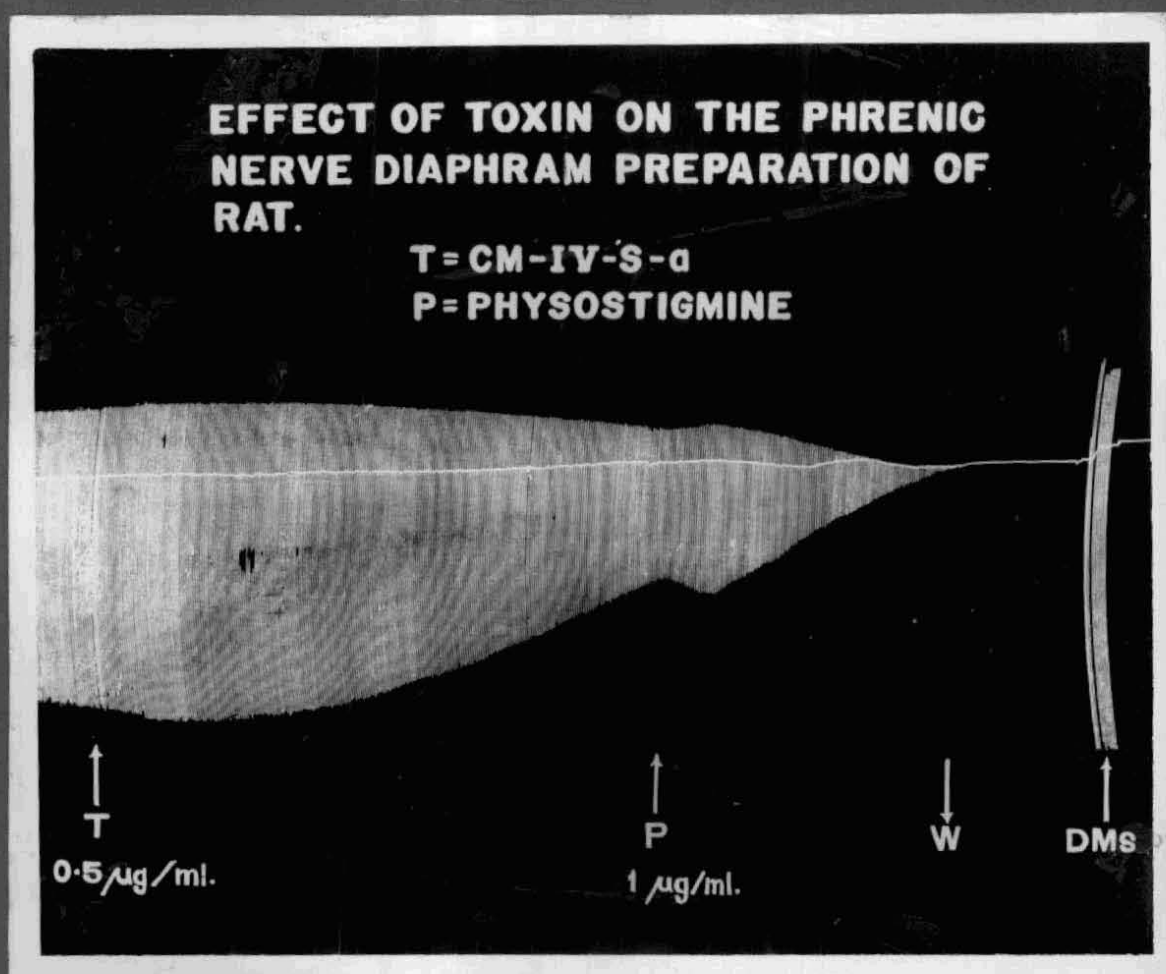


Figure-24

Antagonism by neostigmine (Five experiments) :

The preparation was exposed to the toxin (0.5 $\mu\text{g/ml}$) and 20 minutes after the partial block of the diaphragm, neostigmine in doses (1 $\mu\text{g/ml}$ to 5 $\mu\text{g/ml}$) was added in bath. Only slight transient recovery was obtained. The antagonism did not last long, the block reappeared after the transient antagonism (Figure 24).

4.7.2.2.2 Effect of toxin CM-IV-3a on frog rectus abdominis muscle preparation (Five experiments) :

The control dose response curve for Ach was recorded in the doses 0.5, 1, 2, $\mu\text{g/ml}$. 50% of the response was repeated. Contraction was recorded for 90 seconds. The muscle was washed three times successively at the interval of 5 minutes for 15 minutes with frog-Ringer solution till it completely relaxed before giving the second dose.

The toxin in the dose of 0.1 $\mu\text{g/ml}$ was perfused over the muscle and after two minutes, dose response curve for Ach (0.5, 1, 2, 4, 8 $\mu\text{g/ml}$) was repeated. Ach contractions were found to be inhibited. After one hour, dose response curve for Ach in the doses 2, 4, 8 $\mu\text{g/ml}$ was recorded. Toxin was perfused in higher dose of 0.2 $\mu\text{g/ml}$ and Ach dose response curve was repeated. Ach responses were found to be abolished in the same doses.

EFFECT OF TOXIN ON FROG RECTUS ABDOMINIS MUSCLE PREPARATION

T-CM-IV-S-a

•- $\mu\text{g/ml}$

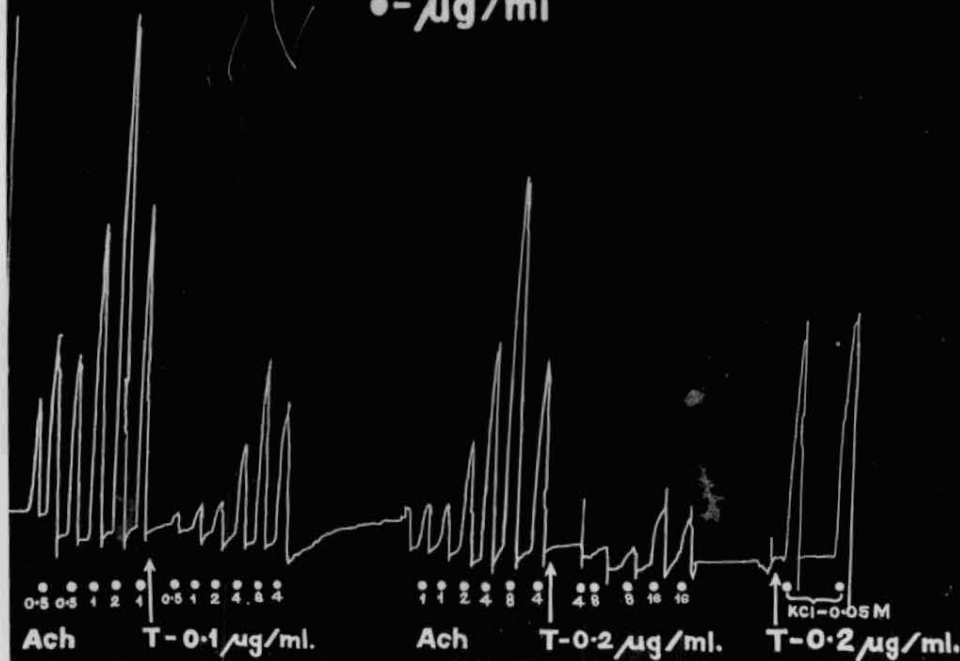


Figure-25

Acetylcholine concentration required to elicit the response was much higher.

Toxin itself did not show contraction on the preparation when exposed to the doses 0.5 μ g to 1 μ g/ml. However, in the presence of toxin (0.2 μ g/ml) potassium chloride responses (0.05 M) were remained unaffected. Observations of the typical experiment are shown in Figure 25.

4.7.2.2.3 Effect of toxin CM-IV-Sa on chick biventer cervicis muscle preparation (Five Experiments) :

The toxin in dose range 1 μ g to 5 μ g/ml was added into the bath and the preparation was stimulated by slightly supramaximal rectangular pulses of 0.5 m-sec duration and the rate 6 per minute at 20 volts. The toxin caused irreversible neuromuscular blockade which did not recover after repeated washings for about two hours and even after addition of neostigmine (5 μ g/ml). Twitch response to acetylcholine (5 μ g/ml) was inhibited during the blockade.

4.7.3 General pharmacology :

4.7.3.1 In vivo studies :

4.7.3.1.1 Effect of toxin CM-IV-Sa on cat blood pressure and respiration (Five experiments) : The responses to

adrenaline (2 $\mu\text{g/kg}$), noradrenaline (2 $\mu\text{g/kg}$), acetylcholine (1 $\mu\text{g/kg}$) and histamine (1 $\mu\text{g/kg}$) were recorded. The toxin in doses 10, 20, 40 and 80 $\mu\text{g/kg}$ was then injected intravenously. Responses to the above mentioned drugs were repeated after the toxin injection and thereafter at an interval of 1 and 2 hours.

The toxin did not produce any appreciable effect on cat blood pressure. However, slight increase in the depth of respiration was noted.

4.7.3.2 In vitro studies :

4.7.3.2.1 Effect of toxin CM-IV-Sa on smooth muscle preparations - Guinea pig ileum, rat uterus (Five experiments each) : The dose response curves for acetylcholine (1,2,4 ng total), histamine (1,2,4 ng total) and 5-hydroxytryptamine (5,10,20 ng total) were obtained. Toxin in doses 1,2,4, 8 μg (total) and 5,10,20 μg (total) was studied for its effects on the dose response curve produced by acetylcholine, histamine and 5-hydroxytryptamine. The toxin was added in the bath and allowed to act for 2 minutes. The toxin in the doses of 100 to 500 μg (total) was added in bath to study its direct action on the tissue.

The toxin did not induce contraction or relaxation of the tissue even upto the doses 500 μg (total).

4.7.3.2.2 Effect of toxin on heart muscle - Rabbit heart (Langendorff) : Effect of adrenaline (1 µg) was recorded and then the heart was perfused with the toxin solution in doses of 50 µg to 500 µg in Ringer-Locke solution.

Toxin did not produce any significant effect on coronary flow, but there was slight increase in the heart rate.

4.8 D i s c u s s i o n :

It is believed that sea snake venom is principally neurotoxic in experimental animals. Symptoms observed in mice and chicks showed that venom produced death due to flaccid paralysis and respiratory failure.

Since the venom is a heterogeneous mixture of different proteins and many other unknown substances and the venom action has been attributed to be toxins (main lethal factor) present in the venom, the major toxic component was isolated in purified form from E. schistosa venom and so called toxin CM-IV-Sa studied further for its pharmacological action.

Toxicity studies were carried out on the venom and the toxin; LD₅₀, ED₅₀ were determined. The toxin

CM-IV-Sa was shown to be three times as toxic as that of crude venom.

The toxin exhibited neuromuscular blockade on rat phrenic nerve diaphragm, chick biventer cervicis and cat gastrocnemius muscle preparation, which was not recovered even after repeated washings for about 2 hours and even after addition of neostigmine solution. After complete blockade, the responses to the direct muscle stimulation were remained unaffected indicating that the toxin did not affect the muscle conduction. The toxin CM-IV-Sa abolished the Ach responses on rectus abdominis muscle and inhibited the twitch response to Ach on chick biventer cervicis. These observations indicated that the toxin interrupted the transmission mainly by post-synaptic mechanism. The results also suggested that the block produced by the toxin was mainly of the nondepolarizing type. This was shown by various observations including the absence of any contraction on rectus abdominis muscle, the flaccid paralysis produced in conscious chicks on intravenous injection and the antagonistic effect of neostigmine.

The neuromuscular block started after a brief latency period. The toxin showed nontypical Wedensky

inhibition on rat phrenic nerve diaphragm. Competitive antagonism of Ach responses on rectus abdominis muscle, head drop in conscious chicks on intravenous injection and the antagonistic effect by neostigmine on rat phrenic nerve diaphragm suggested that the action of the toxin in this respect is similar to curare. But the action showed difference from curare in its slow development, in period of latency, in nontypical Wedensky inhibition, in slowness or absence of reversibility and in incomplete antagonism by neostigmine.

The toxin did not show any significant effect on smooth muscles, heart muscles and blood pressure and respiration.

These all observations suggested that the toxin CM-IV-Sa principally affects the neuromuscular junction mainly by post-synaptic mechanism in a similar manner to other sea snake neurotoxins (studied so far), cobra neurotoxins and α -bungarotoxin.

CHAPTER V

MYOGLOBINURIA IN SEA SNAKE

POISONING

5.1 Introduction :

Mörner (1897) was the first to establish that the muscle pigment is a special substance differing from haemoglobin in the globin fraction. This substance, myoglobin is considered to serve mainly as a store for oxygen in the muscle sarcoplasm and constitutes about 25% of the infants' total haemoglobin.

Myoglobinuria is seen in the diseases like crush injuries, Haff disease, etc.

Reid (1957-1962) from his personal observation of 115 sea snake bite patients at Malaya, found reason to believe that sea snake venom in man is principally myotoxic and myoglobinuria is the main symptom in sea snake poisoning. Myoglobinuria was observed three to six hours after the bite (Reid, 1961). He also reported myonecrosis in dogs injected with E.schistosa venom. But these observations of either myonecrosis or myoglobinuria are yet to be confirmed by others.

Schmidt et al. (1976) studied nephrotoxic action of sea snake venom in mice and concluded that cytotoxic action of this venom is minor one while principal action is neurotoxic.

In the present work, an attempt has been made to examine the urine of the poisoned animals for the presence of myoglobin.

5.2 Materials and Methods :

5.2.1 Animals used :

Ten albino rats of both the sexes (120 g to 150 g), 10 guinea pigs of both the sexes (450 g to 550 g) and 5 dogs (2 males and 3 females; 2.5 kg to 3.5 kg) were used for this experiment.

5.2.2 Collection of urine :

Animals were kept fasting overnight prior injections. Venom was injected intramuscularly with the doses about $1/3$ LD₅₀ by body weight (10 to 15 μ g for rats, 30 to 40 μ g for guinea pigs and 175 to 200 μ g for dogs). Animals were observed for 8 hours after the injection. With this dose of venom, all dogs were died after 8 to 10 hours, 50 % guinea pigs were died after 8 hours, and 30% rats were died after 12 hours. Urine was collected every hour for 8 hours after the injection of the venom. It was tested immediately after collection for the presence of myoglobin.

5.2.3 Testing procedure :

Method described by Blondheim et al. (1958) was

used. Urine was centrifuged and clear supernatant was used for the following tests.

5.2.3.1 Protein test :

This test was carried out to confirm the pigment present in the urine is a protein and not other pigments like alkoetan, porphyrin, etc.

One ml of clear supernatant urine was mixed with 3 ml of 3% sulphosalicylic acid (BSH).

5.2.3.2 Orthotolidine test :

This test was used to test the urine for the presence of either myoglobin or haemoglobin. The method described by Smith (1958) was followed.

Reagents :

1. Alcoholic orthotolidine 4%
2. Working orthotolidine solution (5 ml of 4% alcoholic o-tolidine to 100 ml with 50% v/v acetic acid)
3. Hydrogen peroxide (20 vol.).

Procedure :

Urine (previously kept in boiling water-bath for 5 minutes and then allowed to cool at room temperature) was used. On a white porcelain tile, 4 drops of working

O-tolidine solution and 1 drop of hydrogen peroxide were placed. To this 1 drop of urine was added and the mixture was observed for colour appearance. The blue or green colour within 8 to 60 seconds confirms the presence of either haemoglobin or myoglobin.

5.2.3.3 Saturated ammonium sulphate test :

2.8 g (solid) of ammonium sulphate was added to 5 ml of urine and the mixture was shaken until dissolved and filtered (Whatman No.1) and the filtrate was examined for the colour.

5.3 R e s u l t s :

i) Colour of the supernatant :

Dusky yellow with slight reddish tinge.

ii) Protein test :

Precipitate, showed that other pigments like porphyrin, alkaptan, were absent.

iii) O-tolidine test :

No green or blue colour was observed.

iv) Saturated ammonium sulphate test :

Colour of the filtrate was yellow.

All these results indicated that eventhough protein test was positive, either haemoglobin or myoglobin

was absent in the urine of the animals tested.

5.4 D i s c u s s i o n :

Reid (1957-1962), from his personal observations of sea snake bite patients reported myoglobinuria in sea snake poisoning. Reid (1961) further confirmed this in experimental animal models by showing myonecrosis in dog injected with E.schistosa venom. But these observations are yet to be confirmed by others. Schmidt et al. (1976) studied the nephrotoxic action of sea snake venom and reported very little cytotoxic action.

Studies on enzymatic activities (Chapter II) revealed that E.schistosa venom from the Western coast of India is different in composition from the Malayan coast. Tu (personal communication) suggested that the difference may be due to biological variation in the venom of same species from different coasts.

Reid (1973) is of the opinion that sea snake venom in human subject is principally myotoxic, however, no opportunity have come yet to observe sea snake bite case and neither it has been reported in the literature except two reports (M'Kenzie, 1820; Peal, 1903). Therefore, Reid's observation has not been confirmed clinically. However, in experimental animals like rat, guinea pig, dog, either haemoglobin or myoglobin was not observed.

This observation agrees well with the observation of Schmidt et al. (1976). This suggested that sea snake venom might be acting by different mechanism in experimental animal models and in human beings.

CHAPTER VI

DISCUSSION

Sea snakes are the most abundant and widely dispersed of the World's venomous reptiles. Their venom is known to contain potent neurotoxins which are some of the most toxic substances in the world. Around the coastal seas of Indian and Western Pacific oceans, fishermen encounter these snakes every day. Few death cases have also been noted (personal discussion with fishermen) due to sea snake bite along the Ratnagiri sea coast. Frequent occurrence of sea snakes in the nets and their occasional biting constitutes a real occupational hazard in the fishing folk. Sea snake Enhydryna schistosa is one of the most dangerous and commonly available species along the Western coast of India.

In scientific centres throughout the world, venoms are being increasingly recognized as tools for research. In some research fields, venoms are unique because of a highly specific action on vital body processes. The studies on sea snake venoms are very rare in India. The present studies were undertaken to find out the enzymatic composition of the venom, to isolate and to purify pharmacologically active component and to characterize the same for its neuromuscular blocking property.

Snakes were collected from the Bombay sea Coast from local fishermen. Species were identified using keys

for the identification by Smith (1926) and Wall (1921). Venom was extracted by dissecting out venom glands. About 2 g of venom was collected, which was used throughout the course of present investigations. The protein concentration of the venom gland extract was determined by direct spectrophotometric method and by Lowry's method. Toxicity studies were carried out on mice and chicks and LD₅₀, ED₅₀ values were determined.

It is well established that sea snake venom is a heterogenous mixture of proteins, many of which possess enzyme activities. Mainly hydrolytic enzymes are present in snake venoms. The literature reviewed in Section (2.3) showed that sea snake venom contain several enzymes. Since the venom enzymes have very specific substrate requirements, they find very important tool as a reagent in the elucidation of the complex structures of natural products and in the determination of structure of nucleic acids (Razzell and Khorana, 1959).

Tu and Toom (1971) tested enzyme activities of the venom of E. scbistosa obtained from Malaya and reported following enzymes, namely, hyaluronidase, clotting activity, alkaline phosphatase, phosphodiesterase, deoxyribonuclease, acetylcholinesterase and leucine amino

peptidase. The present studies showed that E.schistosa venom obtained from the Western Indian coast, in addition to phosphodiesterase, phosphomonoesterase, acetylcholinesterase and phospholipase A contain 5'nucleotidase and proteolytic activity.

Protease activity was estimated using casein and denatured haemoglobin as substrates. This activity was reported to be absent in the venom of the same species obtained from Malaya (Tu and Toom, 1971). The difference in the venom of the same species may be due to the difference in the method of extraction of venom or this may be due to the variation in the composition of the venom obtained from different geographical origins. Tu (personal communication, 1976) suggested this difference may be due to the biological variations in the species available on Indian coast and Malayan coast.

Phospholipase A in E.schistosa venom was recognised by Carey and Wright (1961), Tu and Toom (1971). We estimated this activity using egg yolk clearing assay and it was observed that E.schistosa venom is very rich in this enzyme. Bee venom and cobra venom (Condrea and DeVries, 1965) and krait venom (Chhatwal, 1974) are among the richest sources of this enzyme. The studies indicated that sea snake E.schistosa would be another

rich and easily available source of phospholipase A. Formerly it was believed that the hydrolysing activity with synthetic substrates, BAEE and ATEE were present together with protease activity. But Murata et al. (1963) and Soh and Chan (1974) showed that the enzyme responsible for esterolytic activity is different from protease enzyme. It was found that E.schistosa venom from the Western coast of India lacks amino acid esterase activity with BAEE as a substrate, while the protease activity with casein and denatured haemoglobin as substrates was present.

Formerly it was believed that enzymes are responsible for the toxic action of the venom. But with the advent of protein chemistry new chromatographic techniques were developed and enzymes were separated from the toxic action of the venom. The literature reviewed in Section (3.2.1) clearly showed that neurotoxins are the main and the most powerful lethal factors present in the sea snake venoms studied so far, in spite of the presence of large number of enzymes which are shown to be nontoxic (Tu et al., 1970; Uwatoko-Setoguchi et al., 1968; Tu and Toom, 1971).

Our objective was to isolate and purify the biologically active component from E.schistosa venom from

the Western Indian coast and to characterize it for its neuromuscular blocking property.

Tu and Toom (1971) isolated the toxic component from the venom of E.schistosa from the Strait of Malacca by CM-cellulose column chromatography by two-step purification procedure. The preparation was shown to be homogeneous by zonal electrophoresis, ultracentrifugation, isoelectric focusing and gel filtration, and had LD₅₀ toward mice of 44 µg/kg of body weight. The molecular weight of the toxin was 7200 by sedimentation velocity and diffusion experiments. The toxin accounted for 30% of the total venom proteins. Further Karlsson et al. (1972) fractionated the same venom obtained from the same geographical origin, i.e. Strait of Malacca and isolated two principal neurotoxins by gel filtration on Sephadex G-75 followed by gradient chromatography on Bio-Rex 70 in ammonium acetate buffers. The two toxins schistosa 4 and schistosa 5 had LD₅₀ toward mice 85 µg/kg of body weight and had molecular weights 6689 and 6679 respectively calculated from amino acid analysis. The two toxins accounted for 39% of the soluble venom proteins.

In the present studies, the toxin from E.schistosa venom was isolated from the Western Indian coast by

two-step purification procedure, CM-cellulose chromatography followed by gel filtration on Sephadex G-50. The preparation was shown to be homogeneous by disc electrophoresis, immunoelectrophoresis, gel diffusion and gel filtration. The molecular weight of the toxin CM-IV-Sa was observed to be 7000 (approx.) by gel filtration. The toxin was accounted for about 38% of the total venom proteins and had LD₅₀ towards mice 70 µg/kg of body weight.

The toxicity and molecular weight of the toxins isolated in different laboratories from E. schistosa venom were compared in Table 6. On comparison it appears that the toxin CM-IV-Sa might be slightly different from the toxin (Tu and Toom, 1971) and two neurotoxins schistosa 4 and schistosa 5 (Karlsson et al., 1972) isolated from the same venom from the Strait of Malacca. However, this difference may be due to the different laboratory conditions or variations in the animals used or the species of E. schistosa available along the Western coast of India, may be a different subspecies. The variations in the toxins of the given species from different geographical region has already been demonstrated by Miranda et al. (1970). Further work regarding amino acid analysis and structural determination might reveal the exact nature of the toxin.

In many cases, the venom has been simultaneously studied at several different laboratories, thus, it is not unusual to find different names assigned for the same toxin. Tu and Toom (1971) isolated the major toxic component from E.schistosa venom from the Strait of Malacca and named as 'toxin'. Karlsson et al. (1972) isolated two principal neurotoxins from the same venom and named them as schistosa 4 and schistosa 5. The principal toxin isolated from the same species from the Western coast of India was named as toxin CM-IV-Sa. The name is given according to the stages of fractionation. CM-IV for fourth fraction from carboxymethyl cellulose chromatography and Sa stands for first fraction from gel filtration on Sephadex G-50 of the fraction CM-IV.

The action of dried venom of E.schistosa was described by Rogers (1903-1904), Fraser and Elliot (1904-1905). They concluded that the primary action of the snake venom was at the respiratory centre and on the motor end plate. Tu (1959) reported that sea snake venom produced marked inhibition of respiration. Carey and Wright (1961) showed that E.schistosa venom blocked neuromuscular transmission. The detailed mechanism of neuromuscular blocking action by E.schistosa venom was described by Cheymol et al. (1967, 1972). They concluded

that the venoms irreversibly block nicotinic acetylcholine receptors at the postsynaptic membrane.

The postsynaptic action of the isolated sea snake toxin preparations was described on erabutoxins a, b and c (Tamiya and Arai, 1966; Sato et al., 1970; Tamiya and Abe, 1972; Cheymol et al., 1972; Takamizawa, 1970) and laticotoxin a (Sato et al., 1969). The action of the whole venom of L.semifasciata was accounted for the toxins contained in it (Cheymol et al., 1972). Walker and Yech (1974) reported in vitro neuromuscular properties of E.schistosa venom in variety of skeletal muscle preparations and concluded that the action of the venom was different from d-tubocurarine.

LD₅₀ and ED₅₀ of the toxin were determined in mice and chicks and were found to be 70 µg/kg (i.m.) and 120 µg/kg (i.v.) respectively.

The toxin exhibited neuromuscular blockade in rat phrenic nerve diaphragm, chick biventer cervicis and cat gastrocnemius muscle preparation. The block was not recovered even after repeated washings for about 2 hours. Even after block, the responses to direct muscle stimulation were remained unaffected indicating that the toxin did not affect the muscle conduction.

The results suggested that in skeletal muscles the toxin mainly interrupts the transmission by post-synaptic mechanism. This was shown by inhibition of twitch response to acetylcholine on chick biventer cervicis and complete inhibition of Ach response on rectus abdominis muscle. The results also suggested that the block produced by the toxin was mainly of the nondepolarizing type. This was shown by various observations including the absence of any contraction on rectus abdominis muscle, the flaccid paralysis produced on conscious chicks on intravenous injections and the antagonistic effect of neostigmine.

Competitive antagonism of Ach responses on rectus abdominis muscle, head drop in conscious chicks on intravenous injection and the antagonistic to neostigmine suggested that the action of the toxin in this respect is similar to curare, but the action differs from curare in its slow development, in the incomplete antagonism with neostigmine, in eliciting nontypical Wefensky inhibition and in the absence of slowness of reversibility.

In contrast to the cases of experimental animals, a necrotic lesion of the muscle tissue was observed on a human victim of sea snake bite (Reid, 1956; Marsden and Reid, 1961). Myoglobinuria was shown to be a typical

symptom of sea snake bite poisoning in human subject (Reid, 1961). But Reid's observation is yet to be confirmed by others. Schmidt (1976) reported very less cytotoxic action to sea snake venom injected to mice. An attempt was made to examine the urine of the experimental animals injected with E. schistosa venom. The results showed that urine of the poisoned animals did not show either haemoglobin or myoglobin. This observation agrees well with the studies of Schmidt et al. (1976). This suggests that sea snake venom in experimental animals and in human beings might be acting by different mechanisms.

Chang and Lee (1973) demonstrated the action of α -bungarotoxin which combines irreversibly and with high specificity with nicotinic receptors. The specific binding of the neurotoxins to the acetylcholine receptor sites has been accepted as a fact and that the toxin have been found to be an extremely useful tool for the identification, isolation and assay of the Ach receptors (Changeux et al., 1970; Bourgois et al., 1971; Miledi and Potter, 1971; Barnard et al., 1971; Fambrough and Hartzell, 1972; Moody et al., 1973; Schmidt and Raftery, 1973; Menez et al., 1971; Berg et al., 1972; Karlsson et al., 1972 ; Klett et al., 1973; Biesecker, 1973).

The toxin CM-IV-Sa has molecular weight 7000, and has very similar action to that of α -bungarotoxin. Smaller the size, faster the action to diffuse into vital organs (Tu, 1973). In this respect toxin CM-IV-Sa may find more superior than that of α -bungarotoxin.

S U M M A R Y

A N D

C O N C L U S I O N S

Snakes were collected from the Bombay sea coast, identified using keys for the identification of snakes by Smith (1926), Wall (1921). Venom was extracted by dissecting out venom glands.

Toxicity studies were carried out on crude venom and LD₅₀ towards mice was determined. In experimental animals (mice, chicks) venom produced flaccid paralysis and respiratory failure. Reid (1961) reported myoglobinuria as the main symptom in sea snake poisoning in human subjects. An attempt has been made to examine the urine of the animals injected with the venom, but neither haemoglobin nor myoglobin was observed in the urine indicating that sea snake venom might be acting by different mechanism in experimental animals and in human beings.

Protein estimation was carried out by direct spectrophotometric method and by Lowry's method.

Enzymatic activities of phospholipase A, exonuclease (phosphodiesterase, alkaline phosphomonoesterase, 5'nucleotidase, protease, acetylcholinesterase, L-amino acid oxidase and amino acid esterase were estimated. It was shown that E.schistosa venom lacks amino acid esterase and L-amino acid oxidase enzyme activity, while

the venom is found to be very rich in phospholipase A. Thus, this venom would be another rich and easily available source of this enzyme. Tu and Toom (1971) reported that proteolytic activity is absent in E.schistosae venom obtained from Malaya, while it was observed that this activity is present in the same venom from the Western Indian coast. This difference may be due to the variation in the composition of the venom from different geographical origins. Tu (personal communication) suggested that this difference may be due to the biological variations in the species from different coasts.

A principal toxin was purified by two-step purification procedure, CM-cellulose chromatography followed by gel filtration on Sephadex G-50. The preparation was shown to be homogeneous by disc electrophoresis, immunoelectrophoresis, gel diffusion and gel filtration. The molecular weight of the toxin was determined by gel filtration. On comparison, the toxicity and the molecular weight, the toxin CM-IV-Sa appears to be slightly different from the toxin (Tu and Toom, 1971) and two neurotoxins (Karlsson et al., 1972) isolated from the same species from the Strait of Malacca.

LD₅₀, ED₅₀ of the toxin was determined. Toxin did not exhibit any significant action on smooth muscles,

heart muscles and, blood pressure and respiration. However, the toxin CM-IV-Sa exhibited neuromuscular blockade on neuromuscular preparations in vivo as well as in vitro. The results suggested that the toxin interrupts the transmission mainly by postsynaptic mechanism by competing with acetylcholine in similar manner to curare. However, the action showed difference from curare in its slow development, in the period of latency, in slowness or absence of reversibility, in eliciting nontypical Wedensky inhibition and in incomplete antagonism with neostigmine.

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S Y N O P S I S

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Sea snakes belong to the family Hydrophiidae, suborder Proteroglypha and order Ophidia. The family Hydrophiidae is probably the most widely distributed in the world. It is divided into two subfamilies - Hydrophiinae and Laticaudinae. Only the snakes belonging to the subfamily Hydrophiinae are available on the Western coast of India (Wall, 1921). These snakes are exclusively adapted to the marine life and are all venomous and dangerous to man (Smith, 1926; Tu, 1974).

Sea snakes in their native habitat are sluggish. They frequently get entangled in the nets and are caught along with other fishes. Almost every cast of the net results in the capture of several sea snakes. Fisherman carefully throw them back in the sea by catching their tail. Eventhough sea snakes are sluggish and bite only under provocation (Reid, 1957), their bites are quite common and deaths are not unusual among the fishermen who are frequently exposed to the sea. Frequent occurrence of sea snakes in the nets and their occasional bites constitute a real occupational hazard to the fishing industry.

Sea snake venom is a salivary secretion containing proteins, many of which possess enzyme activities, strongly basic nonenzymatic polypeptides and many other unknown substances. Due to the heterogeneous nature the pharmacological study of this venom becomes complicated. Limited study has been carried out so far of the sea snake venoms as compared to the venoms of land snakes. The results of these studies have been reviewed from time to time (Zeller, 1948; Meldrum, 1965; Jimenez-Porras, 1968; Henriques *et al.*, 1971; Lee, 1972; Barne, 1968; Boquet, 1973; Tu, 1973; 1974; Reid, 1973). Hydrophilic venoms are characterized by their neurotoxic action producing flaccid paralysis and death due to respiratory failure (Jimenez-Porras, 1968).

The snake venoms differ from species to species not only in their chemical composition but also with respect to specific pharmacological actions. There is also difference in the venom of the same species collected from different geographical areas (Zeller, 1948; Miranda *et al.*, 1971). It is, therefore, necessary to study the venom of a species from different geographical origin.

In the present work the pharmacological studies were carried out on the venom of Enhyalura schistosa (common sea snake). Aim of the work was to study enzymatic activities of the crude venom, to fractionate the venom by using mild physicochemical methods and to isolate and purify biologically active components and to study the mode of action of the active components.

Sea snakes were obtained from the Bombay sea coast from local fishermen. Species of the snakes were identified as per keys described by Smith (1926) and Wall (1937). Venom was collected by dissecting out venom glands. About 2.0 g of the venom was collected from about 170 sea snakes. It was obtained in the month of March/April 1975 and this was used for present investigations.

The physical and chemical properties of the venom were studied. The various enzymatic activities namely, Phospholipase A (E.C. 3.1.1.4), Exonuclease (Phosphodiesterase) (E.C. 3.1.4.5), Acetylcholinesterase (E.C. 3.1.1.7), L-Amino acid oxidase (E.C. 1.4.3.2), 5'Nucleotidase (E.C. 3.1.3.5), Phosphomonoesterase (Alkaline phosphatase) (E.C. 3.1.3.2), Amino acid esterase and protease were determined in E. schistosa

venom. It is interesting to note that proteolytic activity was found to be present in E.schistosa venom from the Western coast of India, while this activity was reported to be absent in the venom of the same species collected from the Strait of Malacca (Tom et al., 1969).

Monovalent antisera against the venom of E.schistosa was prepared by immunizing rabbits. MLD_{50} and LD_{50} in Kasouli mice and ED_{50} in leghorn chicks were determined using statistical method of Litchfield and Wilcoxon (1949). The crude venom had LD_{50} (i.m.) 210 μ g per kg and ED_{50} (i.v.) 400 μ g/kg.

The venom was fractionated into four protein peaks by CM-cellulose column chromatography. The most retarded of the protein peaks was further purified by gel filtration on Sephadex G-50. The homogeneity of the toxin was tested by disc electrophoresis (Reisfeld et al., 1962), gel diffusion (Ouchterlony, 1949), immunoelectrophoresis (Graber and Williams, 1955) and gel filtration. The toxin was found to be homogeneous and was three times as toxic as the crude venom. The toxic protein was highly basic. Molecular weight of the toxin CM-IV-3a was determined by gel filtration and was found to be approximately 7,000.

Action of the crude venom and the toxic component was studied on different biological preparations in vivo as well as in vitro.

Study of neuromuscular blocking action :

1) In vivo studies :

Chicks.

Cat gastrocnemius muscle.

2) In vitro studies :

a) Neuromuscular preparations -

Rat phrenic nerve diaphragm;

Chick biventer cervicis.

b) Skeletal muscle preparations -

Frog rectus abdominus muscle.

General pharmacology :

In vivo studies :

Cat blood pressure and respiration.

In vitro studies :

Guinea pig ileum,

Rat uterus,

Rabbit heart.

Reid (1961) observed often red coloured urine,

Myoglobinuria in sea snake poisoning cases. The exact

cause of which is still unknown. Attempts were made to observe the myoglobinuria in experimental models such as rats, guinea pigs and dogs. We could not find any trace of myoglobin in the urine of the animals tested.

The work revealed that venom of E. schistosa (common sea snake) from the Western coast of India contains single major neurotoxin of low molecular weight. On comparison, the toxicity and the molecular weight, this toxin CM-IV-Sa appeared to be slightly different from the toxin (Tu and Toom, 1971) and two neurotoxins (Karlsson and Eaker, 1972) isolated from the same species from the Strait of Malacca. The pharmacological studies showed that the prominent action of the toxin is at post-junctional membrane. The toxin competes with the acetylcholine in a manner similar to δ -tubocurarine, but it differs from curare in its slow development, in the imperfect antagonism by neostigmine and in the absence or slowness of reversibility. The enzymatic activities also revealed that E. schistosa venom from the Western coast of India is different from the venom collected from the Strait of Malacca.

The work on sea snake venom has never been attempted in India. Ours is the first investigation of this kind. Our present investigations have opened

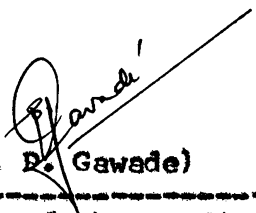
numerous avenues for further studies on the venom of sea snakes. It is to be hoped that as further data becomes available a deeper understanding on the mode of action of these highly toxic venoms would be achieved.

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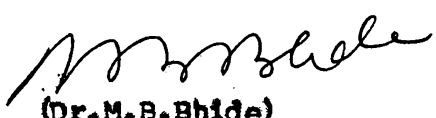
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Forwarded through the Research Guide :-


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..... S. P. GAWADE

Statement under University Rule No.O.412 and O.413

As required by University Ordinance Nos.O.412 and O.413 I state that the work presented in this thesis which I intend to submit is of an original nature and so far, has not been submitted for any degree of this or any other University. Wherever references have been made to the work of others, it has been clearly indicated with the sources of information in the "bibliography".

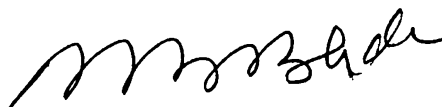
The subject dealt with, has hardly been studied in the country in such details and it is hoped that it will create considerable interest in the subject and promote further work.



(S. P. Gawade)

Signature of the candidate

Certified that the above statement made by the student is correct.



(M. B. Bhide)

Signature of the Research Guide

