Animal, Plant, and Microbial Toxins

Volume 2
Chemistry, Pharmacology, and Immunology

Animal, Plant, and Microbial Toxins

Volume 1 – Biochemistry

Volume 2 – Chemistry, Pharmacology, and Immunology

Animal, Plant, and Microbial Toxins

Volume 2

Chemistry, Pharmacology, and Immunology

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Preface

During the past two decades, research on animal, plant, and microbial toxins has expanded rapidly, and new and exciting information has appeared to clarify both the clinical and therapeutic aspects of intoxication and, even more important, to help us understand more exactly the structure and the mode of action of toxins on a molecular basis.

Because of the interdisciplinary nature of toxin research, it is of vital importance that workers specializing in its different aspects should make a particular effort to exchange and keep up with new developments in this rapidly-expanding field. This has been one of the main purposes of the series of international meetings that have been held over the past ten years. The increasing interest in the results of toxin research can be explained partly by the growing general preoccupation with pollution and its toxic effects, which means that more and more specialists in other fields are finding it necessary to keep in touch with current research into naturally occurring toxins.

The papers in these volumes were presented at the Fourth International Symposium on Animal, Plant and Microbial Toxins, organized by the International Society on Toxicology, held in Tokyo in September, 1974. The editors have decided to include both original reports and review articles, arranged according to disciplines. Editing has been kept to the minimum necessary for adequate comprehension of the materials.

Our labors were greatly lightened by the able assistance of our Associate Editors, Drs. R. Murata, M. Funatsu, and N. Tamiya, who checked a number of manuscripts, and we are deeply indebted to the generosity and energy of Prof. G. Sakaguchi of the University of Osaka Prefecture who singlehandedly read and corrected nearly all the papers. Our profound thanks go to them and also to Miss K. Fujita and Mrs. N. Ando for the zeal with which they tackled the mountains of secretarial work generated by a project of this magnitude. We would like to thank the Japan Snake Institute for the financial help which has made this publication possible. Last but not least, we wish to express our

gratitude to the staff of Plenum Publishing Co., who throughout their dealings with us have displayed an almost saintly degree of patience and understanding.

In conclusion, we sincerely hope that this publication will stimulate and accelerate future research over the whole field of animal, plant, and microbial toxins.

Akira Ohsaka
Kyozo Hayashi
Yoshio Sawai

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The Isolation, Properties, and Amino Acid Sequence of *Laticauda semifasciata* III, a Weak and Reversible Neurotoxin of a Sea Snake, *Laticauda semifasciata*, Venom

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INTRODUCTION

From the sea snake *Laticauda semifasciata* venom, three neurotoxins, erabutoxins a, b, and c, were isolated and sequenced (Tamiya and Arai, 1966; Tamiya and Abe, 1972; Sato and Tamiya, 1971; Endo *et al.*, 1971). More than 95% of the lethal activity of the venom was ascribed to these toxins. Each of them consists of 62 amino acid residues and four disulfide bridges.

The venom also contains a weak and easily reversible neurotoxic component which was named *Laticauda semifasciata* III (LsIII). LsIII is a new-type sea snake venom component with 66 amino acid residues and five disulfide bridges. The present paper describes the isolation, properties, and amino acid sequence of LsIII.

EXPERIMENTS AND RESULTS

CM-Cellulose Column Chromatography. LsIII was isolated from the crude venom by CM-cellulose column chromatography started at a lower pH (5.9)

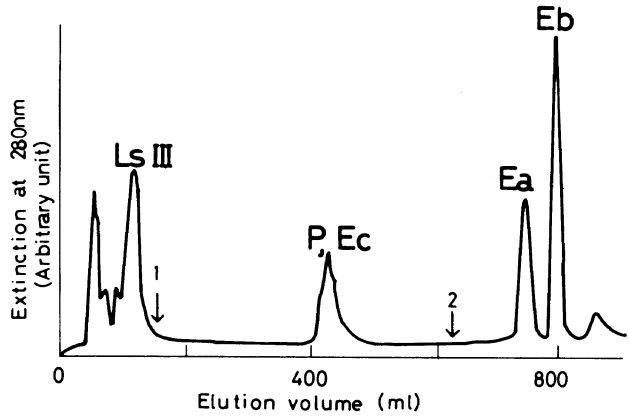


Fig. 1. CM-Cellulose column chromatography of *Laticauda semifasciata* venom. Twelve glands (1.54 g) of *Laticauda semifasciata* were minced with scissors and extracted with 0.01 M sodium-potassium phosphate buffer, pH 5.9 (15.4 ml). The extract was dialyzed in Visking cellulose tube and centrifuged. From 15 ml of the supernatant, 5 ml was chromatographed on a CM-cellulose column (1.7 × 34 cm), which had been equilibrated with the buffer. At arrow 1, the elution buffer was changed to 0.01 M phosphate buffer, pH 6.4. At arrow 2, a linear gradient with NaCl concentration was applied, with 0.01 M phosphate buffer, pH 6.4 (200 ml) in the mixing chamber and the same buffer containing 0.2 M NaCl in the reservoir. The components contained in the venom were as follows: dialyzed extract, 63.2 (100%); *Laticauda semifasciata* III (LsIII), 12.6 (19.9%); phospholipase A and erabutoxin c (P, Ec), 6.57 (10.4%); erabutoxin a (Ea), 7.45 (11.8%); erabutoxin b (Eb), 16.5 (26.1%), where the numerical values represent total extinction at 280 nm.

than that previously described (Tamiya and Arai, 1966; Tamiya and Abe, 1972). An example of the separation of the venom proteins of *L. semifasciata* into their components is shown in Fig. 1. The elution of proteins from the column was followed by the continuous measurement of extinction at 280 nm with a flow monitor, Toyo Uvicon 540M (Toyo Scientific Instruments Co., Tokyo, Japan). The component LsIII content of the venom was 10%-20% as determined by absorption at 280 nm. On rechromatography, LsIII was quantitatively recovered as a single symmetrical elution peak.

Disk Electrophoresis. The disk electrophoresis of the venom components was carried out at pH 4.0 according to Reisfeld *et al.* (1962) with slight modifica-

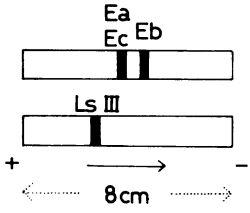


Fig. 2. Disk electrophoretogram of *Laticauda semifasciata* venom components. The electrophoresis was performed at a constant current of 4 mA/gel (15% acrylamide) for 2 hr.

tions described previously by Tamiya and Arai (1966). The result is shown in Fig. 2. LsIII gave a single band which migrated to the cathode more slowly than the erabutoxins.

Molecular Weight Determination

SDS-Polyacrylamide Gel Electrophoresis. The SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (1969) with 0.1% SDS in 10% acrylamide gel. The average mobilities obtained with 10 individual gels are plotted against the known molecular weights expressed on a semilogarithmic scale (Fig. 3). The interpolated molecular weight of LsIII was estimated to be 7300.

Ultracentrifugation. LsIII was dissolved in 0.05 M sodium potassium phosphate buffer, pH 7.0, containing 0.1 M sodium chloride. A solution of $E^{1\,cm}_{280\,nm}$ 17.5 (8.5 mg/ml) was subjected to approach-to-equilibrium sedimentation of Archibald (Schachman, 1959) in the same cell as that described by Yphantis (1960). From the sedimentation equilibrium curve of LsIII obtained after centrifugation at 16.5°C and 20,410 rpm, the molecular weight of 7100 was calculated.

Amino Acid Composition. Both LsIII and RCM-LsIII (S-carboxymethylated LsIII) were hydrolyzed with 6 M hydrochloric acid (0.4 ml) in a sealed glass tube *in vacuo* at 105°C for 24, 48, and 72 hr. The analysis was performed with an automatic amino acid analyzer (type JLC-5AH, Japan Electron Optics

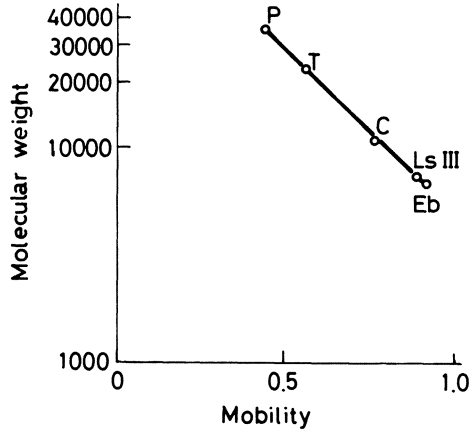


Fig. 3. Molecular weight determination of LsIII by SDS-polyacrylamide gel electrophoresis. The average mobilities obtained with 10 individual gels are plotted. The electrophoresis was performed at a constant current of 8 mA/gel for 5 hr. Erabutoxin b (Eb), α-chymotrypsin chain (C), trypsin (T), and pepsin (P) were used as standards. The mobility of bromophenol blue was taken as unity.

Laboratory Co., Tokyo, Japan). The tryptophan/tyrosine ratio was determined to be 0.66 by UV absorption as described by Goodwin and Morton (1946). The results are given in Table 1. The formula weight obtained by the amino acid analyses agreed well with the molecular weight obtained by SDS disk electrophoresis and by ultracentrifugation. A solution of 1-mg LsIII/ml gave an $E^{1\,cm}_{280\,nm}$ value of 2.06, reflecting its high tryptophan and tyrosine contents.

Isoelectric Focusing. The electrofocusing experiments were carried out with LKB 8100 Ampholine electrofocusing equipment according to the manual (LKB Produkter AB Bromma, Sweden). The results are shown in Fig. 4. The isoelectric point of LsIII is pH. 7.2.

Toxicity of LsIII. The LD_{50} value of LsIII, assayed by intramuscular injection in mice (NIH strain, weighing 18-19 g), was 1.24 μg/g body weight, and in baby chicks (26-40 g) 0.45 μg/g. It is 8-10 times less toxic than erabutoxins

Table 1. Amino Acid Composition[a]

	LsIII	RCM-LsIII[b]		Ea	Eb	Ec
Lysine	3.96	3.95	(4)	4	4	3
Histidine	1.09	1.02	(1)	1	2	2
Arginine	2.10	1.81	(2)	3	3	3
Aspartic acid	6.25	6.18	(6)	5	4	5
Threonine	5.82	5.79	(6)	5	5	5
Serine	5.82	5.70	(6)	8	8	8
Glutamic acid	6.08	6.16	(6)	8	8	8
Proline	4.12	3.65	(4)	4	4	4
Glycine	4.53	4.15	(4)	5	5	5
Alanine	4.18	3.88	(4)	0	0	0
Cystine	8.65	8.00[c]	(10)	8	8	8
Valine	3.01	2.91	(3)	2	2	2
Methionine	–	–	(0)	0	0	0
Isoleucine	2.05	1.88	(2)	4	4	4
Leucine	2.15	1.95	(2)	1	1	1
Tyrosine	3.08	2.76	(3)	1	1	1
Phenylalanine	1.05	0.91	(1)	2	2	2
Tryptophan			(2)[d]	1	1	1
Total			66	62	62	62
Molecular weight						
Formula			7155	6838	6861	6847
SDS electrophoresis			7300			
Ultracentrifuge			7100	7430	7430	

[a] The results are expressed as mole of amino acid per mole of toxin.
[b] Values in parentheses are the nearest integers.
[c] As S-carboxymethylcysteine. Cystine residues tend to give smaller values.
[d] Estimated spectrophotometrically.

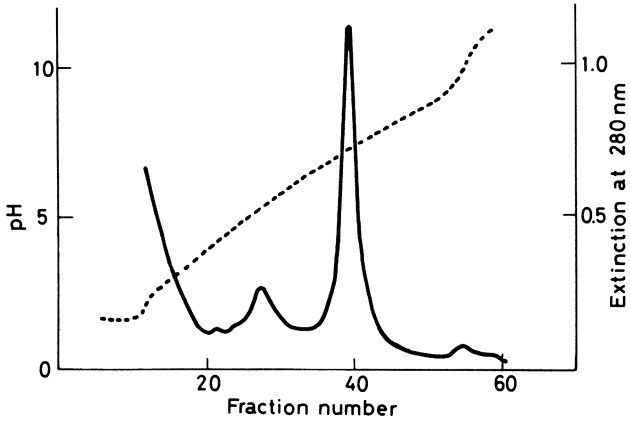


Fig. 4. Isoelectric focusing of LsIII. LsIII (4.8 mg) was subjected to isoelectric focusing with carrier Ampholyte pH 3–10 in a total volume of 100 ml. After 61 hr at 300 V, 2.0-g fractions were collected and measured for the pH (dashed line) and the extinction at 280 nm (solid line).

a, b, and c. The toxic symptoms produced in animals by LsIII were similar to those caused by erabutoxins and other synaptically acting snake toxins.

Mode of Action of LsIII. LsIII (5 μg/ml) inhibited the contraction, by 0.5 μg/ml of acetylcholine, of a frog rectus abdominis muscle by 50% 5 min after addition to the medium. Erabutoxin b gives the same inhibition at 0.4 μg/ml (Fig. 5). LsIII did not affect the contraction of the muscle by KCl (final concentration 0.05 M). The inhibition was rather easily removed by washing the muscle with Ringer solution.

The effect of component LsIII on a chick biventer cervicis nerve-muscle preparation is shown in Fig. 6. Unlike cardiotoxins (Lee *et al.*, 1968), LsIII did not produce any contracture at a concentration as high as 50 μg/ml. The

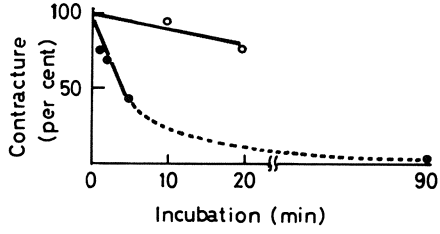


Fig. 5. Effect of incubation with LsIII on the acetylcholine contracture of frog *rectus abdominis* muscle. Each muscle was placed in glucose-Ringer solution (5 ml), through which oxygen was continuously bubbled. The contracture of the muscle by the addition of acetylcholine chloride (final 0.5 μg/ml) was measured before and after the incubation with LsIII. LsIII final 0.83 μg/ml (open circles); LsIII final 5 μg/ml (closed circles).

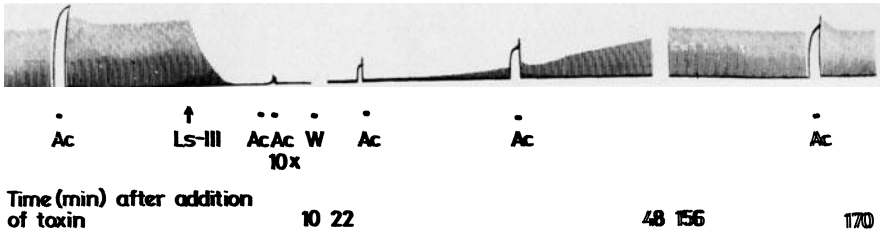


Fig. 6. The effect of LsIII on chick's biventer cervicis nerve-muscle preparations. An isolated biventer cervicis muscle preparation (Ginsborg and Warriner, 1960) from chicks (4–7 days old) was suspended with a tension of 0.5 g in 20-ml Krebs' solution, which was bubbled continuously with $O_2 + CO_2$ (95:5) at 37°C. Indirect stimuli were applied through the tendon at a rate of 6/min with supramaximal rectangular pulses of 0.5 msec. Electrical stimulation was interrupted during testing acetylcholine response (Ac). The dose of ACh was 20 μg/ml, except at Ac 10×, which was 200 μg/ml. At W and then every 10 min, the preparation was washed with Krebs' solution.

twitch response of the muscle to indirect stimulation was blocked within 5 min and the response to acetylcholine (20 μg/ml) was also completely abolished by LsIII. On repeated washing both the twitch response to indirect stimulation and the acetylcholine response were recovered. By contrast, the neuromuscular block produced by erabutoxin b in this muscle preparation is irreversible (Lee *et al.*, 1972). The relationship between the concentration of LsIII and the time for complete neuromuscular block in this preparation is compared with that for erabutoxin b in Fig. 7. When 10 μg/ml of LsIII was added to the medium in which a rat phrenic nerve-diaphragm preparation was suspended, the twitch height elicited by indirect stimulation decreased progressively and complete neuromuscular block took place in 53 ± 8.1 min, whereas the twitch response to direct stimulation remained unaffected (Fig. 8). After washing, the twitch response to indirect stimulation reappeared immediately and complete recovery took place within 30 min.

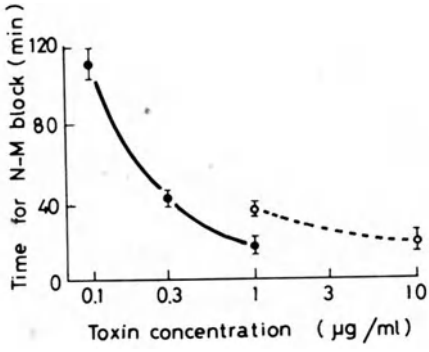


Fig. 7. Relationship between concentration of neurotoxins and time for complete neuromuscular block of the chick's biventer cervicis muscle preparation. The open circles denote the toxin LsIII and the solid circles Eb. The vertical bars denote standard errors for five experiments.

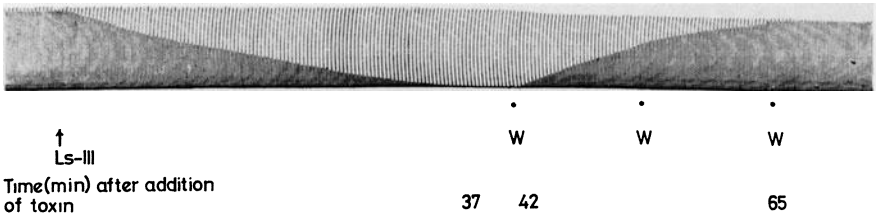


Fig. 8. The effect of LsIII on rat phrenic nerve-diaphragm preparations. A phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946) was suspended with a tension of 2 g in 20-ml Tyrode's solution which was continuously aerated with $O_2 + CO_2$ (95:5) at 37°C. Indirect (0.1 msec) and direct (0.5 msec) supramaximal stimulations were applied alternately every 10 sec. At the arrow, LsIII was added (final concentration; 10 μg/ml). At W, the preparation was washed with Tyrode's solution.

Amino Acid Sequence Analysis. LsIII was reduced with 2-mercaptoethanol and S-carboxymethylated with iodoacetate in the presence of 8 M urea (Sato and Tamiya, 1971). The urea and the excess reagents were removed by gel filtration on a column (1.8 X 65 cm) of Sephadex G-25 (coarse grade) in 0.1 M pyridine. To carry out the sequencing of LsIII with an automatic sequence analyzer (type JAS 47K, Japan Electron Optics Laboratory Co., Tokyo, Japan), it is desirable to obtain relatively large fragments, which may be digested further when necessary (Fig. 9). Citraconylation was carried out as described by Gibbons and Perham (1970) with slight modifications (Maeda and Tamiya, 1974). The tryptic peptides A and B from citraconylated RCM-LsIII were separated by Sephadex G-50 (fine grade) column (1.6 X 74 cm) chromatography. Further enzymic digestion of the peptides was performed with trypsin and α-chymotrypsin in 0.1 M NH_4HCO_3 buffer, pH 7.8, for 16 hr at 37°C. A substrate concentration

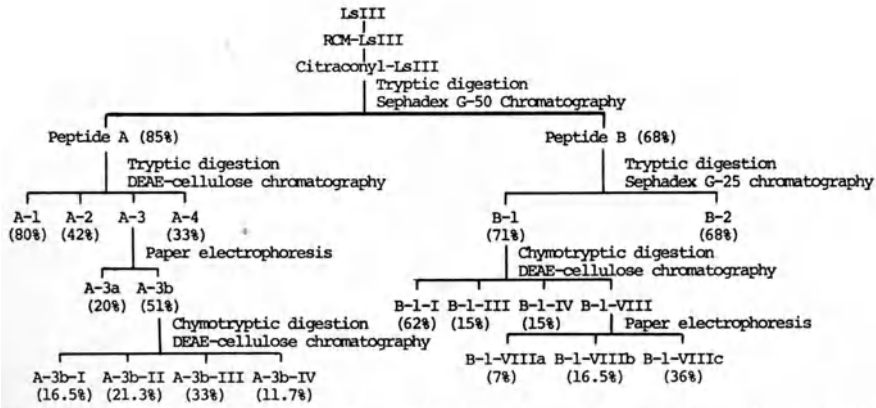
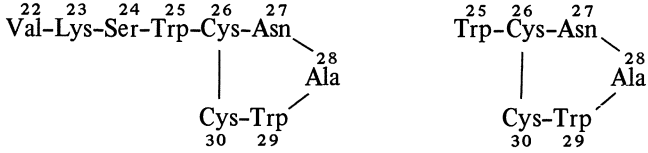


Fig. 9. Enzymatic fragmentation of RCM-LsIII. The percentage values are the yields of the peptides at each digestion step.

was about 5 mg/ml and the enzyme/substrate ratio was 1:50–100 (wt./wt.). The whole scheme of fragmentation and separation of resulting peptides are summarized in Fig. 9.

The N-terminal sequences of RCM-LsIII, peptides A and A-4 were analyzed with the sequence analyzer, and the sequences of small peptides were determined by the direct manual PTH method according to Iwanaga *et al.* (1969). In some cases the amino acid released was subtractively identified by amino acid analysis of a portion of the peptide remaining after a round of Edman degradation. The whole amino acid sequence of LsIII is shown in Fig. 10.

Location of Disulfide Bridges. LsIII (7 mg) was digested with pepsin on 0.01 M HCl (1.0 ml) at an enzyme/substrate ratio of 1:25 (wt./wt.) at 37°C for 40 hr. Two peptides, S-3 and S-4, were eluted later than the salts with 0.1 M acetic acid on application of the digest to a column (1.4 × 58 cm) of Sephadex G-25 (fine grade). Both were Ehrlich positive, containing two tryptophan residues per molecule judged from the UV absorption values. Their amino acid composition revealed their structure to be as follows:



Peptide S-3 Peptide S-4

The isolation of these peptides suggested the disulfide linkage between half-cystine residues at positions 26 and 30, making an extra loop. The positions of other disulfide bridges are assumed to be the same as other venom components

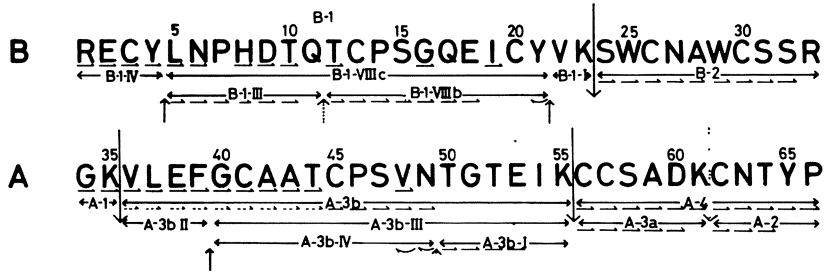


Fig. 10. Amino acid sequence of LsIII. Peptides are shown by bars with names. Solid horizontal arrows indicate the amino acids detected by Edman degradation and dotted horizontal arrows the amino acids cleaved off by the sequence analyzer for the purpose of obtaining a residual peptide. Curved arrows indicate amino acids detected by carboxypeptidase A digestion. Downward and upward arrows indicate the bonds cleaved by trypsin and α-chymotrypsin, respectively, and dotted vertical arrows show the partial cleavages by the enzymes.

from the sequence homology (Endo *et al.*, 1971; Yang *et al.*, 1970; Takechi and Hayashi, 1972; Ohta and Hayashi, 1973).

DISCUSSION

During the last several years more than 40 "neurotoxins" and "cardiotoxins" have been isolated from the venom of *Hydrophiidae* and *Elapidae* snakes and sequenced. These neurotoxins are classified into two groups, namely short-chain and long-chain toxins (Strydom, 1973). The former consist of 60–62 amino acid residues with four disulfide bridges, and the latter of 71–74 amino acids with five disulfide bridges. The cardiotoxins, including cytotoxins and a direct lytic factor, belong to the short-chain group (Lee, 1972).

Laticauda semifasciata III as reported here consists of 66 amino acid residues and is the first sea snake venom component with five disulfide bridges thus far isolated. The toxic symptoms produced by LsIII in mice and in baby chicks were similar to those produced by erabutoxins from the same venom, but LsIII was 8–10 times less toxic than the latter. LsIII inhibited the acetylcholine contracture of frog rectus abdominis muscle, and abolished the twitch response to indirect stimulation in the rat phrenic nerve-diaphragm preparation. LsIII did not affect the response to direct stimulation. However, the effect of LsIII on isolated muscles was easily removed by washing. Unlike cardiotoxins, LsIII did not produce contracture of the skeretal muscle even at a high concentration. All of these findings indicate that LsIII is not a cardiotoxin but a postsynaptically acting neurotoxin, affinity of which to acetylcholine-receptor sites may be much lower than those of other neurotoxins. It is noteworthy that this weakly neurotoxic component accounts for about 10%, by weight, of the venom protein.

The amino acid sequence of LsIII is given in Fig. 10. The sequence is clearly homologous to those of all the other neurotoxins and cardiotoxins isolated and sequenced so far from *Hydrophiidae* and *Elapidae* snakes (Table 2). LsIII shares tryptophan-29, arginine-37, and glycine-38 with other postsynaptic neurotoxins in addition to eight half-cystine residues, tyrosine-25, glycine-44, and proline-50 which are common to all neuro- and cardiotoxins.

LsIII is different, however, from other postsynaptic neurotoxins, especially in the following five features.

1. LsIII is a neutral protein with an isoelectric point of pH 7.2, whereas the other neurotoxins are highly basic proteins.

2. Ten half-cystine residues of LsIII are at the same positions as in long-chain toxins, making an additional loop between 30-34 (26-30 in Fig. 10). However the tail found in long-chain toxins is missing in LsIII.

3. Aspartic acid-31, which is common to other neurotoxins, is replaced by aspargine.

Table 2. Amino Acid Sequences of Venom Components from *Hydrophiidae* and *Elapidae*[a]

	Species, Toxins	5							10							15							20							25							30							35							
1.	*Laticauda semifasciata* Erabutoxin c	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	S	E	S	C	Y	H	K	Q	W	S	D		F			R	G	T									
2.	*Laticauda semifasciata* Erabutoxin b	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	S	E	S	C	Y	H	K	Q	W	S	D		F			R	G	T									
3.	*Laticauda semifasciata* Erabutoxin a	R	I	C	F	N	Q	H	S	S	Q	P	Q	T	T	K	T	C	P	S	G	S	E	S	C	Y	N	K	Q	W	S	D		F			R	G	T									
4.	*Laticauda laticaudata* Laticotoxin a'	R	R	C	F	N	H	P	S	S	Q	P	Q	T	N	K	S	C	P	P	G	E	N	S	C	Y	N	K	Q	W	R	D		H			R	G	T									
5.	Laticotoxin α	R	R	C	F	N	H	P	S	S	Q	P	Q	T	N	K	S	C	P	P	G	E	N	S	C	Y	N	K	Q	W	R	D		H			R	G	T									
6.	*Enhydrina schistosa* 5	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C	A			E	S	S	C	Y	K	K	T	W	S	D		H			R	G	T									
7.	*Enhydrina schistosa* 4	M	T	C	C	N	Q	Q	S	S	Q	P	K	T	T	T	N	C	A			E	S	S	C	Y	K	K	T	W	S	D		H			R	G	T									
8.	*Dendroaspis polylepis* α	R	I	C	Y	N	H	Q	S	T	T	R	A	T	T	K	S	C	E			E	N	S	C	Y	K	K	Y	W	R	D		H			R	G	T									
9.	*Naja nivea* Toxin β	M	I	C	H	N	Q	Q	S	S	Q	R	P	T	I	K	T	C	P		G	E	T	N	C	Y	K	K	R	W	R	D		H			R	G	T									
10.	*Naja nivea* Toxin δ, *Naja haje* Toxin α	L	Q	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	R	W	R	D		H			R	G	S									
11	*Naja melanoleuca* d	M	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	Q	W	S	D		H			R	G	T									
12.	*Naja nigricollis* Toxin α	L	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	T	C	P		G	E	T	N	C	Y	K	K	V	W	R	D		H			R	G	T									
13.	*Hemachatus haemachatus* II	L	E	C	H	N	Q	Q	S	S	Q	P	P	T	T	K	S	C	P		G	D	T	N	C	Y	N	K	R	W	R	D		H			R	G	T									
14.	*Hemachatus haemachatus* IV	L	E	C	H	N	Q	Q	S	S	Q	T	P	T	T	Q	T	C	P		G	E	T	N	C	Y	K	K	Q	W	S	D		H			R	G	S									
15.	*Naja naja atra* Cobrotoxin	L	E	C	H	N	Q	Q	S	S	Q	T	P	T	T	T	G	C	S	G	G	E	T	N	C	Y	K	K	R	W	R	D		H			R	G	Y									
16.	*Bungarus multicinctus* α Bungarotoxin	I	V	C	H		T	T	A	T	I	P	S	S	A	V	T	C	P	P	G	E	N	L	C	Y	R	K	M	W	C	D	A	F	C	S	S	R	G	K								
17.	*Dendroaspis polylepis* γ	R	T	C		N			K	T	F	S	D	Q	S	K	I	C	P	P	G	E	N	I	C	Y	T	K	T	W	C	D	A	W	C	S	Q	R	G	K								
18.	*Dendroaspis polylepis* δ	R	T	C		N			K	T	F	S	D	Q	S	K	I	C	P	P	G	E	N	I	C	Y	T	K	T	W	C	D	A	W	C	S	Q	R	G	K								
19.	*Ophiophagus hannah* A	T	K	C	Y				V	T	P	D	V	K	S	Q	T	C	P	A	G	E	N	I	C	Y	T	K	T	W	C	D	A	W	C	S	T	R	G	K								
20.	*Ophiophagus hannah* B	T	K	C	Y				V	T	P	D	A	T	S	Q	T	C	P	D	G	E	N	I	C	Y	T	K	T	W	C	D	N	F	C	S	S	R	G	K								
21.	*Naja nivea* toxin α	I	R	C	F				I	T	P	D	V	T	S	Q	A	C	P	D	G		H	V	C	Y	T	K	M	W	C	D	N	F	C	G	M	R	G	K								
22.	*Naja melanoleuca* b	I	R	C	F				I	T	P	D	V	T	S	Q	I	C	A	D	G		H	V	C	Y	T	K	T	W	C	D	N	F	C	A	S	R	G	K								
23.	*Naja naja siamensis* 3	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	A	F	C	S	I	R	G	K								
24.	*Naja naja naja* 4	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	S	R	G	K								
25.	*Naja naja naja* 3	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	I	R	G	K								
26.	*Naja naja* Toxin A	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	I	R	G	K								
27.	*Naja naja* Toxin B	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	G	F	C	S	S	R	G	K								
28.	*Naja naja* Toxin C	I	R	C	F				I	T	P	D	I	T	S	K	D	C	P	N	G		H	V	C	Y	T	K	T	W	C	D	A	F	C	S	I	R	G	K								
29.	*Laticauda semifasciata* III	R	E	C	Y					L	N	P	H	D	T	Q	T	C	P	S	G	Q	E	I	C	Y	V	K	S	W	C	N	A	W	C	S	S	R	G	K								
30.	*Naja naja atra* Cardiotoxin	L	K	C		N			K	L	V	P	L	F	Y	K	T	C	P	A	G	K	N	L	C	Y		K	M	I-	M	V	A					T	P	K	V								
31.	*Naja naja* Cytotoxin II	L	K	C		N			K	L	V	P	L	F	Y	K	T	C	P	A	G	K	N	L	C	Y		K	M	Y	M	V	A					T	P	K	V								
32.	*Naja naja* Cytotoxin I	L	K	C		N			K	L	I	P	L	A	Y	K	T	C	P	A	G	K	N	L	C	Y		K	M	Y	M	V	S					N	K	T	V								
33.	*Naja nigricollis* 14 Cardiotoxin	L	K	C		N			Q	L	I	P	P	F	W	K	T	C	P	K	G	K	N	L	C	Y		K	M	T	M	R	A					A	P	M	V								
34.	*Naja naja* (Cambodia) Cardiotoxin	L	K	C		N			K	L	I	P	I	A	S	K	T	C	P	A	G	K	N	L	C	Y		K	M	F	M	M	S					D	L	T	I								
35.	*Hemachatus haemachatus* DLF (12B)	L	K	C	H	N			K	L	V	P	F	L	S	K	T	C	P	E	G	K	N	L	C	Y		K	M	T	M	L	K					M	P	K	I								
36	*Naja haje* Cardiotoxin	L	K	C	H				K	L	V	P	P	V	W	K	T	C	P	E	G	K	N	L	C	Y		K	M	F	M	V	S					T	S	T	V								
37.	*Naja melanoleuca* Cardiotoxin	L	E	C		N			K	L	V	P	I	A	H	K	T	C	P	A	G	K	N	L	C	Y		Q	M	Y	M	V	S					K	S	T	I								
38.	*Dendroaspis angusticeps* F_{VII}	T	M	C	Y	S	H	T	T	T	S	R	A	I	L	T	N	C	G	E			N	S	C	Y	R	K	S	R	R	H	P						P	K	M								

	40				45				50				55				60				65			70					75				Total	Reference
1.	I	I	E	R	G	C			G	C	P	T	V	K	P	G	I	N	L	S	C	C	E	S	E	V	C	N	N				62	Tamiya and Abe, 1972
2.	I	I	E	R	G	C			G	C	P	T	V	K	P	G	I	K	L	S	C	C	E	S	E	V	C	N	N				62	Sato and Tamiya, 1971
3.	I	I	E	R	G	C			G	C	P	T	V	K	P	G	I	K	L	S	C	C	E	S	E	V	C	N	N				62	Sato and Tamiya, 1971
4.	I	T	E	R	G	C			G	C	P	T	V	K	P	G	I	K	L	T	C	C	Q	S	E	D	C	N	N				62	Sato *et al.*, unpublished
5.	I	T	E	R	G	C			G	C	P	Q	V	K	S	G	I	K	L	T	C	C	Q	S	D	D	C	N	N				62	Sato *et al.*, unpublished
6.	R	I	E	R	G	C			G	C	P	Q	V	K	S	G	I	K	L	E	C	C	H	T	N	E	C	N	N				60	Fryklund *et al.*, 1972
7.	R	I	E	R	G	C			G	C	P	Q	V	K	P	G	I	K	L	E	C	C	H	T	N	E	C	N	N				60	Fryklund *et al.*, 1972
8.	I	I	E	R	G	C			G	C	P	K	V	K	P	G	V	G	I	H	C	C	Q	S	D	K	C	N	Y				60	Strydom, 1972
9.	I	I	E	R	G	C			G	C	P	S	V	K	K	G	V	G	I	Y	C	C	K	T	D	K	C	N	R				61	Botes, 1971
10.	I	T	E	R	G	C			G	C	P	S	V	K	K	G	I	E	I	N	C	C	T	T	D	K	C	N	N				61	Botes *et al.*, 1971, 1969
11.	I	I	E	R	G	C			G	C	P	S	V	K	K	G	V	K	I	N	C	C	T	T	D	R	C	N	N				61	Botes, 1972
12.	I	I	E	R	G	C			G	C	P	T	V	K	P	G	I	K	L	N	C	C	T	T	D	K	C	N	N				61	Eaker and Porath, 1967
13.	I	I	E	R	G	C			G	C	P	T	V	K	P	G	I	N	L	K	C	C	T	T	D	R	C	N	N				61	Strydom and Botes, 1971
14.	R	T	E	R	G	C			G	C	P	T	V	K	P	G	I	K	L	K	C	C	T	T	D	R	C	N	K				61	Strydom and Botes, 1971
15.	R	T	E	R	G	C			G	C	P	S	V	K	N	G	I	E	I	N	C	C	T	T	D	R	C	N	N				62	Yang *et al.*, 1969
16.	V	V	E	L	G	C	A	A	T	C	P	S	K	K	P	Y	E	E	V	T	C	C	S	T	D	K	C	N	H	P	P	K	R	Q	P	G	74	Mebs *et al.*, 1972
17.	R	V	E	L	G	C	A	A	T	C	P	K	V	K	A	G	V	E	I	K	C	C	S	T	D	D	C	D	K	F	Q	F	G	K	P	R	72	Strydom, 1972
18.	I	V	E	L	G	C	A	A	T	C	P	K	V	K	A	G	V	E	I	K	C	C	S	T	D	N	C	N	K	F	K	F	G	K	P	R	72	Strydom, personal communication
19.	R	V	D	L	G	C	A	A	T	C	P	I	V	K	P	G	V	E	I	K	C	C	S	T	D	N	C	N	P	F	P	T	W	R	K	R	P	73	Joubert, 1973
20.	R	I	N	L	G	C	A	A	T	C	P	K	V	K	P	G	V	D	I	K	C	C	S	T	D	N	C	N	P	F	P	T	W	K	R	R	H	73	Joubert, 1973
21.	R	V	D	L	G	C	A	A	T	C	P	K	V	K	P	G	V	N	I	K	C	C	S	R	D	N	C	N	P	F	P	T	R	K	R	S		71	Botes, 1971
22.	R	V	D	L	G	C	A	A	T	C	P	T	V	K	P	G	V	N	I	K	C	C	S	T	D	N	C	N	P	F	P	T	R	N	R	P		71	Botes, 1972
23.	R	V	D	L	G	C	A	A	T	C	P	T	V	K	T	G	V	D	I	Q	C	C	S	T	D	N	C	N	P	F	P	T	R	K	R	P		71	Karlsson, personal communication
24.	R	V	D	L	G	C	A	A	T	C	P	T	V	R	T	G	V	D	I	Q	C	C	S	T	D	N	C	N	P	F	P	T	R	K	R	P		71	Karlsson, personal communication
25.	R	V	D	L	G	C	A	A	T	C	P	T	V	R	T	G	V	D	I	Q	C	C	S	T	D	N	C	N	P	F	P	T	R	K	R	P		71	Karlsson, personal communication
26.	R	V	D	L	G	C	A	A	T	C	P	T	V	R	T	G	V	D	I	Q	C	C	S	T	D	D	C	D	T	F	P	T	R	K	R	P		71	Nakai *et al.*, 1971
27.	R	V	D	L	G	C	A	A	T	C	P	T	V	R	T	G	V	D	I	Q	C	C	S	T	D	D	C	D	T	F	P	T	R	K	R	P		71	Hayashi, personal communication
28.	R	V	D	L	G	C	A	A	T	C	P	T	V	K	T	G	V	D	I	Q	C	C	S	T	D	D	C	D	T	F	P	T	R	K	R	P		71	Hayashi, personal communication
29.	V	L	E	F	G	C	A	A	T	C	P	S	V	N	T	G	T	E	I	K	C	C	S	A	D	K	C	N	T	Y	P							66	Maeda and Tamiya, 1974
30.	P	V	K	R	G	C	I	D	V	C	P	K	S	S	L	V	L	K	Y	V	C	C	N	T	D	R	C	N										60	Narita and Lee, 1970
31.	P	V	K	R	G	C	I	D	V	C	P	K	S	S	L	V	L	K	Y	V	C	C	N	T	D	R	C	N										60	Takechi *et al.*, 1972
32.	P	V	K	R	G	C	I	D	V	C	P	K	N	S	L	V	L	K	Y	E	C	C	N	T	D	R	C	N										60	Hayashi *et al.*, 1971
33.	P	V	K	R	G	C	I	D	V	C	P	K	S	S	L	L	I	K	Y	M	C	C	N	T	D	K	C	N										60	Botes, unpublished
34.	P	V	K	R	G	C	I	D	V	C	P	K	S	N	L	L	V	K	Y	V	C	C	N	T	D	R	C	N										60	Botes, unpublished
35.	P	I	K	R	G	C	T	D	A	C	P	K	S	S	L	L	V	K	V	V	C	C	N	K	D	K	C	N										61	Fryklund *et al.*, 1973
36.	P	V	K	R	G	C	I	D	V	C	P	K	N	S	A	L	V	K	Y	V	C	C	S	T	D	K	C	N										60	Botes, personal communication
37.	P	V	K	R	G	C	I	D	V	C	P	K	S	S	L	L	V	K	Y	V	C	C	N	T	D	R	C	N										60	Botes, personal communication
38.	V	L	G	R	G	C			G	C	P	P	G	D	D	N	L	E	V	K	C	C	T	S	P	D	K	C	N	Y								61	Viljoen and Botes, 1973

[a] 1–29, Neurotoxins; 30–38, cardiotoxins; 1–7 and 29, *Hydrophiidae* 8–28, 30–30, *Elapidae*. The amino acids surrounded by solid lines are found in common at the same positions and those surrounded by the dotted lines have similar groups at the same positions.

4. The usual positive charge at position 53 is missing in LsIII.

5. The usual hydroxyl group at position 9 is missing in LsIII. Some of these five features may possibly explain the weak and reversible neurotoxicity of LsIII, but the question of which of them is or are essential remains for further research.

To elucidate the structure-function relationships of snake toxins, many efforts have been made to find the minimum structural requirements by the following two ways. One is chemical modification studies with group-specific reagents and the other is comparison of the primary structures of related toxic components. The study of the effect of chemical modification of a specific group, however, must be accompanied by the evidence of conformational reservation of the toxin molecule. The second method relies on the chance that all of the nonessential residues are replacable by others.

The elucidation of the structures of weakly acting toxins or inactive components, which are synthesized by the animals themselves, provides a new approach to the clarification of the essential structure of the active toxins.

SUMMARY

A weakly neurotoxic component (LsIII) has been isolated by CM-cellulose column chromatography from the venom of a sea snake, *L. semifasciata.* The LsIII content was about 10%-20% of the venom protein as determined by UV absorption at 280 nm. LsIII is homogeneous on rechromatography and disk electrophoresis. The isoelectric point of LsIII is pH.7.2. The molecular weight of LsIII was shown to be 7100 by ultracentrifugation and 7300 by SDS-polyacrylamide gel disk electrophoresis.

The LD_{50} of LsIII by intramuscular injection is 1.24 μg/g body weight for mice and 0.45 μg/g for baby chicks. LsIII is about 8-10 times less toxic than erabutoxins a, b, and c, all of which are contained in the same venom. Experiments with isolated muscle preparations from three different species indicated that LsIII is an easily reversible postsynaptically acting neurotoxin.

The primary structure of LsIII has been determined. LsIII consists of 66 amino acid residues and five disulfide bridges, one of which is located between residues 26 and 30. The weak and reversible neurotoxicity of LsIII was discussed from the comparison of its transitional structure with those of neuro- and cardiotoxins of sea snakes and *Elapidae* snakes thus far isolated and sequenced.

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Structural Studies of the Toxic Components from *Naja naja oxiana* Venom

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INTRODUCTION

The snakes of the family *Elapidae* and *Hydropidae* contain certain neurotoxins in their venom which specifically block the acetylcholine receptors on the post-synaptic membrane. These neurotoxins are basic polypeptides with molecular weights of 7000-8000 and with four or five intramolecular disulfide bridges (Yang, 1974). Besides, neurotoxins of *Naja naja oxiana* venom also contain a group of basic polypeptides which contain methionine, are rich in lysine and hydrophobic amino acids, and poor in glycine and arginine (Condrea, 1974). Like neurotoxins of the 60-4 structural type (Karlsson *et al.*, 1972), these polypeptides have 60-61 amino acid residues and four intramolecular disulfide bridges. As a rule they are considerably less toxic than neurotoxins, but exert cytotoxic and cardiostatic effects. To understand the mechanism of action of neurotoxins and cytotoxins it would be of help to have more knowledge of those structures. It seemed useful to us to determine the amino acid sequence of the above-mentioned compounds (Grishin *et al.*, 1973, 1974a, b).

MATERIALS AND METHODS

Crude venom of the Middle-Asian cobra, *N. n. oxiana*, was obtained from Kirghiz Serpentarium. Trypsin, chymotrypsin, and carboxypeptidases A and B

were purchased from Worthington, thermolysin from Serva, and carboxypeptidase C from Roth. Carboxymethylcellulose CM-32 was a product of Watman, the Sephadexes were the products of Pharmacia, ion exchangers Bio-Rex 70 were from Bio Rad, and Chromo-Beads P4 were from Technicon. Cyanogen bromide was synthesized immediately before use. Other reagents were chromatographically pure.

Chromatographic fractionation of the crude venom on Sephadex G-75 and CM-cellulose was performed as described earlier (Turakulov *et al.*, 1971). Fraction 3 which contained cytotoxin was rechromatographed on a cation exchanger, Bio-Rex 70, using ammonium acetate gradient.

Reduction and carboxymethylation was carried out according to Crestfield *et al.* (1963). Amino acid composition was determined on a BC 201 Bio-Cal amino acid analyzer. Tryptic, chymotryptic, and thermolytic hydrolyses were carried out in 0.1 M NH_4HCO_3 (pH 8.5) for 4 hr at 37° C (the enzyme to substrate ratio was 1:50). Neurotoxin II and cytotoxin tryptic hydrolysates were fractionated on a 0.9 × 60 cm column packed with Chromo-Beads P4 with a continuous pH and concentration gradient of volatile pyridine–acetate buffer. The effluent was analyzed on a Technicon II automatic analyzer. Cyanogen bromide cleavage of carboxymethylated cytotoxin was carried out in 70% formic acid with a 100% excess of the reagent (22 hr, room temperature). The resulting products were fractionated on Sephadex G-25 in 0.1 M NH_4HCO_3 buffer. In all the other cases, isolation and purification of peptides were done by paper chromatography and electrophoresis in standard buffer systems. Amino acid sequence determinations were carried out as described elsewhere (Vinogradova *et al.*, 1973). N-terminal sequences of the carboxymethylated cytotoxin and its cyanogen bromide fragment were determined on a Beckman model 890 automatic sequencer.

RESULTS AND DISCUSSION

Ion exchange chromatography on carboxymethylcellulose CM-32 of the main fraction obtained by gel filtration of the crude venom (Turakulov *et al.*, 1971) gave practically homogeneous neurotoxins I and II. Cytotoxin was obtained by chromatography on Bio-Rex 70 of fraction 3 (Fig. 1). Amino acid composition of the neurotoxins and cytotoxin are listed in Table 1. The number of Trp residues was calculated from the alkaline hydrolysis data and molar absorption at 280 nm.

Sequential determinations of the carboxymethylated toxins were made by conventional methods. For first fragmentation, we used tryptic hydrolysis. Tryptic digestion of carboxymethylated neurotoxin I proceeded quite specifically. From the hydrolysate, we obtained eight peptides (Table 2). By chromatography

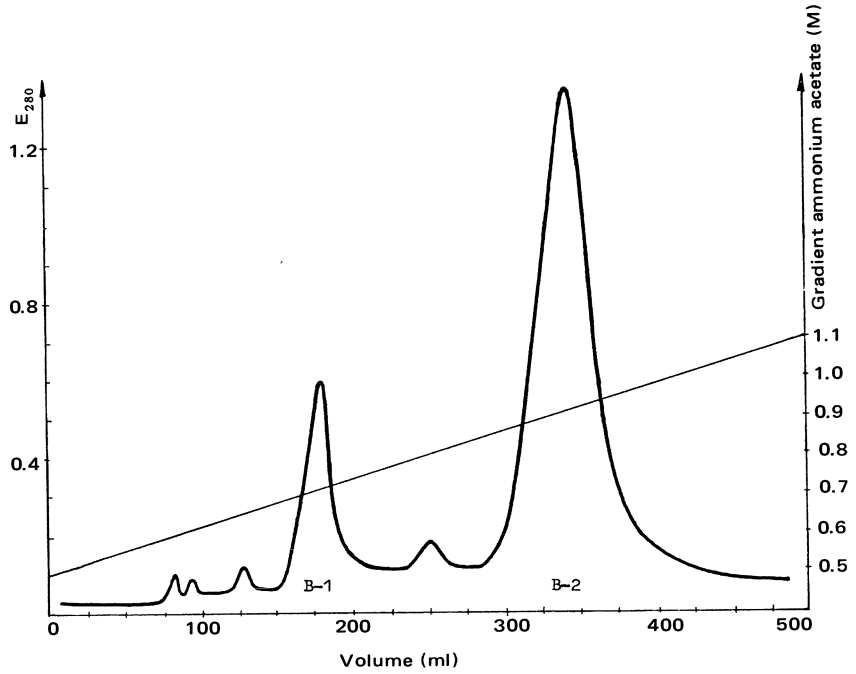

Fig. 1. Chromatography on Bio-Rex 70 of fraction 3. 1.5 × 28 cm column in 0.2 M ammonium acetate, pH 6.5. Gradient elution was from 0.5 M to 1.1 M ammonium acetate solution; flowrate, 45 ml/hr.

or paper electrophoresis, we could not isolate the 20-membered peptide in amounts sufficient for analysis. However chymotryptic hydrolysis of carboxymethylated neurotoxin I gave us nine peptides which afforded enough information for establishing the amino acid sequence of neurotoxin I (Table 3).

Neurotoxin I from *N. n. oxiana* venom belongs to the group of the so-called long-chain neurotoxins with five intramolecular disulfide bonds. It somewhat differs from all known neurotoxins of this class by the complete absence of phenylalanine residues in its chain and such low contents of basic amino acid residues (Fig. 2). Its amino acid sequence between Cys(3) and Cys(15) is also rather different from that usually found in toxins of this kind. It should also be noted that at position 51, where neurotoxins usually have Lys or Arg residues, this one has glutamic acid (Glu). Possibly this structural differences are the reason of the physiological peculiarities observed for this neurotoxin (for example, its toxicity is lower than that usually exhibited by other toxins; its LD_{50} for mice is 0.56 mg/kg). According to amino acid analysis, tryptic hydrolysis of carboxymethylated neurotoxin II should give 11 sufficiently short peptides. Ion exchange chromatography was found to be the best approach to fractionation of the tryptic hydrolysate. Chromo-Beads P4 packing (22-k diameter) in-

Table 1. The Amino Acid Composition of Toxins and Cyanogen Bromide Peptides of Cytotoxin from *Naja naja oxiana* Venom

Amino acid	Neurotoxin I	Neurotoxin II	Cytotoxin	CB-1	CB-2	CB-3
Asp	6.01(6)	8.12(8)	5.12(5)	1.21(1)	–	3.84(4)
Thr	9.04(9)	6.03(6)	1.96(2)	1.02(1)	–	1.01(1)
Ser	3.94(4)	3.96(4)	2.92(3)	1.13(1)	–	2.02(2)
Glu	6.02(6)	6.04(6)	0.08	–	–	–
Pro	7.08(7)	4.09(4)	5.04(5)	2.21(2)	–	2.84(3)
Gly	4.02(4)	5.09(5)	2.12(2)	1.32(1)	–	1.14(1)
Ala	4.05(4)	0.13	3.07(3)	1.03(1)	–	2.12(2)
½ Cys	9.96(10)	8.01(8)	8.02(8)	3.04(3)	–	5.03(5)
Val	1.88(2)	1.90(2)	6.75(7)	0.98(1)	–	5.61(6)
Met	–	0.01	1.74(2)	(1)	(1)	–
Ile	4.78(5)	1.86(2)	0.89(1)	–	–	0.96(1)
Leu	1.89(2)	1.91(2)	5.84(6)	3.42(4)	–	1.90(2)
Tyr	2.81(3)	0.92(1)	1.79(2)	0.82(1)	–	0.95(1)
Phe	0.02	0.03	2.04(2)	1.02(1)	(1)	–
His	0.87(1)	1.91(2)	0.86(1)	–	–	1.02(1)
Lys	6.08(6)	5.03(5)	9.94(10)	5.65(6)	–	3.95(4)
Arg	1.98(2)	4.06(4)	1.12(1)	0.02	–	0.93(1)
Trp	1.76(2)	1.81(2)	–	–	–	–
Total	73	61	60	24	2	34

Table 2. Amino Acid Composition of Tryptic Peptides from Carboxymethylated Neurotoxin I

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8
Asp	–	–	–	–	–	0.9(1)	0.9(1)	2.6(3)
Thr	–	–	–	–	1.0(1)	4.1(4)	1.0(1)	1.0(1)
Ser	–	–	0.2	–	–	1.2(1)	1.0(1)	1.0(1)
Glu	1.0(1)	–	1.0(1)	0.2	–	1.8(2)	0.2	0.2
Pro	1.0(1)	–	–	0.9(1)	–	2.9(3)	–	2.0(2)
Gly	–	1.0(1)	0.2	–	0.2	1.0(1)	1.0(1)	–
Ala	–	–	–	–	–	1.1(1)	1.0(1)	–
Val	–	–	–	–	–	–	–	–
Cm-Cys	–	–	–	–	0.8(1)	1.7(2)	1.7(2)	2.6(3)
Ile	–	–	–	–	1.0(1)	1.6(2)	–	–
Leu	–	–	–	–	–	0.9(1)	–	–
Tyr	–	–	–	–	0.8(1)	0.8(1)	–	–
His	–	–	–	–	–	–	–	0.7(1)
Lys	1.0(1)	1.0(1)	0.9(1)	0.2	1.1(1)	1.1(1)	–	1.0(1)
Arg	0.9(1)	–	–	1.0(1)	–	–	0.8(1)	–
Trp	–	–	–	–	–	–	(2)	–
Total	4	2	2	2	5	20	10	12
Yield, %	35	44	21	26	84	39	24	48

Table 3. Amino Acid Composition of Chymotryptic Peptides from Carboxymethylated Neurotoxin I

Amino acid	Ch-1	Ch-2	Ch-3	Ch-4	Ch-5	Ch-6	Ch-7	Ch-8	Ch-9
Asp	–	–	3.8(4)	1.1(1)	–	–	4.0(4)	1.1(1)	–
Thr	–	1.7(2)	1.0(1)	2.7(3)	1.8(2)	0.9(1)	1.0(1)	–	1.9(2)
Ser	0.1	–	1.2(1)	1.2(1)	2.1(2)	–	1.2(1)	–	1.3(1)
Glu	1.0(1)	–	2.0(2)	2.1(2)	2.0(2)	–	1.1(1)	–	1.1(1)
Pro	0.9(1)	–	2.7(3)	2.7(3)	1.0(1)	–	1.9(2)	–	0.8(1)
Gly	0.1	–	0.1	1.1(1)	3.1(3)	–	–	0.1	1.3(1)
Ala	–	–	–	0.9(1)	1.9(2)	–	–	1.0(1)	2.0(2)
Val	–	–	–	–	1.6(2)	–	–	–	0.8(1)
Cm-Cys	–	–	2.6(3)	1.9(2)	3.0(3)	1.0(1)	2.7(3)	1.0(1)	1.8(2)
Ile	–	–	0.9(1)	1.7(2)	0.8(1)	1.0(1)	1.0(1)	–	–
Leu	–	–	–	1.0(1)	1.0(1)	–	–	–	–
Tyr	–	–	–	0.9(1)	0.8(1)	0.9(1)	–	–	1.0(1)
His	–	–	0.7(1)	–	–	–	1.0(1)	–	–
Lys	0.9(1)	1.0(1)	2.8(3)	1.0(1)	0.9(1)	–	1.9(2)	–	–
Arg	1.0(1)	–	0.8(1)	–	1.0(1)	–	–	–	–
Trp	–	(1)	–	–	–	–	–	(1)	–
Total	4	4	20	19	22	4	16	4	12
Yield, %	10	28	13	14	12	10	9	12	14

creased the resolution of the column considerably so that a greater part of peptides was eluted in well defined peaks (Fig. 3). Fractions I, III, V, VII, IX, and X actually were individual peptides.

Other peptides were isolated and purified by paper electrophoresis and chromatography. We isolated and identified 10 tryptic peptides (Table 4). Then carboxymethylated neurotoxin II was subjected to chymotryptic hydrolysis. The resulting chymotryptic digest was fractionated and purified by electrophoresis and paper chromatography. Therefrom we obtained four chymotryptic peptides, which permitted us to arrange the tryptic fragments (Table 4). However, the tryptic and chymotryptic hydrolyses did not allow us to isolate the peptide containing both Trp residues. Therefore, we resorted to thermolytic hydrolysis which gave us an 8-membered peptide containing the two Trp residues. The sequence of this peptide permitted us to place the Trp residues in the toxin chain.

Neurotoxin II from *N. n. oxiana* venom is a short-chain neurotoxin with four intramolecular disulfide bridges (Fig. 4) and with a very interesting Trp-Trp sequence in positions 27-28.

Amino acid sequence of the carboxymethylated cytotoxin was determined on the automatic amino acid sequencer. The N-terminal sequence of the compound identified by the instrument using the quadrol program was as follows:

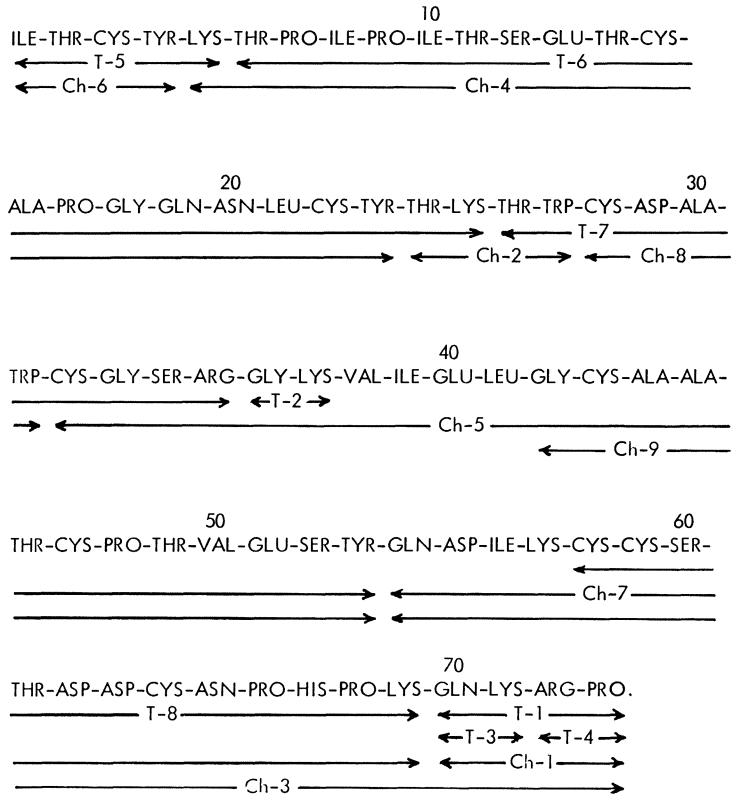

Fig. 2. The amino acid sequence of neurotoxin I from *Naja naja oxiana* venom; T, tryptic peptides; Ch, chymotryptic peptides.

Leu-Lys-Cys-Lys-Lys-Leu-Val-Pro-Leu-Phe-X-X-X-Cys-Pro-Ala-Gly-X-X-Leu-X-Tyr-X-Met-Phe-Met. . . . To obtain larger peptide fragments, we hydrolyzed the carboxymethylated cytotoxin with cyanogen bromide. The produced peptides were separated by gel filtration on Sephadex G-25. The amino acid sequence of the cyanogen bromide fragments is given in Table 1. Leu was found to be the N-terminal amino acid in the CB-1 peptide; consequently this peptide should be the N-terminal fragment of the cytotoxin molecule, as is also consistent with its amino acid composition. Peptide CB-2 for its N-terminal amino acid had Phe; thus the CB-2 sequence should be Phe-Met. N-terminal amino acid sequence of the CB-3 peptide was established by means of the sequencer using the *N*,*N*-dimethylallylamine peptide programme and was found to be as follows: Val-Ala-Ala-Pro-His-Val-Pro-Val-Lys-Arg-Gly-Cys-X-Asp-Val-Cys-Pro. . . . The total amino acid sequence of the CB-3 peptide was deduced from its chymo-

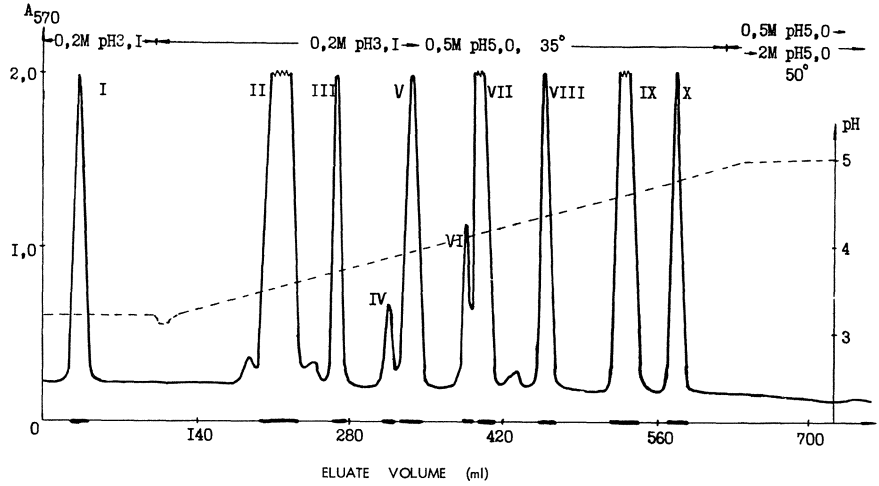

Fig. 3. Fractionation on Chromo-Beads P4 resin of the tryptic digest of S-carboxymethylated neurotoxin II. 0.9 X 100 cm column in 0.2 M pyridine acetate, pH 3.1. Gradient of concentration and pH from 0.2 M, pH 3.1 to 2.0 M, pH 5.0, pyridine acetate buffer; flow-rate, 28 ml/hr; fraction volume, 2.8 ml.

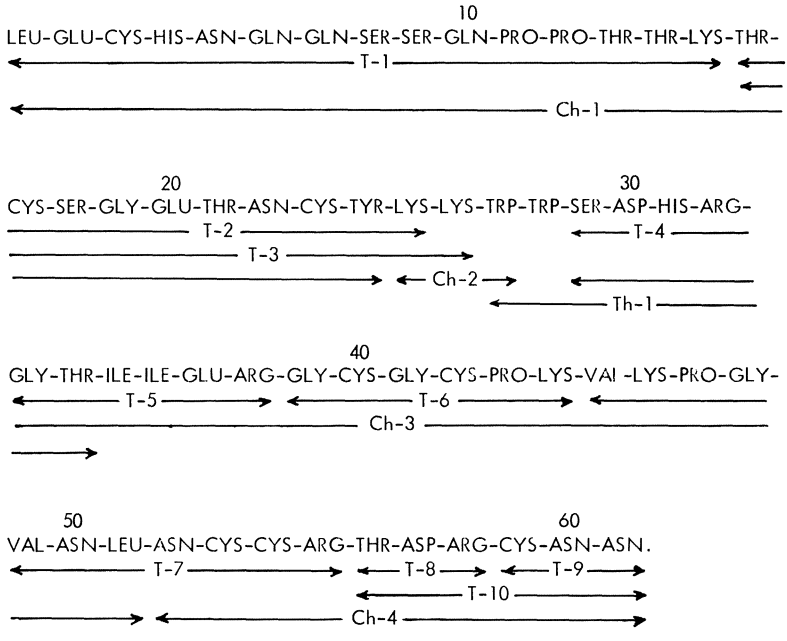

Fig. 4. The amino acid sequence of neurotoxin II from *Naja naja oxiana* venom: T, tryptic peptides; Ch, chymotryptic peptides; Thy, thermolytic peptide.

Table 4. Amino Acid Composition of Tryptic and Chymotryptic Peptides from Carboxymethylated Neurotoxin II

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10	Ch-1	Ch-2	Ch-3	Ch-4
Asp	1.0(1)	1.1(1)	1.0(1)	1.0(1)	–	–	2.0(2)	1.0(1)	1.9(2)	2.9(3)	2.1(2)	–	2.1(2)	3.9(4)
Thr	1.9(2)	1.9(2)	1.8(2)	–	0.9(1)	–	–	1.0(1)	–	1.0(1)	4.1(4)	–	1.2(1)	1.1(1)
Ser	1.9(2)	1.1(1)	1.2(1)	0.9(1)	–	–	–	0.2	0.2	0.1	3.1(3)	–	1.0(1)	–
Glu	4.1(4)	1.2(1)	1.1(1)	–	1.0(1)	–	–	–	–	–	4.8(5)	–	1.0(1)	–
Pro	2.0(2)	–	–	–	–	1.0(1)	1.0(1)	–	–	–	1.9(2)	–	2.0(2)	–
Gly	–	1.1(1)	1.1(1)	–	1.0(1)	2.0(2)	1.0(1)	0.2	0.3	0.2	1.2(1)	–	4.2(4)	–
Val	–	–	–	–	–	–	1.8(2)	–	–	–	–	–	1.9(2)	–
Cm-Cys	0.8(1)	1.8(2)	2.0(2)	–	–	2.1(2)	2.2(2)	–	1.0(1)	1.1(1)	2.9(3)	–	2.0(2)	2.8(3)
Ile	–	–	–	–	1.5(2)	–	–	–	–	–	–	–	1.7(2)	–
Leu	1.0(1)	–	–	–	–	–	0.9(1)	–	–	–	0.9(1)	–	1.0(1)	–
Tyr	–	0.8(1)	0.7(1)	–	–	–	–	–	–	–	0.7(1)	–	–	–
His	1.0(1)	–	–	1.0(1)	–	–	–	–	–	–	0.8(1)	–	0.8(1)	–
Lys	1.0(1)	1.0(1)	1.7(2)	0.1	–	1.0(1)	0.8(1)	–	–	–	1.0(1)	(2)	2.1(2)	–
Arg	–	–	–	1.0(1)	0.9(1)	–	0.8(1)	0.9(1)	–	0.9(1)	0.2	–	2.0(2)	1.8(2)
Trp	–	–	–	–	–	–	–	–	–	–	–	(1)	–	–
Total	15	10	11	4	6	6	11	3	3	6	24	3	23	10
Yield, %	30	12	24	2	76	96	30	28	12	30	24	72	16	32

Table 5. Amino Acid Composition of Tryptic Peptides from Carboxymethylated Cytotoxin

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10	T-11
Asp	–	–	–	–	–	1.0(1)	–	1.1(1)	1.0(1)	–	2.9(3)
Thr	–	–	–	–	0.9(1)	–	–	–	–	–	0.9(1)
Ser	–	–	1.0(1)	1.0(1)	–	–	–	–	–	1.7(2)	–
Pro	–	–	0.8(1)	1.0(1)	1.0(1)	–	1.8(2)	0.8(1)	0.9(1)	–	–
Gly	–	–	–	–	1.1(1)	–	–	0.9(1)	1.1(1)	–	–
Val	–	–	1.1(1)	1.1(1)	–	–	2.8(3)	1.0(1)	0.8(1)	1.0(1)	0.7(1)
Ala	–	–	–	–	1.0(1)	–	2.0(2)	–	–	–	–
Cm-Cys	–	0.8(1)	–	–	0.8(1)	0.8(1)	–	1.8(2)	1.8(2)	–	2.7(3)
Ile	–	–	–	–	–	–	–	1.0(1)	0.9(1)	–	–
Leu	1.0(1)	–	1.9(2)	1.8(2)	–	1.0(1)	–	–	–	2.0(2)	–
Met	–	–	–	–	–	–	1.5(2)	–	–	–	–
Tyr	–	–	–	–	–	0.8(1)	–	–	–	–	0.8(1)
Phe	–	–	1.0(1)	0.9(1)	–	–	0.9(1)	–	–	–	–
His	–	–	–	–	–	–	0.8(1)	–	–	–	–
Lys	1.0(1)	1.8(2)	2.0(2)	1.0(1)	1.2(1)	1.0(1)	0.9(1)	1.1(1)	1.0(1)	1.0(1)	1.0(1)
Arg	–	–	–	–	–	–	–	0.9(1)	–	–	–
Total	2	3	8	7	6	5	12	9	8	6	10

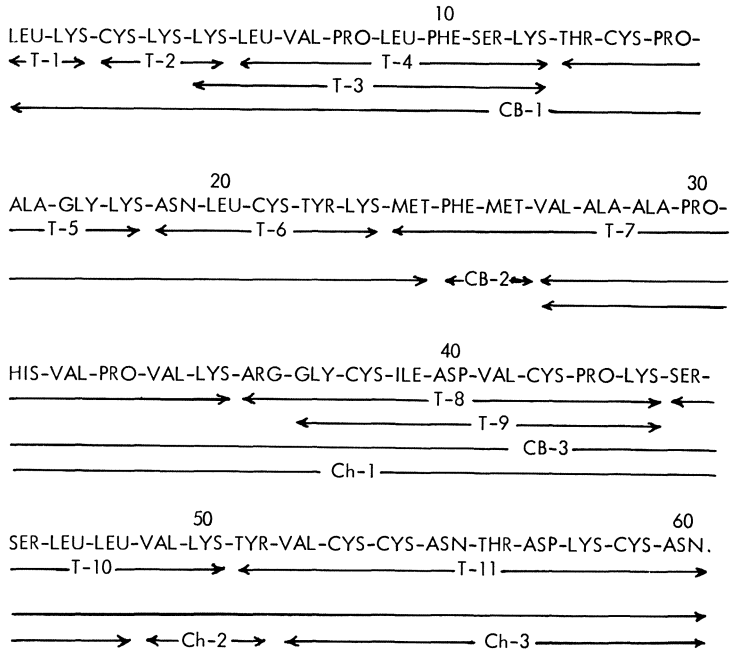

Fig. 5. The amino acid sequence of cytotoxin from *Naja naja oxiana* venom: T, tryptic peptides; CB, cyanogen bromide peptides; Ch, chymotryptic peptides of the CB-3.

tryptic peptides and the peptides from the cytotoxin tryptic digest. Chymotryptic peptides were separated chromatographically and electrophoretically. The tryptic digest of the carboxymethylated cytotoxin was fractionated on Chromo-Beads P4 under the conditions similar to those used for neurotoxin II. Eleven peptides were isolated from the digest; their amino acid composition is shown in Table 5. All of the described results allowed us to construct the amino acid sequence of the cytotoxin (Fig. 5).

We can now conclude that the cytotoxin from the *N. n. oxiana* venom is homologous with the cytotoxins and cardiotoxins from the venoms of other cobras. As we can see from Fig. 5, its main structural peculiarity is the Lys residue in position 4.

SUMMARY

Neurotoxins I and II and a cytotoxin have been isolated from *N. n. oxiana* snake venom and their primary structures were elucidated. Neurotoxin I has been shown to have an amino acid sequence of 73 residues, neurotoxin II has a sequence of 61 residues, and the cytotoxin has 60 residues.

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Structural Nature of Presynaptic Neurotoxins from the Venoms of the Australian Tiger Snake, *Notechis scutatus scutatus*, and the Taipan *Oxyuranus scutellatus scutellatus*

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INTRODUCTION

The flaccid paralysis characteristic of envenomation by elapid and hydrophid snakes is due to protein toxins which block transmission across the cholinergic neuromuscular junction either by (1) a postsynaptic curarelike action on the nicotinic acetylcholine receptors of the muscle motor endplate, or (2) a presynaptic interference with the release of acetylcholine from the motor nerve terminals.

All elapid and hydrophid venoms examined thus far contain at least one, and usually several, postsynaptic neurotoxins of the curarimimetic type. In a review published about a year ago Karlsson (1973) listed the amino acid sequences of 27 curarimimetic neurotoxins isolated from the venoms of sea snakes, cobras, mambas, and a species of krait, along with the sequences of six cytotoxic or cardiotoxic and/or lytic proteins isolated from various cobra venoms. By now the list of known sequences certainly exceeds fifty. All of these molecules contain either 60–62 amino acids and four disulfide bridges (the short-chain neurotoxins and the cytotoxin group) or 71–74 amino acids and five disulfide bridges

(the long-chain neurotoxins). They are homologous with respect to amino acid sequence and seem to have a common disulfide pairing, suggesting grossly similar molecular structures. Crystallographic investigations now in progress should soon provide some accurate three-dimensional models. Owing to their strict specificity, the curarimimetic neurotoxins have become very important tools for the isolation and study of the nicotinic acetylcholine receptor.

Comparatively little is known about the structures of presynaptic neurotoxins, and discussions of possible target molecules remain on a purely hypothetical level. The classical examples of presynaptic neurotoxins are those produced by various serological types of the anaerobic bacterium *Clostridium botulinum*. The action of these toxins appears to be exclusively presynaptic, and is characterized by essentially irreversible inhibition of both spontaneous and evoked transmitter release from motor-nerve terminals (Burgen, 1972). However, the awesome toxicity of these substances has severely hindered proper biochemical fractionation work even with the modest mg quantities of material required for structural analysis with the present "nanomole" scale methodology.

Presynaptic neurotoxins exhibiting functional similarity to the *botulinum* toxins have been isolated from three different elapid venoms. The venom toxins are 5–6 orders of magnitude less potent than those from *C. botulinum* and can be studied in ordinary biochemical laboratories without governmental involvement and without significant risks to personnel.

β-Bungarotoxin from the venom of the banded krait, *Bungarus multicinctus* (Lee *et al.*, 1972), was described as containing 180 amino acids and 10 disulfide bridges, corresponding to a "formula weight" of about 20,000. The authors did not comment on the large discrepancy between the latter and their reported molecular weight of 28,500. The toxin has a mouse LD_{50} of 14 μg/kg and appears to be exclusively presynaptic in action (Chang *et al.*, 1973a), but differs from botulinum toxin in causing an initial increase in both the frequency and amplitude of miniature end plate potentials (m.e.p.ps) prior to the blockage of transmitter release. Furthermore, competition experiments suggests that β-bungarotoxin and botulinum toxin act at different, but apparently overlapping or interacting sites (Chang *et al.*, 1973b).

Notexin, isolated in this laboratory from the venom of the Australian tiger snake, *Notechis scutatus scutatus* (Karlsson *et al.*, 1972) is a basic protein consisting of 119 amino acids in a single peptide chain crosslinked by seven disulfide bridges. Notexin has a mouse lethal dose of 25 μg/kg and acts presynaptically, suppressing both spontaneous and evoked acetylcholine releases (Harris *et al.*, 1973). However, upon prolonged *in vivo* application the toxin has a direct dystrophic action on the muscle cell.

Taipoxin, isolated in this laboratory from the venom of the Australian taipan, *Oxyuranus scutellatus scutellatus* is a complex glycoprotein of uncertain

molecular weight. Taipoxin has a mouse LD_{50} of only 2 μg/kg and appears to be exclusively presynaptic in action (Kamenskaya and Thesleff, 1974), suppressing both spontaneous and evoked releases of acetylcholine. In contrast to β-bungarotoxin, neither notexin nor taipoxin causes any initial "bursts" of m.e.p.ps.

Presynaptic neurotoxicity is not confined to elapid venoms. Crotoxin, from the venom of the South American rattlesnake, *Crotalus durissus terrificus* is a protein complex with a molecular weight of about 30,000 (Gralén and Svedberg, 1938). Crotoxin has a mouse LD_{50} of 82 μg/kg and is predominantly presynaptic in action, but in contrast to the toxins mentioned above, its main effect is to stimulate rather than suppress spontaneous release of the transmitter. Following an initial reduction in m.e.p.ps frequency, crotoxin evokes large spontaneous potentials followed by explosive bursts of m.e.p.ps (Brazil, 1972).

Fractionation of the crotoxin complex has been accomplished in two different results. Habermann and Rübsamen (1971) and Rübsamen *et al.* (1971) resolved the complex into a very basic, weakly toxic phospholipase A and a nontoxic acidic protein called crotapotin, along with small amounts of undefined material. Recombination of crotapotin and the phospholipase restored the neurotoxicity of crotoxin, concommitant with a four- to five-fold inhibition of the phospholipase A activity. Hendon and Fraenkel-Conrat (1971), on the other hand, obtained an *acidic* A component showing the phospholipase A activity but lacking hemolytic activity, and a basic hemolytic B component with very little phospholipase activity! Neither A nor B showed neurotoxic activity, but a 1:2 mixture was nearly as toxic as crotoxin.

The venom of the Italian black widow spider, *Latrodectus mactans tridecimguttatus*, causes "avalanches" of m.e.p.ps, totally exhausting the supply of acetylcholine and depleting the nerve terminals of synaptic vesicles (Longenecker *et al.*, 1970; Clark *et al.*, 1972). The toxicity of the venom appears to be associated with several proteins of rather high molecular weight (Granata *et al.*, 1972) but no characterization data are yet available.

In the present paper we present the complete amino acid sequence of the tiger snake neurotoxin, notexin. Notexin is strikingly similar to a basic phospholipase A isolated in this laboratory from *Naja nigricollis* venom. The latter enzyme is neurotoxic, showing both the presynaptic and dystrophic effects observed with notexin, albeit about 50 times less potent (J. B. Harris, personal communication). Furthermore, notexin is highly homologous in sequence to both porcine pancreatic phospholipase A (de Haas *et al.*, 1970a) and a nontoxic, nonbasic phospholipase A from *Naja melanoleuca* venom (Jouberg, 1974).

Fractionation studies with the complex glycoprotein, taipoxin, indicate that the toxicity is associated with subunits of the same size and structural type as notexin. Amino-terminal sequence data indicate that these subunits are also homologous to phospholipases, suggesting that all of the presynaptic snake

venom toxins mentioned above have evolved from an ancestral "phospholipase" structure that has been highly conserved in the pancreatic and/or salivary secretions of vertebrates.

MATERIALS AND METHODS

Tiger snake (*N. s. scutatus*) and taipan (*O. s. scutellatus*) venoms were purchased in desiccated form from Eric Worrell's Australian Reptile Park, P.O. Box 192, Gosford, N.S.W. 2250, Australia. Lyophilized *N. nigricollis* venom was purchased from Miami Serpentarium Laboratories, Miami, Florida, U.S.A.

Isolation of the Neurotoxins. Notexin and the basic *N. nigricollis* phospholipase (*N. nigricollis* PLase) were isolated by gel filtration on Sephadex G-75 in 0.1 M ammonium acetate followed by cation-exchange chromatography of the appropriate fractions on columns of Bio-Rex 70 equilibrated with 0.2 M ammonium acetate at pH 6.5 and 7.3, respectively. Elution was done with a concave gradient of 0.09 vs 1.4 M ammonium acetate. Taipoxin was isolated by gel filtration on Sephadex G-75 followed by column-zone electrophoresis (Porath, 1956) in beds of water-pyridine extracted cellulose powder (Porath, 1964).

Assays of Phospholipase Activity. Assays of phospholipase activity were done by titration, according to Dole and Meinertz (1960), of fatty acids liberated from egg yolk emulsions (Wahlström, 1971).

Toxicity Assays. Toxicity assays were done by intravenous injection (caudal vein) in female white mice weighing 20–25 mg, using 3–6 mice at each dose level. The electrophysiological investigations of notexin and taipoxin were done as described elsewhere (Harris *et al.*, 1973; Kamenskaya and Thesleff, 1974).

Amino Acid Analysis. Amino acid analyses were done with a Durrum D-500 analyzer following hydrolysis for 24 or 72 hr with 6 N HCl containing 10 mg/ml reagent grade phenol at 110°C in thoroughly evacuated ampoules. Glucosamine was determined in the amino acid analyses of the 24-hr hydrolysates, using a recovery factor of 54% obtained with free N-acetylglucosamine "hydrolyzed" under the same conditions.

Carbohydrate Analysis. Carbohydrate analyses were done by gas chromatography following methanolysis and re-N-acetylation as described by Clamp *et al.* (1967).

Sequence Analysis of Notexin. The reduced and S-carboxymethylated and the performic acid oxidized derivatives of notexin were digested overnight at 37°C in 0.1 M ammonium bicarbonate buffer, pH 8, with a staphylococcal protease (SP) exhibiting a high specificity for glutamoyl peptide bonds (Rydén *et al.*, 1974). The enzyme is similar to that described earlier by Houmard and Drapeau (1972). Following group separation of the digests on Sephadex G-50

at pH 1.9, where the enzyme is not active, the peptides were purified by column-zone electrophoresis, gel filtration on Sephadex G-25, and high-voltage electrophoresis on paper as required.

Sequential degradations were done by the direct phenylisothiocyanate method (Edman, 1970), essentially as described by Iwanaga *et al.* (1969). The water-soluble (cysteic acid, His, and Arg) and ethyl acetate-soluble phenylthiohydantoins were identified by paper electrophoresis and thin-layer chromatography, respectively, as described earlier (Fryklund *et al.*, 1972).

Molecular Weights. The molecular weights of the reduced and S-carboxymethylated taipoxin subunits were estimated by gel filtration on appropriately calibrated columns of Sepharose 6B in 6 M guanidine hydrochloride according to the method of Fish *et al.* (1969) as described by Rydén (1971) and Karlsson *et al.* (1972).

RESULTS

Isolation of Notexin. Notexin can be isolated directly from the crude tiger snake venom in a single chromatographic operation, as previously described (Karlsson *et al.*, 1972). However, we now perform an initial group separation on Sephadex G-75 (Fig. 1a) to simplify isolation of the postsynaptic toxins of lower molecular weight. Fraction II in Fig. 1a accounts for 58% of the crude venom and 63% of the total venom protein. Gradient chromatography of fraction II on Bio-Rex 70 yields the pure notexin, which corresponds to peak 4 in Fig. 1b. In the particular run shown notexin accounted for 6.7% of the total venom protein. Gradient chromatography of the lower molecular weight fraction III (14% of the dried crude venom and 16% of the total venom protein) gave the pattern shown in Fig. 1c, where the numbered peaks, 1–5, seem to have postsynaptic toxicity. Peak 4 is definitely a curarimimetic neurotoxin containing 73 amino acids and 5 disulfide bridges. The amino-terminal sequence is Leu-Ile-Cys-Tyr-Met-Gly-Lys-, and the molecule terminates in -Arg-His-Pro-Pro at the carboxyl end. This toxin, which we tentatively refer to as Notechis III-4, seems to be a near relative of α-bungarotoxin.

Similarities between Notexin and a Basic Phospholipase from *Naja nigricollis* Venom. The basic *N. nigricollis* PLase was isolated by a two-step procedure identical to that described above for notexin, except that the gradient run was done at pH 7.3 rather than pH 6.5. This basic phospholipase is similar to the phospholipase AI isolated earlier in this laboratory (Wahlström, 1971) from *N. nigricollis* venom of different origin, and shows about the same toxicity in mice. The reduced and S-carboxymethylated derivative of the basic PLase is cleaved in better than 90% yield at both of its methionine residues upon treatment overnight in 70% formic acid with a 30-fold molar excess of cyanogen

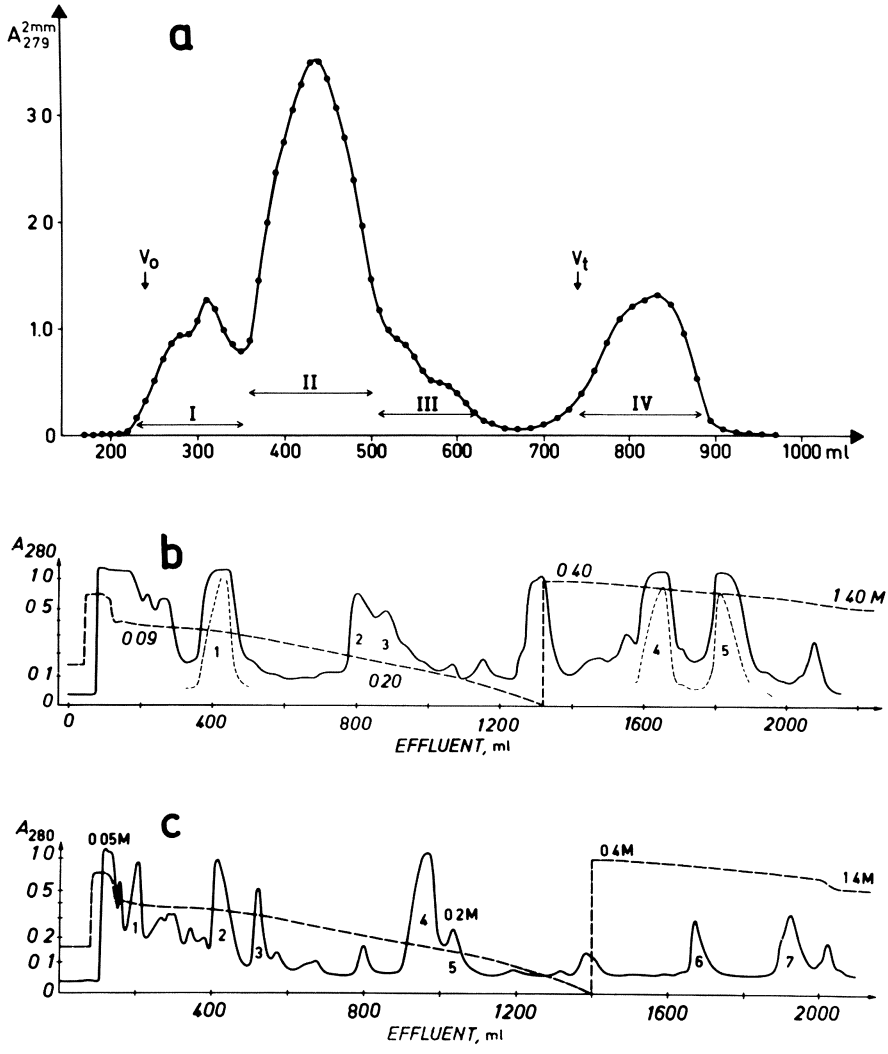

Fig. 1. Fractionation of tiger snake venom. (a) Gel filtration of 2.1-g crude venom on a column (3.2 × 92 cm) of Sephadex G-75 in 0.1 M ammonium acetate. (b) Gradient chromatography of gel filtration fraction II on a column (3.2 × 27 cm) of Bio-Rex 70 equilibrated with 0.2 M ammonium acetate at pH 6.5. The sample was applied in 0.05 M buffer and elution was done at 80 ml/hr with a 2-liter concave gradient of 0.09 M vs 1.4 M ammonium acetate. The numbers affixed to the conductivity trace indicate the approximate concentration of ammonium acetate in the effluent. The solid trace is the absorbance at 280 nm recorded from a flow cell of 10-mm optical path. The dashed curves in peaks 1, 4, and 5 were recorded from a tandem cell of 2.5-mm pathlength. (c) Gradient chromatography of gel filtration fraction III. (Column and conditions exactly as described for b.)

Table 1. Amino Acid Compositions of Notexin and the Basic *Naja nigricollis* Phospholipase

Amino acid	Notexin	*Naja nigricollis* PLase
Tryptophan	2	3
Lysine	11	10
Histidine	3	3
Arginine	5	5
Aspartic acid	18	16
Threonine	3	4
Serine	3	4
Glutamic acid	7	5
Proline	5	4
Glycine	10	12
Alanine	9	9
Half-cystine	14	14
Valine	4	5
Methionine	2	2
Isoleucine	4	3
Leucine	4	6
Tyrosine	10	9
Phenylalanine	5	4
Total	119	118
Formula weight	13,578	13,169
Mouse LD_{100}, μg/kg	25	500
Relative PLase activity (egg yolk)	0.05	1.0

bromide, yielding the fragments 1-8, 9-57, and 58-118. Composition data for these and various subfragments confirm the integral values given in Table 1 for the intact enzyme, which is very similar in amino acid composition to notexin. The basic *N. nigricollis* PLase is about one half as toxic as the basic phospholipase component of the crotoxin complex (Rübsamen *et al.*, 1971) and shows both the presynaptic and dystrophic activities of notexin (Harris *et al.*, 1973), but is about 30-fold less potent. Furthermore, as measured by the titration of fatty acids liberated from egg yolk emulsions, notexin has significant phospholipase activity, about 5% that of the basic *N. nigricollis* PLase.*

Sequence Analysis of Notexin. The amino acid sequence shown in Fig. 2 was established by familiar methods, the only novel feature of the work being the use of the staphylococcal protease (Rydén *et al.*, 1974) for the main fragmentation of the peptide chain. A detailed account of the sequence analysis will be reported elsewhere (Halpert and Eaker, 1975), and only a brief outline will be presented here.

*Note Added in Proof: In the *absence* of deoxycholate. In the *presence* of deoxycholate notexin is *more* active than the *nigricollis* enzyme toward ovolecithin.

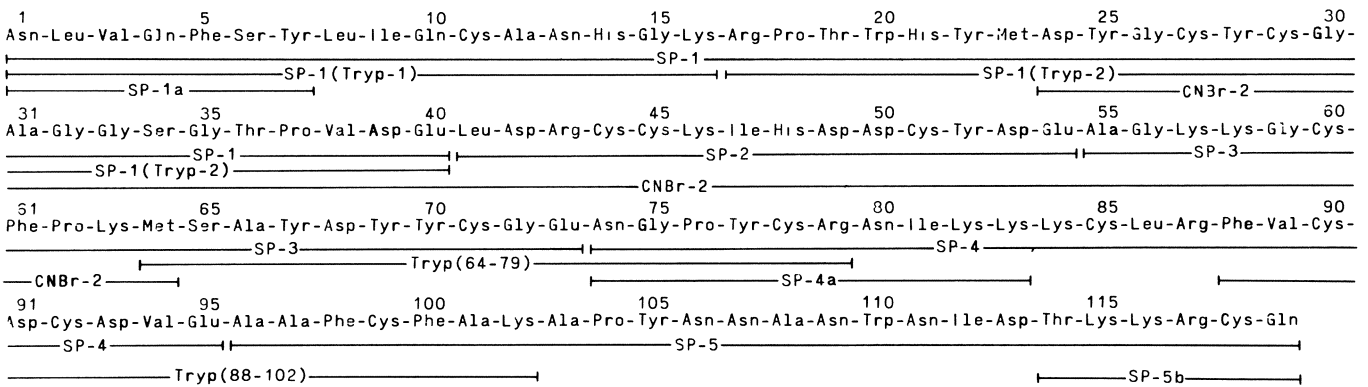

Fig. 2. Complete amino acid sequence of notexin indicating the tryptic peptides and a cyanogen bromide fragment used to align the five major peptides obtained by cleavage of the reduced and S-carboxymethylated or performic acid oxidized protein with a staphylococcal protease (SP) specific for glutamoyl peptide bonds. Departures from strict glutamoyl specificity were indicated by low-yield splits following Tyr-7, Lys-83, and Asp-113.

The amino-terminal sequence Asn-Leu-Val-Gln-Phe- was established by manual degradation as reported earlier (Karlsson *et al.*, 1972). A run done on the reduced and S-carboxymethylated derivative in an automatic Beckman Sequencer by Dr. Lowell H. Ericsson (University of Washington, Seattle) extended the sequence through Tyr-22, with ambiguities regarding only residues 17 and 21. Residue 23 appeared to be methionine. Digestion of the reduced and S-carboxymethylated notexin with carboxypeptidase A liberated one residue of glutamine from the carboxyl terminus.

Peptide SP-1, the largest of the five fragments obtained by cleavage at the four glutamoyl peptide bonds, was assigned to the amino terminus on the basis of composition, thereby representing residues 1–40. Cleavage of this peptide with trypsin yielded the peptide SP-1(Tryp-1) corresponding in composition to the known sequence 1–16, and the larger peptide SP-1(Tryp-2) evidently representing residues 17–40. Seven cycles of Edman degradation on the latter peptide established the sequence $\overset{17}{\text{Arg}}$-Pro-Thr-Trp-$\overset{21}{\text{His}}$-Tyr-$\overset{23}{\text{Met}}$. Nine cylces of degradation on the cyanogen bromide fragment CNBr-2, evidently representing residues 24–64, established the sequence $\overset{24}{\text{Asp}}$-Tyr-Gly-Cys-Tyr-Cys-$\overset{30}{\text{Gly}}$-Ala-Gly-. Digestion of the peptide SP-1(Tryp-2) with carboxypeptidase A in 0.2 M pyridine acetate at pH 5.0 liberated one residue each of Glu and Asp, indicating the terminal sequence -Asp-$\overset{40}{\text{Glu}}$. The remainder of the sequence of SP-1 was established by 11 cycles of degradation on the chymotryptic subfragment (not indicated in Fig. 2) corresponding to residues 29–40.

The sequence of residues 41–54 was established by 13 cycles of degradation on the peptide SP-2. The sequence of residues 55–64 was established by 10 cycles of degradation on the peptide SP-3. The remainder of the sequence of SP-3 was done by a 8-cycle degradation of the cyanogen bromide subfragment corresponding to residues 65–73.

Fifteen cycles of degradation of the peptide SP-4 established the sequence of residues 74–88, and nine cycles on the tryptic peptide Tryp(88–102) gave the sequence through Ala-96, which was amino terminal in the C-terminal peptide SP-5. Seventeen cycles of degradation on the latter gave the sequence through Ile-112. The remainder of the sequence was established by a 5-cycle degradation of the peptide SP-5b. The sequence $\overset{111}{\text{Asn}}$-Ile-Asp-$\overset{114}{\text{Thr}}$ was confirmed with a peptide arising from a chymotryptic split at Trp-110.

The sequence is supported by much additional redundant data. Every single residue in the molecule has been positively identified either as a phenylthiohydantoin or as a free amino acid obtained by carboxypeptidase digestion or by analysis (without hydrolysis) of the residue remaining after degradation of a peptide through its penultimate amino acid. Unless some particularly labile amide(s) has been lost in the course of the peptide fractionation, the assignment of the Gln and Asn vs Glu and Asp residues should therefore be correct. We

have weak suspicions only in the case of residue 24, which might be Asn, although it was clearly Asp in CNBr-2.

Homology between Notexin and Phospholipases. As shown in Fig. 3, there is a high degree of homology between notexin and porcine pancreatic phospholipase A (de Haas *et al.*, 1970a) throughout the first half and the last third of the sequences, while the molecules show identity in only seven positions in the 36-residue stretch between Lys-56 and Cys-93. Twelve of the 14 half-cystine residues in notexin align nicely with the twelve half-cystines of the pancreatic enzyme, the remaining two at positions 45 and 100 (homology numbering) being represented by deletions. If notexin has the same disulfide pairing as the phospholipase (de Haas *et al.*, 1970b) the seventh bridge would connect these two residues. Notexin shows still higher homology with the *N. melanoleuca* phospholipase, as illustrated in Fig. 4. But again, only 9 of 34 positions are identical in the stretch between Lys-57 and Cys-92 (homology numbering). However, this does not necessarily imply that the structural features responsible for the neurotoxicity of notexin reside in that region, since the two phospholipases are also very different in the same stretch.

Isolation and Properties of Taipoxin. Fraction II, obtained simply by gel filtration of crude taipan venom on Sephadex G-75 at neutral pH (Fig. 5), is the

```
 1                5                  10                  15
Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile-Lys-CYS-Ala-Ile-Pro-Gly-Ser-
Asn-   -Val-   -   -Ser-Tyr-Leu-   -Gln-   -   -Asn-His-   -Lys-

           20                  25                  30
His-Pro-Leu-Met-Asp-Phe-Asn-Asn-Tyr-Gly-CYS-Tyr-CYS-Gly-Leu-Gly-
Arg-   -Thr-Trp-His-Tyr-Met-Asp-   -   -   -   -   -Ala-   -

        35                  40                  45
Gly-Ser-Gly-Thr-Pro-Val-Asn-Glu-Leu-Asn-Arg-CYS-----Glu-His-Thr-
   -   -   -   -   -Asp-   -   -Asp-   -   -CYS-Lys-Ile-His-

     50                  55                  60
Asp-Asn-CYS-Tyr-Arg-Asp-Ala-Lys-Asn-Leu-Asn-Asp-Ser-CYS-Lys-Phe-
   -Asp-   -   -Asp-Glu-   -   -Ala-Gly-Lys-Lys-Gly-   ---------

 65                  70                  75                  80
Leu-Val-Asp-Asn-Pro-Tyr-Thr-Glu-Ser-Tyr-Ser-----Tyr-CYS-Ser-Ser-
-----------Phe-   -Lys-Met-Ser-Ala-   -Asp-Tyr-   -   -Gly-Glu-

                 85                  90                  95
Asn-Thr-Glu-Ile-Thr-CYS-Asn-Ser-Lys-Asn-Asn-Ala-CYS-Glu-Ala-Phe-
   -----Gly-Pro-Tyr-   -Arg-Asn-Ile-Lys-Lys-Lys-   -Leu-Arg-   -

          100                 105                 110
Ile-CYS-Asn-----Asp-Arg-Asn-Ala-Ala-Ile-CYS-Phe-Ser-Lys-Ala-Pro-
Val-   -Asp-CYS-   -Val-Glu-   -   -Phe-   -   -Ala-   -   -   -

       115                 120                 125
Tyr-Asn-Lys-Glu-His-Lys-Asn-Leu-Asn-Thr-Lys-Lys-Tyr-CYS----
   -   -Asn-Ala-Asn-Trp-   -Ile-Asp-   -   -   -Arg-   -Gln
```

Fig. 3. Alignment showing homology between notexin (lower sequence) and porcine pancreatic phospholipase A (upper sequence). Blanks (- -) in the notexin sequence signify identity with the phospholipase. Deletions (- - -) have been assigned to maximize homology. The sequences show identity in 54 of the 127 positions in the homology alignment. The His residue at position 47 in the pancreatic phospholipase (homology numbering) appears to be part of the catalytic site of the enzyme (Volwerk *et al.*, 1974).

```
 1                5                   10                  15
Asn-Leu-Val-Gln-Phe-Ser-Tyr-Leu-Ile-Gln-CYS-Ala-Asn-His-Gly-
   -   -Tyr-   -   -Lys-Asn-Met-   -His-   -Thr-Val-Pro-----
 16               20                  25                  30
Lys-Arg-Pro-Thr-Trp-His-Tyr-Met-Asp-Tyr-Gly-CYS-Tyr-CYS-Gly-
Asn-   -Ser-Trp-   -   -Phe-Ala-Asn-   -   -   -   -   -   -
 31               35                  40                  45
Ala-Gly-Gly-Ser-Gly-Thr-Pro-Val-Asp-Glu-Leu-Asp-Arg-CYS-CYS-
Arg-   -   -   -   -   -   -   -   -Asp-   -   -   -   -   -
 46               50                  55                  60
Lys-Ile-His-Asp-Asp-CYS-Tyr-Asp-Glu-Ala-Gly-Lys-Lys-----Gly-
Gln-   -   -   -Asn-   -   -Gly-   -   -Glu-   -Ile-Ser-   -
 61               65                  70                  75
CYS-Phe-Pro-Lys-Met-Ser-Ala-Tyr-Asp-Tyr-Tyr-----CYS-Gly-Glu-
   -Trp-   -Tyr-Ile-Lys-Thr-   -Thr-   -Asp-Ser-   -Gln-Gly-
 76               80                  85                  90
Asn-Gly-Pro-Tyr-CYS-Arg-Asn-Ile-Lys-Lys-Lys-CYS-Leu-Arg-Phe-
Thr-Leu-Thr-Ser-   -Gly-Ala-Ala-Asn-Asn-----   -Ala-Ala-Ser-
 91               95                  100                 105
Val-CYS-Asp-CYS-Asp-Val-Glu-Ala-Ala-Phe-CYS-Phe-Ala-Lys-Ala-
   -   -   -   -   -Arg-Val-   -   -Asn-   -   -   -Arg-   -
106              110                 115                 120
Pro-Tyr-Asn-Asn-Ala-Asn-Trp-Asn-Ile-Asp-Thr-Lys-Lys-Arg-CYS-Gln
   -   -Ile-Asp-Lys-   -Tyr-   -   -   -Phe-Asn-Ala-   -   -
```

Fig. 4. Alignment showing homology between notexin (upper sequence) and a phospholipase A from *Naja melanoleuca* venom. Blanks (- -) in the lower sequence signify identity with the upper one. Two deletions (- - -) have been asigned to each sequence to maximize homology. The sequences as written show identity in 63 of 121 positions.

most lethal material yet isolated from any snake venom. Following removal of a few percent of impurities by column-zone electrophoresis (Fig. 6) the material which we have named taipoxin migrates as a homogenous substance in polyacrylamide gel electrophoresis at pH 7-9 and shows a single sharp band at pH 5.25 in isoelectric focusing.

Below pH 4 the taipoxin dissociates and can be separated into two distinct

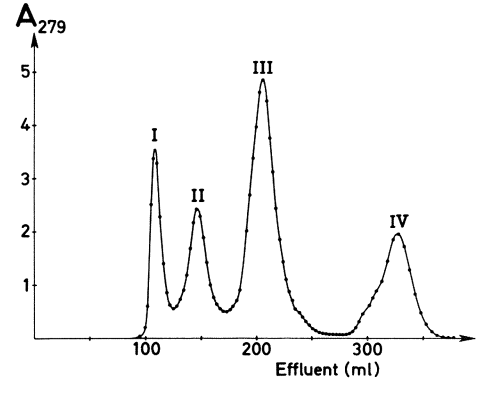

Fig. 5. Gel filtration of 207-mg crude taipan venom on a 2 X 94 cm column of Sephadex G-75 in 0.1 M ammonium acetate at neutral pH.

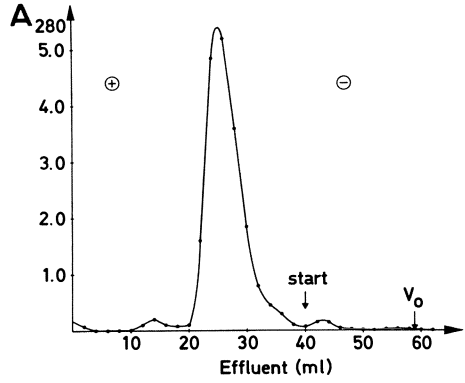

Fig. 6. Zone electrophoresis of 30-mg taipoxin (peak II in Fig. 5) on a 1 × 96 cm column of water-pyridine extracted cellulose powder in 0.05 M N-ethylmorpholine-acetic acid buffer, pH 7.5. The total interstitial volume (V_0) of the bed was 59 ml. The sample was applied in 2 ml 0.025 M buffer and was displaced downward 20 ml to the starting position indicated. The electrophoresis was run for 15 hr at 1000 V at 10–12°C. The current was 7.5 mA.

fractions by gel filtration in 1 M propionic acid, or simply 0.1 M acetic acid as shown in Fig. 7. The amino acid compositions of taipoxin and the α- and β-fractions thus obtained are compared in Table 2. The α- and β-fractions are similar with regard to the content of Lys, Thr, Gly, Met, Leu, and especially half-Cys, but are otherwise distinctly different. As determined by amino acid analysis, the α- and β- fractions account for 60% and 40% of the protein (amino acid) content of taipoxin, respectively. This ratio has been very reproducible among different runs with different batches of venom.

As determined by the gas-liquid chromatographic method (Clamp *et al.*, 1967) the α-fraction contains several kinds of carbohydrate residues, while the β-fraction contains none (Table 2). We have not yet done a total carbohydrate

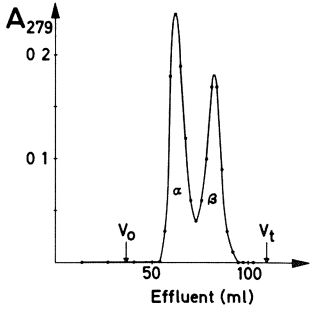

Fig. 7. Gel filtration of 3-mg electrophoretically purified taipoxin (Fig. 6) on a 1 × 144 cm column of Sephadex G-75 in 0.1 M acetic acid.

Table 2. Amino Acid Compositions of Taipoxin and Its α- and β-Fractions

Amino acid	Residues per 100 residues		
	Taipoxin	α(G-75)	β(G-75)
Tryptophan[a]	–	2.00[b]	2.44[b]
Lysine	4.59	4.73	4.89
Histidine	2.24	1.83	3.01
Arginine	5.66	5.08	6.51
Aspartic acid	13.49	14.70	12.14
Threonine	5.27	5.57	5.41
Serine	4.97	5.55	3.27
Glutamic acid	9.29	8.22	9.88
Proline	4.98	4.27	3.61
Glycine	7.73	7.99	7.72
Alanine	7.35	6.88	8.36
Half-cystine[c]	11.22	12.09	11.77
Valine	3.44	2.87	4.69
Methionine	2.05	2.05	1.79
Isoleucine	3.30	3.73	2.51
Leucine	4.67	4.46	4.75
Tyrosine	6.09	6.85	5.60
Phenylalanine	3.73	3.13	4.07
Glucosamine	1.70[b]	2.63[b]	0
Total	100.07	99.98	99.98
Fucose	–	0.55	0
Mannose	–	1.34	0
Galactose	–	2.12	0
N-Acetylglucosamine	–	2.37	0
N-Acetylneuraminic acid	–	2.30	0

[a] Estimated spectrophotometrically.
[b] Not included in summation.
[c] Determined as cysteic acid.

analysis of the intact taipoxin, but since the α-fraction contains 93% of the glucosamine present in taipoxin as determined in conjunction with amino acid analysis we assume that it contains all of the carbohydrate. If so, the taipoxin complex is 8%–9% carbohydrate.

Both the α- and β-fractions are toxic (Table 3) and have the same kind of activity as intact taipoxin (Kamenskaya and Thesleff, 1974), but are, on a weight basis, only 4% and 40% as potent, respectively. The β-fraction has about the same toxicity as notexin. Since the β-fraction accounts for 40% of the protein content of taipoxin, the β-fraction is at most 16% and probably not more than 10% as toxic as whole taipoxin on a molar basis. We have not yet observed any significant increase in toxicity upon remixing the α- and β-fractions.

Molecular Weight and Stoichiometry of the Taipoxin Complex. The partial specific volume ($\bar{v}$) calculated for the protein part of taipoxin from the amino acid composition data is 0.70. Assuming a value of 0.60 for the partial specific volume of the 9% of carbohydrate, the $\bar{v}$ of taipoxin was estimated to be about 0.69. Insertion of this value into the data equation obtained in a single ultracentrifuge experiment leads to a value of 57,000 for the molecular weight of the taipoxin complex. The α- and β-fractions have not been examined in the ultracentrifuge. The molecular weights estimated for the intact taipoxin and the α- and β-fractions by gel filtration on a calibrated column of Sephadex G-75 in 0.2 M ammonium acetate were 41,800, 30,200, and 11,400, respectively (Table 3). Although these latter values are internally consistent, at least one and probably all of them are too low. Gel filtration of the reduced and S-carboxymethylated β- fraction on a thoroughly calibrated column of Sepharose 6B in 6 M guanidine hydrochloride as described in the Methods section indicates a peptide chain length of 123 residues, which would correspond to a molecular weight of nearly 14,000. This latter value for the molecular weight of the β-chain(s) is strongly supported by other evidence given in the next section, and is 21% higher than the G-75 value of 11,400. Multiplication of the G-75 values for the α-fraction and taipoxin by the factor 1.21 gives molecular weights of 37,000 and 51,000, respectively, the latter being reasonably compatible with the ultracentrifuge determination. At any rate, the possibility that taipoxin might be a 1 : 1 complex of a "β-chain" with molecular weight of 14,000 and an "α-chain with a molecular weight of 30,000–40,000 is totally ruled out by the following observations. Since the β-fraction accounts for 40% of the protein, the protein part of the "α-component" of such an αβ complex would have a molecular weight of 21,000 (185 amino acid residues), but according to the results obtained in

Table 3. Material Balance, Toxicity, and Molecular Weight Data for Taipoxin and Its α- and β-Fractions

Parameter	Crude venom	Taipoxin	α(G-75)	β(G-75)
Crude venom, mg/g	1000	180	110	70
Percent of total amino acid content of taipoxin		100	60	40
Percent of total carbohydrate content of taipoxin		100	93[a]	0
Mouse LD_{50}, μg/kg		2		
Mouse LD_{100}, μg/kg	12	5	200	20
Molecular weight				
Centrifuge ($\bar{v}$ = 0.69)		57,000		
Sephadex G-75		41,800	30,200	11,400

[a]Based on glucosamine values obtained with amino acid analyzer.

gel filtration experiments with reduced and S-carboxymethylated material on Sepharose 6B in 6 M guanidine hydrochloride, the peptide chains of the α-fraction are no larger than the β-components.

Heterogeneity of the α- and β-Fractions. Although the β-fraction is very homogeneous with regard to peptide chain length, it shows two well-separated bands in polyacrylamide gel electrophoresis at alkaline pH, and we have recently accomplished preparative separation of the corresponding components by chromatography on SE-Sephadex at pH 5.5 in a concentration gradient of ammonium acetate. The amino acid compositions of these two components, which we have called β_1 and β_2, are compared in Table 4. The β_2 component might well be homogeneous with the integral amino acid composition shown. In the case of the β_1 component the values of 4.5 and 5.5 for serine and leucine were reproducible in three determinations and are definitely significant, possibly indicating a 1 : 1 mixture of two components differing by a Ser/Leu substitution. This "microheterogeneity" of the β-fraction is not caused by individual varia-

Table 4. Amino Acid Compositions of Taipoxin Components β_1 and β_2

Amino acid	β_1		β_2	
Tryptophan	–	3[a]	–	3[a]
Lysine	7.00	7	5.26	5
Histidine	2.85	3	3.72	4
Arginine	6.14	6	7.00	7
Aspartic acid	14.1	14	13.9	14
Threonine	5.97	6	7.00	7
Serine	4.52	4.5	2.89	3
Glutamic acid	13.9	14	12.6	13
Proline	4.93	5	4.19	4
Glycine	8.24	8	8.98	9
Alanine	10.1	10	9.98	10
Half-cystine[b]	13.7	14	14.1	14
Valine	6.25	6	5.96	6
Methionine	1.75	2	1.70	2
Isoleucine	3.02	3	2.98	3
Leucine	5.48	5.5	6.87	7
Tyrosine	6.25	6	5.64	6
Phenylalanine	5.03	5	4.89	5
Total		122		122
Residues in peptide chain (from Sepharose 6B in 6M GuCl)[c]		123		123
Formula weight	13,856		13,864	

[a] Estimated from spectrum.
[b] Determined as cysteic acid.
[c] Reduced and S-carboxymethylated derivatives.

```
                1                   5                  10                  15
porcine A       Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile-Lys-CYS-Ala-Ile-Pro-Gly-Ser-His-
N.mel. A        Asn-   -Tyr-   -   -Lys-Asn-Met-   -His-   -Thr-Val-Pro-----Asn-Arg-
basic N.nig. A  Asn-    Tyr-   -   -Lys-Asn-Met-   -His-   -Thr-Val-Pro---------Arg-
NOTEXIN         Asn-   -Val-   -   -Ser-Tyr-Leu-   -Gln-   -Ala-Asn-His-Gly-Lys-Arg-
TAIPOXIN β      Asn-   -Val-   -   -Gly-Phe-Met-   -Glx-   -Ala-_____________________
TAIPOXIN αp     Asn-   -Leu-   -   -Gly-Phe-Met-   -_____________________________
```

Fig. 8. Amino-terminal sequences suggesting homology between two subunits of taipoxin, three phospholipase A enzymes, and notexin.

tion, since the particular results shown were obtained with venom from a single snake.

The inhomogeneity of the α-fraction was first revealed in a simple experiment wherein the material was reduced and alkylated in 6 M guanidine hydrochloride followed by dialysis against distilled water. Material began to precipitate out after a few hours and the dialysis was continued until all of the precipitate had settled out, leaving a clear supernatant. Amino acid analysis indicates that the precipitate, which we tentatively call α_p, is very similar in size and amino acid composition to the β-components and contains one residue of glucosamine. The material recovered from the supernatant, which we call α_s, also has a high content of half-cystine and aspartic acid, but is very poor in arginine compared to α_p and the β-components and has a much lower content of aromatic amino acids. This α_s component(s) contains 4 residue of glucosamine per 100 residues of amino acids and apparently accounts for at least 80% of the carbohydrate in the whole α-fraction. We cannot give a reliable estimate of the molecular weight of the α_s material, because although it elutes slightly ahead of α_p and the β-components in the Sepharose-guanidine system we are not sure whether the peptide chain is longer or whether the carbohydrate contributes to the size of the random coil.

Amino-Terminal Sequences of Taipoxin Fractions. Edman degradation of the reduced and S-carboxymethylated β-fraction (containing both β_1 and β_2) gave the amino-terminal sequence indicated in Fig. 8. The α_p material shows Leu instead of Val in position 3, but is otherwise identical to β through residue 9, where the degradation was discontinued. The α_s material showed *many* different end groups and the degradation was therefore discontinued after two cycles.

DISCUSSION

The presence in tiger snake venom of postsynaptic as well as presynaptic toxins was mentioned in our initial publication on notexin (Karlsson *et al.*, 1972) and was confirmed by the electrophysiological investigation of Datyner and Gage (1973). The postsynaptic toxin Notechis III-4 (peak 4 in Fig. 1c) is

similar in sequence to α-bungarotoxin. The small shoulder on the trailing edge of peak III in the gel filtration pattern of taipan venom (Fig. 5) also seems to show postsynaptic neurotoxicity.

The presynaptic neurotoxin, notexin, is clearly homologous in structure to two phospholipases of very different origin. Size, composition, and preliminary amino-terminal sequence data (Fig. 8) strongly suggest that most of the subunits of the taipoxin complex also have the same type of structure, there still being considerable uncertainty regarding the nature of the α_s component(s) bearing most of the carbohydrate. The total molecular weight and stoichiometry of taipoxin remain to be established, along with the nature of the interaction between the components designated α_s and α_p. Since these have so far been separated only after reduction and S-carboxymethylation, we cannot at present rule out the possibility of a disulfide link between them. On the basis of our experiences with taipoxin, we suspect that β-bungarotoxin (Lee *et al.*, 1972) also contains more than one peptide chain and that one of the subunits should be of the "phospholipase" type.

In addition to the structural similarity with phospholipases, notexin, taipoxin, and the α- and β-fractions of taipoxin all actually show weak phospholipase activity. This raises the possibility that the neurotoxic action of these substances might involve hydrolysis of some special phospholipid structure in the nerve terminal membrane. The recent discovery by Volwerk *et al.* (1974) of the specific reaction of *p*-bromophenacyl bromide with the active site histidine of pancreatic phospholipase should provide the means to settle the above question. The catalytic histidine of the phospholipase corresponds to histidine residue 48 in the notexin sequence. If the latter residue reacts with the reagent and the neurotoxic activity is lost the toxic action might be catalytic in nature.* Reaction of the histidine without loss of toxicity would eliminate the latter possibility and would provide an excellent means to introduce a radioactive label.

We doubt the catalysis hypothesis, because it almost seems that the high presynaptic toxicity arises at the expense of the phospholipase activity. Pieterson *et al.* (1974) have recently proposed that in addition to the catalytic site, which appears to be functional even in the proenzyme, the active phospholipase contains a special "recognition site" which allows the molecule to interact with organized lipid–water interfaces. We suggest that this lipid recognition site, along with some modification conferring very high specificity toward some unique feature of the nerve-terminal membrane, is responsible for the blocking action of the presynaptic toxins of the "phospholipase" type.

*See Halpert, J., Eaker, D., and Karlsson, E. (1976). The role of phospholipase activity in the action of a presynaptic neurotoxin from the venom of *Notechis scutatus scutatus* (Australian tiger snake). *FEBS Letters* **61**: 72–76.

SUMMARY

The presynaptic neurotoxin, notexin, from the venom of the Australian tiger snake *N. s. scutatus* is homologous in sequence to porcine pancreatic and snake venom phospholipases. The presynaptic neurotoxin, taipoxin, from the venom of the Australian taipan *O. s. scutellatus* is a complex glycoprotein with a molecular weight of 40,000–60,000. The complex contains subunits of about the same size and structural nature as notexin, along with a different sort of subunit bearing most of the carbohydrate.

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Isolation and Amino Acid Sequence of Proteinase Inhibitors from the Venoms of *Hemachatus haemachatus* and *Naja nivea*

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INTRODUCTION

Some snake venoms contain polypeptide inhibitors which inactivate mammalian serine-proteinases, including kallikreins, trypsin, plasmin, and α-chymotrypsin (Takahashi *et al.*, 1972). Such proteinase inhibitors are distributed mainly in the venoms of members of *Viperidae* and *Elapidae* (Takahashi *et al.*, 1974a). One of the inhibitors, inhibitor II, isolated from *Vipera russelli* venom is a polypeptidic substance, consisting of 60 amino acid residues. Its established primary strucutre is very similar to that of the pancreatic basic trypsin inhibitor (Kunitz type) (Takahashi *et al.*, 1974b,c). This report describes the isolation and determination of the amino acid sequence of proteinase inhibitors from the venoms of *Hemachatus haemachatus* (Ringhals cobra) and *Naja nivea* (Cape cobra).

MATERIALS AND METHODS

The venom of *H. haemachatus* (Ringhals cobra, lot no. 33286) was obtained from Calbiochem. Co., San Diego. *N. nivea* (Cape cobra) venom was a generous gift of Dr. E. J. J. Joubert, National Chemical Research Laboratory, Pretoria,

South Africa. Bovine trypsin (three-times recrystallized), bovine α-chymotrypsin (three-times recrystallized) and DFP-treated carboxypeptidases A (68 U/mg) and B (98 U/mg) were purchased from Worthington Biochemical Corp., Freehold. α-Bungarotoxin was a preparation made by Dr. D. Mebs, Zentrum der Rechtsmedizin, Frankfurt. α-*N*-toluenesulfonyl-L-arginine methylester (TAME) and α-*N*-acetyltyrosine ethylester (ATEE) were the products of the Protein Research Foundation, Minoh, Osaka. Sephadex G-75, G-50 (medium), G-25 (superfine), and SE-Sephadex C-25 (2.3 meq/g) were purchased from Pharmacia, Uppsala, Sweden.

Disk Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Davis (1964). For determination of molecular weight, sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was made by the method of Weber and Osborn (1969). Whale sperm myoglobin, bovine chymotrypsinogen A, ovalbumin, and horse heart cytochrome c from Schwartz-Mann, New York, were used as marker proteins. The gels were stained with Coomassie brilliant blue R250.

Assay of Proteinase Inhibitor. For determination of inhibitory activity, a mixture of enzyme and inhibitor in 0.9 ml of 0.4 M Tris-HCl buffer, pH 8.5, was preincubated for 5 min, and then 0.1 ml of 0.1 M substrate (TAME or ATEE) was added. After 10 min, the amount of substrate hydrolyzed was determined by the hydroxamate method (Roberts, 1958). One inhibitory unit is defined as the amount causing reduction of substrate hydrolysis by 1 μmol/min.

Amino Acid Composition. Samples, each containing 0.01 to 0.05 μmol, were hydrolyzed in evacuated, sealed tubes with 0.5 ml of twice-distilled, 5.8 N constant-boiling HCl for 24, 48, and 72 hr at 110°C. The acid was removed by evaporation under reduced pressure (0.01 mmHg) in a desiccator containing KOH pellets. All analyses were performed in a JEOL amino acid analyzer, model JLC-5AH, by the method of Spackman *et al.* (1958). For peptide fragments, only 24-hr hydrolysis was performed.

Enzymatic Digestion. An S-carboxymethyl (S-CM) derivative of each proteinase inhibitor was prepared by the method of Crestfield *et al.* (1963) and digested at 37°C for 6 hr in 0.2 M ammonium bicarbonate buffer, pH 8.5, with a weight ratio of enzyme to substrate of 1:50. All digestions were terminated by adding 10% acetic acid and the materials were freeze dried.

Fractionation and Purification of Peptides. Many peptides were fractionated first by gel filtration on a Sephadex G-25 column (1.5 × 120 cm) with 10% acetic acid as eluant. Further purification of impure peptides was accomplished by paper chromatography in 1-butanol-pyridine-acetic acid-water (15:10:3:12, by volume) or by high-voltage paper electrophoresis at pH 6.4 (pyridine-acetic acid-water, 100:4:900, vol./vol.) and at pH 3.4 (pyridine-acetic acid-water, 1:10:289, vol./vol.). Peptides were detected with a buffered ninhydrin reagent

(Takahashi *et al.* 1974c) and eluted with a mixture of equal volumes of 30% acetic acid and 10% pyridine.

Amino Acid Sequence. The N-terminal sequences of most peptides and the intact inhibitors were determined by Edman's phenylthiohydantoin (PTH) procedure (Edman, 1970). PTH-derivatives were determined semiquantitatively by measuring the absorbance at 269 nm before chromatography. The other details of Edman's sequence analysis were previously described (Takahashi *et al.* 1974c). With samples of less than 0.01 μmol, the Edman-dansyl technique (Gray, 1972) with a slight modification (Kimura, 1974) was used and dansyl amino acids were identified by two-dimensional thin-layer chromatography on polyamide sheets (purchased from Cheng and Chin Trading Co.) with the solvents of Woods and Wang (1967) and Kimura (1974). The determination of the C-terminal residue was performed by a modification (Braun and Schroeder, 1967) of the method of Akabori *et al.* (1957). Amide groups in peptides were detected either by direct identification of PTH-derivatives after Edman degradation, or by estimation of the net charge of the peptides during electrophoresis at pH 5.4.

Abbreviations and Symbols. The following abbreviations were used for various peptides: T, tryptic and C, chymotryptic peptides. Horizontal arrows pointing to the right or left below the amino acid residues denote that the sequences were determined by direct Edman degradation or the Edman-dansyl method and hydrazinolysis, respectively.

RESULTS

Isolation of Proteinase Inhibitors from *Hemachatus haemachatus* Venom. Lyophilized venom (300 mg) was dissolved in 5 ml of 0.1 M ammonium bicarbonate buffer, pH 7.9, and applied to a Sephadex G-75 column (3.0 × 142 cm), equilibrated with the same buffer. Elution was made with the equilibration buffer and 8-ml fractions were collected at 4°C at a flowrate of 45 ml/hr. The elution profile is shown in Fig. 1. Four major peaks with absorption at 280 nm were separated, and one of the peaks strongly inhibited the trypsin activity. The fractions indicated by the solid bar were collected and lyophilized. For further purification, the dried material was dissolved in 2 ml of 0.04 M ammonium formate buffer, pH 4.7, and subjected to ion-exchange chromatography on SE-Sephadex C-25. A column (2.5 × 25 cm) was equilibrated with 0.04 M ammonium formate buffer, pH 4.7, and eluted with a linear gradient obtained by placing 1 liter each of the equilibration buffer in the mixing vessel and 0.5 M ammonium acetate buffer, pH 9.5, in the reservoir. Then, the column was eluted with a gradient, formed with 1 liter each of 0.5 and 1.0 M buffer, pH 9.5, to elute all the adsorbed materials. Fractions of 10 ml were collected at a flowrate of 60

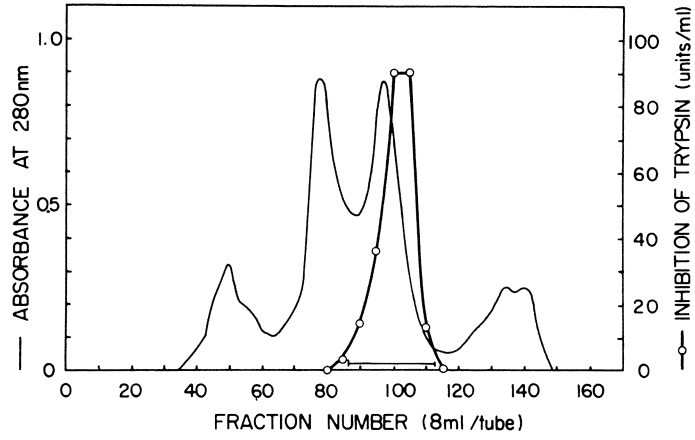

Fig. 1. Gel filtration of *Hemachatus haemachatus* venom on a Sephadex G-75 column. The experimental conditions were described in the text.

ml/hr at 4°C. As seen in the chromatogram in Fig. 2, two peaks with the trypsin inhibitory activity were separated. Fraction numbers 54-65 (I) and 96-111 (II) were collected, freeze dried and sublimated *in vaccuo* (0.01 mmHg) at 40°C. The overall yields of the components, designated as inhibitors I and II, isolated from 300 mg of lyophilized venom were about 1.5 and 5 mg, respectively.

Isolation of Proteinase Inhibitors from *Naja nivea* Venom. Procedures similar to those mentioned in the previous section were used to isolate the proteinase inhibitor from *N. nivea* venom. Figure 3 shows the gel filtration pattern of the venom (2.43 g) on a Sephadex G-75 column. The fractions containing the trypsin inhibitory activity were collected and lyophilized. The dried material (964 mg) was dissolved in 10 ml of 0.04 M ammonium acetate buffer, pH 4.7 and subjected to ion-exchange chromatography on an SE-Sephadex C-25 column. The elution pattern is shown in Fig. 4. Two major and a few minor peaks with the trypsin inhibitory activity were separated. Fraction numbers 145-149 (Ia), 150-170 (Ib), and 240-270 (II) were collected, lyophilized, and sublimated at 40°C. Among these materials, inhibitors Ia and Ib appeared to be pure on gel electrophoresis, while inhibitor II contained some additional components. Inhibitor II was purified further by rechromatography on an SE-Sephadex C-25 column, as shown in Fig. 5. The fractions containing inhibitor II (indicated by the solid bar in Fig. 5) were collected, lyophilized, and sublimated. The over-all yields of these three components isolated from 2.43 g of the lyophilized venom were about 20 mg (Ia), 9 mg (Ib), and 25 mg (II), respectively.

Purities of the Isolated Proteinase Inhibitors. Figure 6a and b show the electrophoretic patterns of the purified inhibitors in disk polyacrylamide gel with or without SDS. Under an acidic condition, these inhibitors each gave a

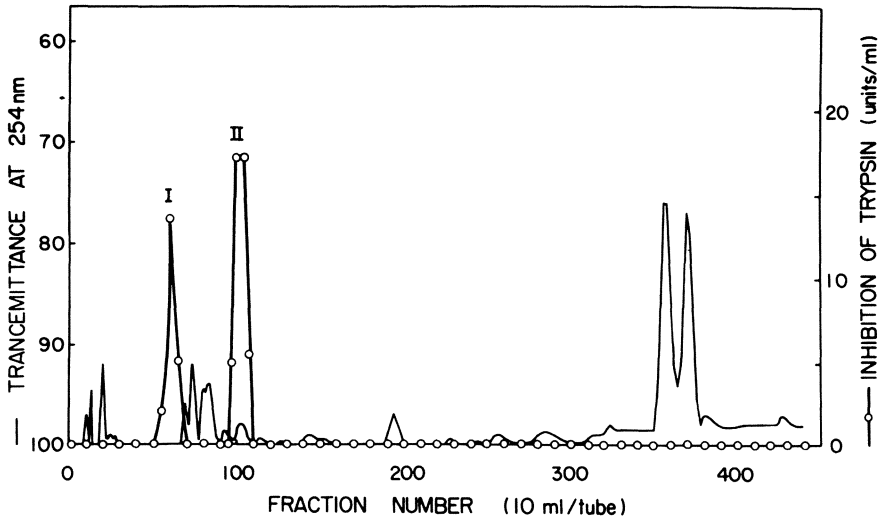

Fig. 2. Separation of fractions containing the proteinase inhibitors on an SE-Sephadex C-25 column. The experimental conditions were described in the text.

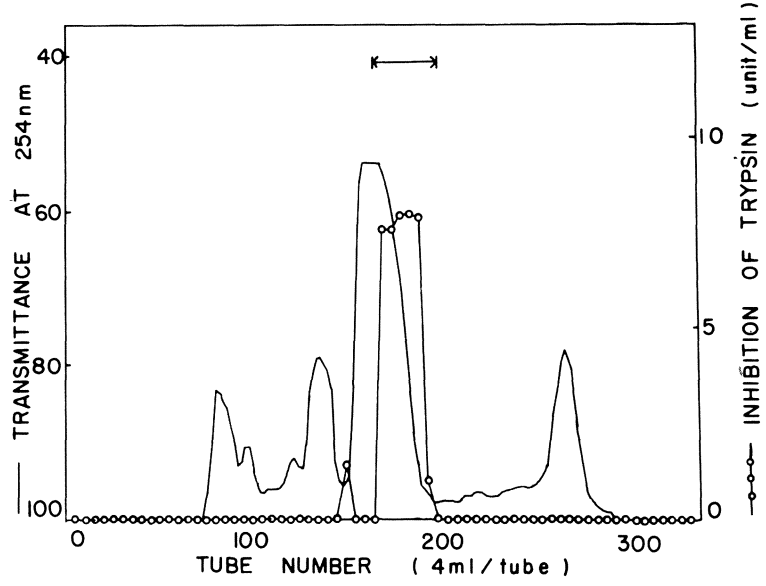

Fig. 3. Gel filtration of *Naja nivea* venom on a Sephadex G-75 column. The lyophilized venom (2.43 g) was applied to a column of Sephadex G-75 (5 X 138 cm) previously equilibrated with 0.1 M ammonium bicarbonate buffer, pH 7.9. Elution was made by the same buffer and 14-ml fractions were collected at a flowrate of 42 ml/hr. The fractions indicated by the solid bar were combined and lyophilized.

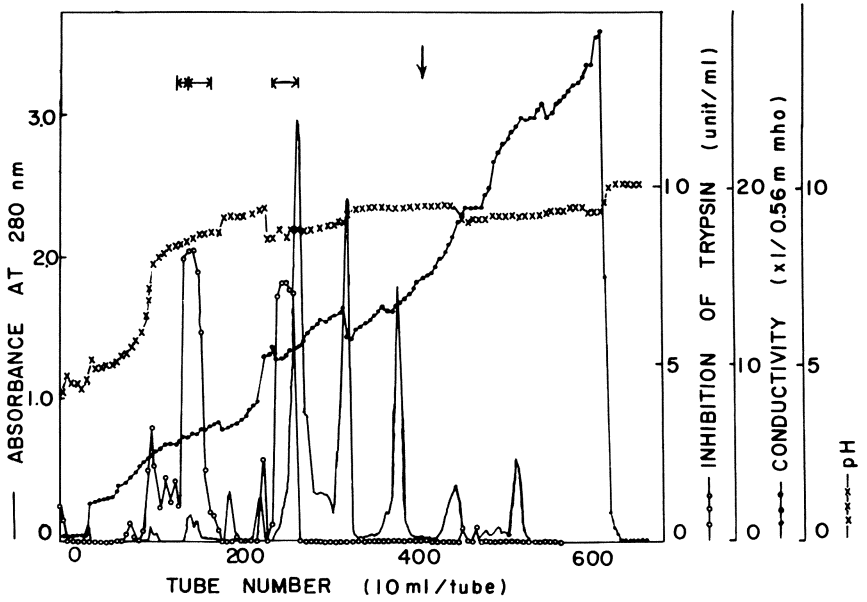

Fig. 4. Separation of fractions containing the proteinase inhibitors on an SE-Sephadex C-25 column. The lyophilized material (964 mg) obtained by gel filtration (Fig. 3) was applied to a column of SE-Sephadex C-25 (2.5 × 57 cm), equilibrated with 0.04 M ammonium acetate buffer, pH 4.7. Then, linear-gradient elution was started with 800 ml each of the equilibration buffer in the mixing vessel and 0.2 M ammonium acetate buffer, pH 9.5, in the reservoir. The buffer in the reservoir was replaced by 0.5 M, pH 9.5, as indicated with an arrow. Fractions of 10 ml were collected at a flowrate of 30 ml/hr. The fractions indicated by the solid bars were combined, lyophilized, and sublimated at 40°C.

single homogeneous band. On SDS-gel electrophoresis at pH 7.2, each also gave a single band stained with Coomassie brilliant blue; their mobilities in the gels were almost the same, suggesting similar molecular weights. From these results, all of the inhibitors isolated here were judged to be essentially pure.

Molecular Weight of Proteinase Inhibitor II from *Hemachatus haemachatus* Venom. A series of marker proteins were run under the same conditions; semilogarithmic plotting of mobility against molecular weight gave a straight line (Fig. 7). The molecular weight of inhibitor II of *H. haemachatus* venom, determined from the plot, was estimated to be 6900. This value was in good agreement with the minimum molecular weight of inhibitor II calculated from its amino acid composition. Since other proteinase inhibitors isolated from *N. nivea* venom had similar mobilities to that of *H. haemachatus* inhibitor II in SDS gel under the same conditions, their molecular weights also seemed to be in a range of about 7000 ± 1000.

Amino Acid Compositions of Venom Proteinase Inhibitors. Table 1 shows the amino acid compositions of six proteinase inhibitors isolated from *Viperidae* and *Elapidae* venoms. Each inhibitor consisted of 51–60 amino acid residues and the compositions were different from one inhibitor to another. For example, no methionine residue was detected in hydrolysates of Russell's viper inhibitor I or II (Takahashi *et al.*, 1974), *H. haemachatus* inhibitor II or *N. nivea* inhibitor Ia, whereas both inhibitors Ib and II from *N. nivea* venom contained methionine. No carbohydrate was found in any of the inhibitors in SDS gel by the method of Zacharius *et al.* (1969). These analytical data on the amino acids in the inhibitors were in good agreement with the integral molar ratio. This may be taken as strong evidence for the homogeneities of the polypeptides. Moreover, the minimum molecular weight calculated from the compositions without taking into account the partial amide contents of dicarboxylic acids ranged from 6300 to 6800 (Table 1).

Inhibition Spectra of Proteinase Inhibitors on Various Proteinases. Table 2 shows the inhibitory effects of one of the purified inhibitors from Russell's viper

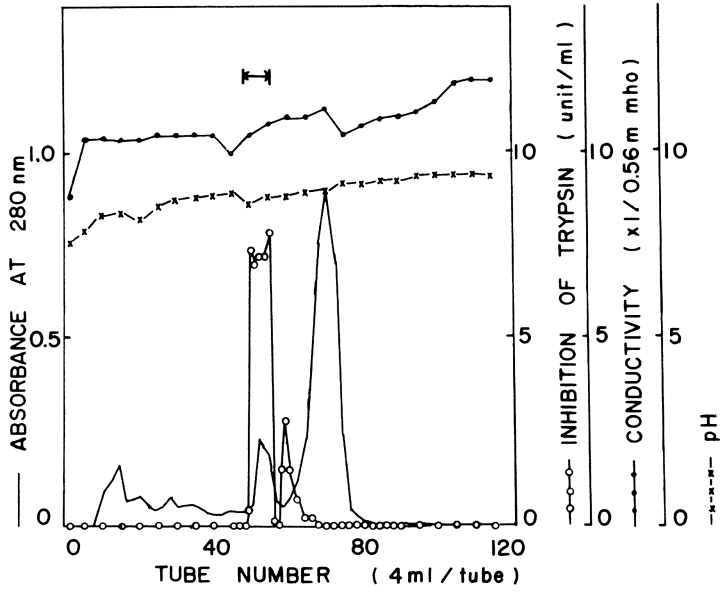

Fig. 5. Further purification of the fractions containing proteinase inhibitor II on an SE-Sephadex C-25 column. The material (146 mg) obtained from the pooled fractions (tubes 240–270 in Fig. 4) was rechromatographed on an SE-Sephadex C-25 column (2.0 × 36 cm) under the same conditions as described in Fig. 4. The fractions indicated by the solid bar were combined and lyophilized.

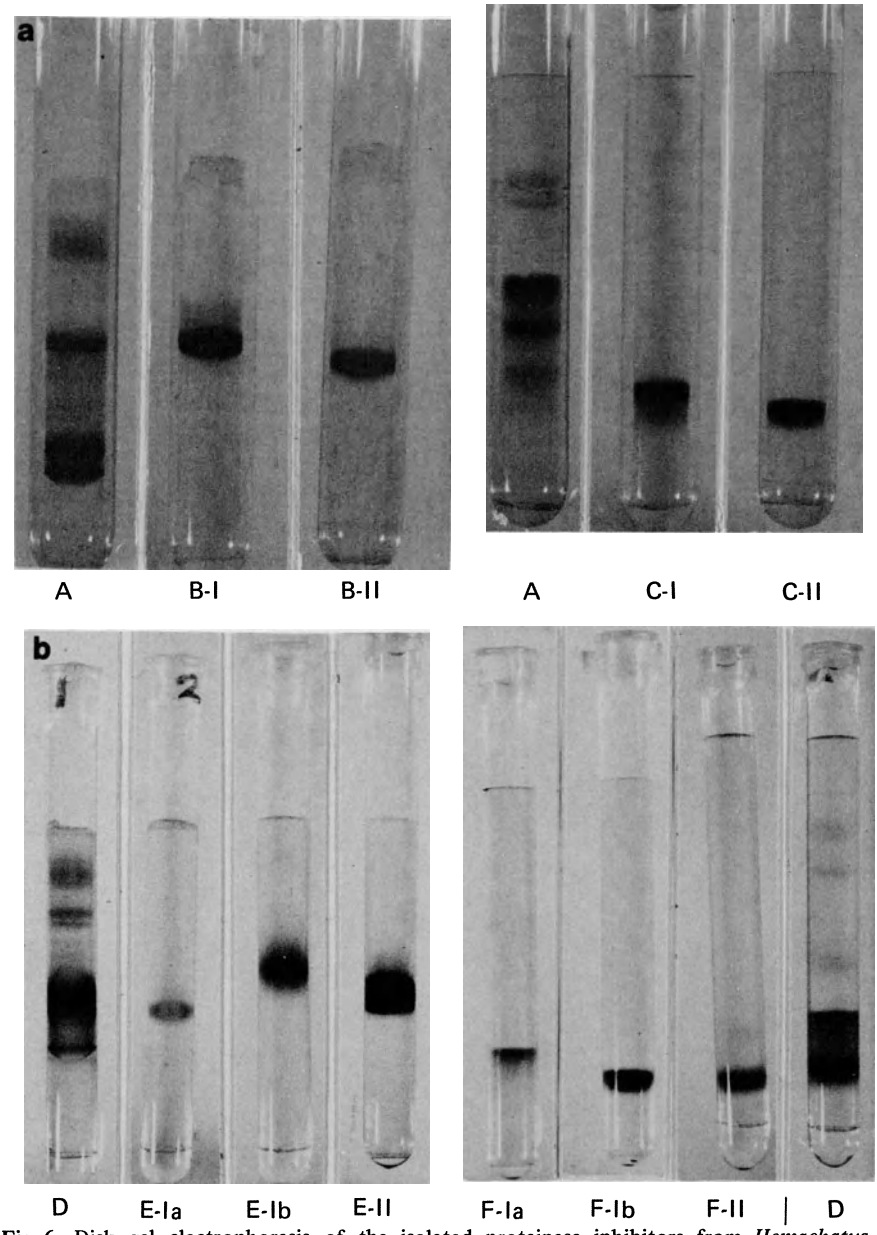

Fig. 6. Disk gel electrophoresis of the isolated proteinase inhibitors from *Hemachatus haemachatus* venom (a) and from *Naja nivea* venom (b) in the absence and presence of SDS, respectively. Electrophoresis was done in 7.5% polyacrylamide gel for 1.2–4 hr at 6 mA/tube. (A) *Hemachatus haemachatus* venom (HHV); (B) HHV inhibitors I and II at pH 4.5; (C) HHV inhibitors I and II in SDS gel at pH 7.2; (D) *Naja nivea* venom (NNV); (E) NNV inhibitors Ia, Ib, and II at pH 4.5; (F) NNV inhibitors Ia, Ib, and II in SDS gel at pH 7.2.

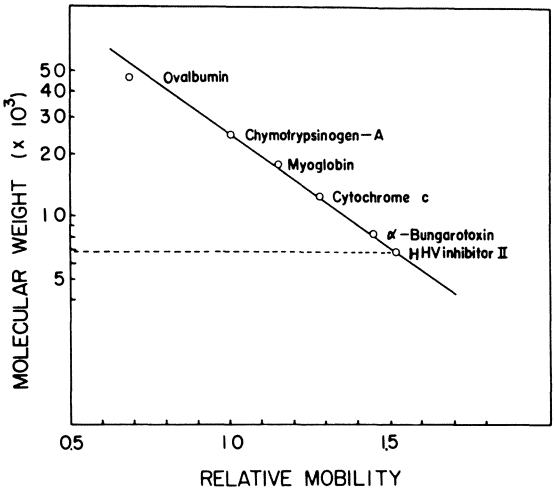

Fig. 7. A plot of the logarithms of the molecular weights of HHV inhibitor II and marker proteins against their relative mobilities in 7.5% gel containing 0.1% SDS. Electrophoresis was performed at 6 mA/tube.

venom on the activities of so-called serine proteinases, thiol proteinases (bromelain, papain and ficin), metal proteinases (thermolysin and snake venom proteinases), and exopeptidases (Takahashi *et al.* 1974b). Each of these enzymes and inhibitor II in different amounts were mixed. The mixtures were preincubated and then the changes in enzyme activities were measured on appropriate substrates. Russell's viper inhibitor II inactivated all of bovine pancreatic serine proteinases including trypsin, α-chymotrypsin, and kallikrein. It also inhibited human and bovine plasmin and plasma kallikrein. However, no inhibitory effect was observed on bovine or human thrombin, snake venom bradykinin-releasing enzyme or reptilase, all of which are known as so-called serine proteinases such as trypsin, nor on metal proteinases, thiol proteinases, or carboxypeptidase A or B. These inhibition spectra appeared to be very similar to those of pancreatic basic trypsin inhibitor (Kunitz type).

Amino Acid Sequence Studies on Proteinase Inhibitor II from *Hemachatus haemachatus* Venom. To elucidate the complete amino acid sequence of inhibitor II from *H. haemachatus* venom, the S-CM derivative (0.64 μmol) was digested by TPCK-treated trypsin under the conditions described in Materials and Methods. The resulting peptides were dissolved in 1 ml of 10% acetic acid, but some insoluble material appeared. This was separated by centrifugation and washed with 10% acetic acid. The washed material, named T-ppt, gave a single spot on paper electrophoresis at pH 3.4. The supernatant containing soluble tryptic peptides was separated by gel filtration on a Sephadex G-25 (superfine, 2.5 X 150 cm) column. The peptides thus obtained were purified further by

Table I. Amino Acid Compositions[a] of Proteinase Inhibitors Isolated from the Venoms of Russell's Viper, *Hemachatus haemachatus*, and *Naja nivea*

Amino acid	Residues/mole					
	Russell's viper, II	Russell's viper, I	*Hemachatus haemachatus*, II	*Naja nivea*, II	*Naja nivea*, Ia	*Naja nivea*, Ib
Lys	3	2	2	3	2	4
His	2	2	1	2	2	1
Ammonia	(12)	(11)	(7)	(6)	(7)	(6)
Arg	7	5	5	7	5	4
Asp	8	7	5	5	4	5
Thr	3	2	3	3	3	3
Ser	2	2	1	1	1	2
Glu	5	5	6	6	6	4
Pro	2	5	2	2	2	3
Gly	8	7	6	6	5	3
Ala	2	3	5	5	4	4
½ Cys	6	4	6	6	4	6
Val	1	1	1	2	1	3
Met	0	0	0	1	0	1
Ile	1	2	3	3	3	3
Leu	3	1	4	3	3	3
Tyr	3	1	3	2	2	1
Phe	4	3	4	3	3	3
Trp	0	0	0	–	–	–
Total	60	52	57	60	50	53

[a]The compositions were calculated by extrapolation or from the average values estimated on samples after 24-, 48-, and 72-hr hydrolysates.

high-voltage paper electrophoresis at pH 3.4 or pH 6.4. Through these procedures, seven major tryptic peptides were obtained.

The amino acid compositions and yields of the tryptic peptides are given in Table 3. The total composition of whole inhibitor II could be explained by the sum of the total residues in these peptides.

The sequences of these tryptic peptides were determined by the direct Edman degradation and dansyl-Edman methods, and the results are summarized in Table 4. T-2 contained the sane N-terminal sequence of Arg-Pro-Asp-Phe- as that of whole inhibitor II, and T-5-1 was the only peptide containing C-terminal glycine, which was identical to that of the C-terminal amino acid of the whole inhibitor. Thus, the former must be located in the N-terminal and the latter in the C-terminal portions of the whole inhibitor. The largest fragment, T-ppt,

consisted of 25 amino acid residues and the N-terminal sequence of the first 14 residues was established as shown in Table 4.

To obtain a second set of peptides to align the five tryptic peptides, T-5-2, T-ppt, T-5-3, T-4-1, and free arginine (T-5-4), chymotryptic digestion of the whole inhibitor was performed. The resulting fragments were separated by combination of gel filtration on a Sephadex G-25 column (superfine, 1.9 X 114 cm) with 10% acetic acid as eluant, and high-voltage paper electrophoresis at pH 3.4 and pH 6.4. Nine peptides were isolated and the total composition accounted for that of the whole inhibitor (Table 5). The extent of the amino acid sequence determinations of the chymotryptic peptides is shown in Table 6. Of the nine peptides, C-4-5, C-3-10, C-3-7, and C-2-2 apparently constituted the overlaps of the tryptic peptides, T-2, T-5-2, T-ppt, T-5-3, T-4-1, and T-5-4. These results provided all of the alignments of the tryptic peptides, as summarized in Fig. 8.

Table 2. Inhibitory Effects of Russell's Viper Venom Inhibitor II on Various Proteinases

Enzyme	Inhibitor II	Basic pancreatic trypsin inhibitor
Bovine plasma kallikrein	+	+
Bovine trypsin	+	+
Bovine α-chymotrypsin	+	+
Bovine plasmin	+	
Human plasmin	+	+
Hog pancreatic kallikrein	+	
Human thrombin[a]	–	–
Bovine thrombin[a]	–	
Snake venom bradykinin releasing enzyme[b]	–	
Reptilase[a]	–	
Bromelain[c]	–	
Papain[c]	–	–
Ficin[c]	–	
Nagase[c]	–	
Thermolysin[c]	–	
Carboxypeptidase A[d]	–	–
Carboxypeptidase B[e]	–	–

[a] Determined with bovine fibrinogen as substrate.
[b] Determined with TAME as substrate.
[c] Determined with denatured casein as substrate.
[d] Determined with Cbz-glycyl-phenylalanine as substrate.
[e] Determined with hippuryl-arginine as substrate.

Table 3. Amino Acid Compositions of Tryptic Fragments of *Hemachatus haemachatus* Venom Inhibitor II

Amino acid	Residues/mole						
	T-2	T-5-2	T-ppt	T-5-3	T-4-1	T-5-4	T-5-1
Lys	0.9(1)	–	–	1.0(1)	–	–	–
His	–	–	0.8(1)	–	–	–	–
Ammonia	3.1	–	6.1	–	–	–	–
Arg	0.9(1)	1.1(1)	1.0(1)	–	1.0(1)	1	–
Asp	1.4(1)	–	2.8(3)	–	1.0(1)	–	0.9(1)
Thr	1.1(1)	–	–	–	0.9(1)	–	–
Ser	–	–	1.0(1)	–	–	–	–
Glu	1.8(2)	–	3.0(3)	–	1.0(1)	–	–
Pro	1.7(2)	–	–	–	–	–	–
Gly	1.4(1)	–	3.7(4)	–	–	–	1.0(1)
Ala	1.2(1)	1.3(1)	3.0(3)	–	–	–	–
½ Cys	1.8(2)	–	1.7(2)	–	0.8(1)	–	0.8(1)
Val	–	–	–	–	–	–	1.2(1)
Met	–	–	–	–	–	–	–
Ile	–	1.0(1)	1.0(1)	–	1.0(1)	–	–
Leu	1.5(2)	–	2.1(2)	–	–	–	–
Tyr	–	0.4(1)	1.5(2)	–	–	–	–
Phe	0.9(1)	–	2.0(2)	0.7(1)	–	–	–
Total	15	4	25	2	6	1	4
Yield, (%)	28	26	43	25	13	–	18
Purification[a]	S-E	S-E	ppt	S-E	S-E	S-E	S-E

[a]Purification procedures were: electrophoresis at pH 3.4 (E); gel filtration on Sephadex G-25 (superfine) (S).

DISCUSSION

As previously reported (Takahashi *et al.* 1974a), the venoms of several snakes of the *Elapidae* family show inhibitory activities on mammalian serine proteinases. From the venoms of *H. haemachatus* and *N. nivea*, several materials inhibiting trypsin have actually been isolated by gel filtration on Sephadex G-75 and ion exchange chromatography on SE-Sephadex C-25. These inhibitors seem to have similar molecular weights of about 6500. However, the amino acid compositions of the isolated inhibitors are slightly different, as exemplified by the differences in the contents of half-cystine, valine, arginine, methionine and lysine.

The amino acid sequence of *H. haemachatus* venom inhibitor II established

Table 4. Amino Acid Sequences of Tryptic Fragments of *Hemachatus haemachatus* Venom Inhibitor II

Peptide	Positions	Amino acid sequence[a] (partial or complete)
Whole inhibitor II		H-Arg-Pro-Asp-Phe-Cys-Glu-Leu-Pro-Ala-Glu-Thr-Gly-Leu-Cys-Lys-Ala-Tyr-Ile- GlyOH
T-2	1–15	H-Arg-Pro-Asp-Phe-(Cys, Glu, Leu, Pro, Ala, Glu, Thr, Gly, Leu, Cys)-Lys
T-5-2	16–19	Ala-Tyr-Ile-Arg
T-ppt	20–44	Ser-Phe-His-Tyr-Asn-Leu-Ala-Ala-Gln-Gln-Cys-Leu-Gln-Phe-(Gly_4, Asx_2, Tyr, Ala, Cys, Ile)-Arg
T-5-3	45–46	Phe-Lys
T-4-1	47–52	Thr-Ile-Asp-Glu-Cys-Arg
T-5-4	53	Arg
T-5-1	54–57	Thr-Cys-Val-GlyOH

[a]The amino acids identified by direct Edman degradation are indicated by arrows.

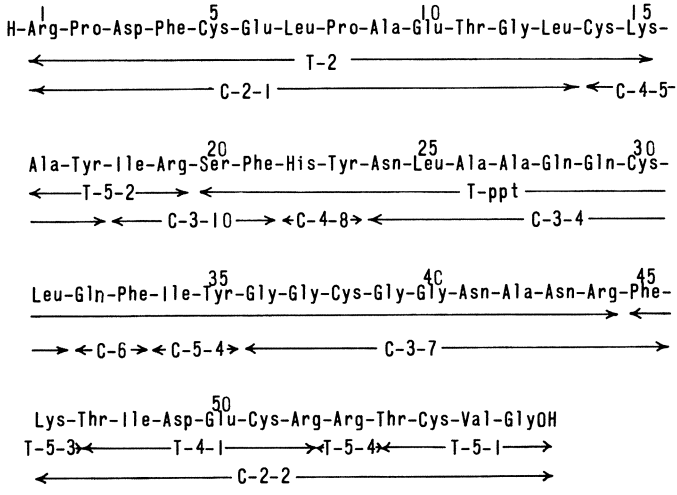

Fig. 8. Amino acid sequence of proteinase inhibitor II isolated from the venom of *Hemachatus haemachatus*. The peptides obtained from digests with trypsin (T) and α-chymotrypsin (C) are shown by arrows.

Table 5. Amino Acid Compositions of Chymotryptic Fragments of *Hemachatus haemachatus* Venom Inhibitor II

Amino acid	Residues/mole								
	C-2-1	C-2-2	C-3-4	C-3-7	C-3-10	C-4-5	C-4-8	C-5-4	C-6
Lys	–	1.1(1)	–	–	–	0.8(1)	–	–	–
His	–	–	–	–	–	–	0.9(1)	–	–
Ammonia	–	–	–	–	–	–	–	–	–
Arg	1.1(1)	2.1(2)	–	0.7(1)	0.9(1)	–	–	–	–
Asp	1.3(1)	1.1(1)	1.0(1)	2.0(2)	–	–	–	–	–
Thr	1.1(1)	1.9(2)	–	–	–	–	–	–	–
Ser	–	–	–	–	1.1(1)	–	–	–	1.1(1)
Glu	2.3(2)	1.1(1)	2.2(2)	–	–	–	–	–	–
Pro	1.8(2)	–	–	–	–	–	–	–	–
Gly	0.7(1)	1.2(1)	–	3.8(4)	–	–	–	–	–
Ala	1.3(1)	–	2.3(2)	1.6(1)	–	1.1(1)	–	–	–
½ Cys	0.9(1)	1.5(2)	0.7(1)	0.5(1)	–	0.6(1)	–	–	–
Val	–	1.3(1)	–	–	–	–	–	–	–
Ile	–	1.0(1)	–	–	0.9(1)	–	–	1.0(1)	–
Leu	1.7(2)	–	1.8(2)	–	–	–	–	–	–
Tyr	–	–	–	–	–	0.8(1)	1.0(1)	0.8(1)	–
Phe	1.1(1)	–	–	0.8(1)	1.0(1)	–	–	–	1.0(1)
Total	13	12	8	10	4	4	2	2	2
Yield, (%)	29	20	10	9	16	12	14	8	16
Purification[a]	S-E	S-E	S-E	S-E	S-E	S-E	S-E	S-E	S

[a]Purification procedures were: electrophoresis at pH 3.4 (E); gel filtration on Sephadex G-25 (superfine) (S).

here is of surprisingly high homology not only to Russell's viper inhibitor II but also to the pancreatic basic trypsin inhibitor (Kassel and Laskowski, 1965) and snail isoinhibitor K (Dietl and Tschesche, 1974). The comparison of the sequences of these inhibitors is given in Fig. 9 and Table 7. The homology amounts to nearly 60% of the structure, though snakes, snails, and cattle are of fairly different phylogenetic descent. The six half-cystine residues of these inhibitors are in the same positions in the amino acid sequences of these polypeptides. Moreover, the homologies are especially well preserved in the region of the reactive site (indicated with a star in Fig. 9) and in the contact area directed against the serine proteinase (Huber *et al.*, 1971). Thus, the tertiary structure of snake venom proteinase inhibitors may be very similar to that of the Kunitz-type inhibitor.

Although the whole amino acid sequence of inhibitor II isolated from *N. nivea* venom is under investigation, the sequence data obtained thus far

Table 6. Amino Acid Sequences of Chymotryptic Fragments of *Hemachatus haemachatus* Venom Inhibitor II

Peptide	Positions	Amino acid sequence[a] (partial or complete)
C-2-1	1–13	H–Arg–Pro–Asp–Phe–Cys–Glu–Leu–Pro–Ala–Glu–Thr–Gly–Leu
C-2-2	46–57	Lys–Thr–Ile–Asp–Glu–Cys–Arg–Arg–Thr–Cys–Val–GlyOH
C-3-4	24–31	Asn–Leu–Als–Ale–Gln–Glx–Cys–Leu
C-3-7	36–45	Gly–Gly–Cys–Gly–Gly–Asx–Ala–Asx–Arg–Phe
C-3-10	18–21	Ile–Arg–Ser–Phe
C-4-5	14–17	Cys–Lys–Ala–Tyr
C-4-8	22–23	His–Tyr
C-5-4	34–35	Ile–Tyr
C-6	32–33	(Glx, Phe)

[a]The amino acids identified by direct Edman or dansyl-Edman method are indicated by arrows.

Table 7. Overall Homologies[a] of Proteinase Inhibitors from Snake Venoms, Snails, and Cattle

Proteinase inhibitor	Percent identity (or chemical similarity)						
	PBT I	CT I	RVV II	HHV II	Toxin I	Toxin K	Snail IK
Pancreatic basic trypsin inhibitor	100	46	52	60	44	58	59
Colostrum trypsin inhibitor	–	100	60	54	46	51	60
Russell's viper inhibitor II	–	–	100	65	44	48	55
Hemachatus haemachatus inhibitor II	–	–	–	100	51	67	66
Dendroaspis polylepis toxin I[b]	–	–	–	–	100	65	45
Dendroaspis polylepis toxin K[b]	–	–	–	–	–	100	49

[a]Chemical similarities are defined as Arg = Lys, Asp = Asn, Glu = Gln, Asp = Glu, Asn = Gln, Ser = Thr, Val = Ile, Ile = Leu, Tyr = Phe = Trp, in addition to identities.
[b]Data from D. J. Strydom (1973), *Nature New Biol.* 243: 88.

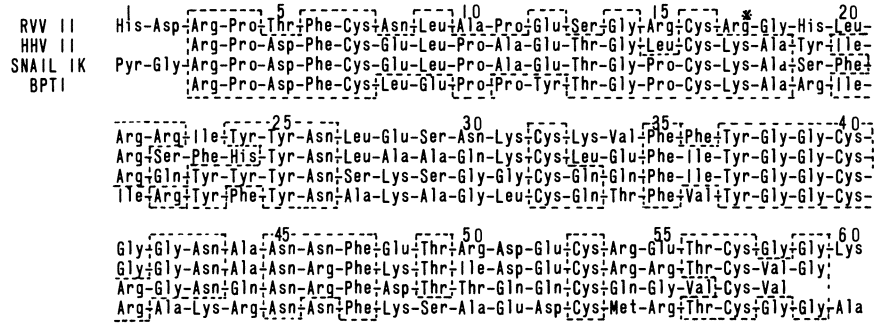

Fig. 9. Sequence homology among trypsin-kallikrein inhibitors from the venoms of Russell's viper (RVV) and *Hemachatus haemachatus* (HHV), and from snails and bovine pancreas (BPTI).

indicate that this inhibitor II has almost identical structure to that of *H. haemachatus* inhibitor II mentioned here.

SUMMARY

Several proteinase inhibitors inhibiting the activity of trypsin were isolated from crude venoms of *H. haemachatus* and *N. nivea*. All of the inhibitors were electrophoretically pure and had molecular weights of about 6500. They each contained 50-60 amino acid residues.

The complete amino acid sequence of inhibitor II isolated from *H. haemachatus* venom (HHV) was established by Edman degradation and the standard enzymatic techniques. HHV inhibitor II consisted of 57 amino acid residues with arginine and glycine at the NH_2- and COOH-termini, respectively. It had a molecular weight of 6300 and contained six half-cystines in disulfide linkages. The overall primary structure of HHV inhibitor II was very similar to not only that of Russell's viper inhibitor II, previously established, but also those of pancreatic basic trypsin inhibitor (Kunitz type) and snail isoinhibitor K. There was about 60% homology in their amino acid sequences. Especially, the regions structurally and functionally important for the Kunitz-type inhibitor molecule were found to be of extremely high sequence homology to HHV inhibitor II. These findings suggest a very slow rate of molecular evolution of the proteinase inhibitor.

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Complete Amino Acid Sequence of Snake Venom Phospholipase A_2

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INTRODUCTION

Phospholipase A_2 (phosphatide acyl-hydrolase, EC 3.1.1.4) catalyzes hydrolysis of fatty acyl ester at the β position of phospholipid. This enzyme is widely distributed in the venoms of snakes, bees, and scorpions as well as in animal tissues. Recently, a number of forms of phospholipase A_2 have been highly purified from various species of *Elapidae* (Braganca *et al.*, 1969; Salach *et al.*, 1971; Shiloah *et al.*, 1973a,b; Wahlström, 1971; Sakhibov *et al.*, 1970; Lo and Chang, 1971), *Hydrophiidae* (Tu *et al.*, 1970), *Viperidae* (Salach *et al.*, 1971; Shiloah *et al.*, 1970), and *Crotalidae* (Wells and Hanahan, 1969; Wu and Tinker, 1969; Augustin and Elliott, 1970). We have also isolated two forms of phospholipase A_2 found in the venom of *Agkistrodon halys blomhoffii,* Japanese trivial name "Mamushi" (Iwanaga and Kawauchi, 1958, 1959; Kawauchi *et al.*, 1971a). Acidic phospholipase A_2, designated as A_2-II, consists of a single polypeptide chain with a molecular weight of about 13,700 (Kawauchi *et al.*, 1971a). It contains N-terminal pyroglutamic acid, C-terminal cystine, and a total of 126 amino acid residues (Kawauchi *et al.*, 1971b). Following these works, we reported the gross structure of A_2-II, in addition to the complete or partial amino acid sequences of CNBr-peptide fragments and tryptic peptides (Samejima *et al.*, 1970). In this paper, we present the complete amino acid sequence of phospho-

lipase A_2-II of *A. halys blomhoffii* venom. The sequence is complete except for assignment of a few amide groups.

MATERIALS AND METHODS

Phospholipase A_2-II was isolated from the venom of *A. halys blomhoffii* by the method previously reported by Kawauchi *et al.* (1971a) with a modification in the chromatography step. Trypsin (TPCK-treated) and α-chymotrypsin (3-times recrystallized) were the products of Sigma Chemical Co., St. Louis. Thermolysin was a generous gift from Dr. K. Morihara, Shionogi Research Laboratory, Osaka. Carboxypeptidases A and B (both di*iso*propylphosphorofluoridate treated) were from Worthington Biochemical Corp., Freehold.

The cyanogen bromide cleavage of whole protein and reduction-alkylation were performed as previously reported by Samejima *et al.* (1970). Tryptic, thermolytic, and chymotryptic digests of the alkylated CNBr-peptide fragment, Br-I-CM, were prepared at an enzyme to protein ratio of 1 : 50 in 0.2 N ammonium bicarbonate, pH 8.5. The digests were fractionated by ion-exchange chromatography on Dowex 50-X2 and gel filtration on Sephadex G-25 (superfine). In many cases, the peptides were further purified by paper chromatography or high-voltage paper electrophoresis at pH 3.5 and 6.5. The peptides were examined for purity by thin-layer chromatography and electrophoresis on a cellulose plate (Merck Co., Darmstadt) according to the method of Kosakowski and Böck (1970).

Amino acid compositions were determined, after acid hydrolysis in 5.7 N HCl (110°C, 24 hr, in evacuated sealed tubes), on a JEOL amino acid analyzer, model JLC-5AH, according to the method of Spackman *et al.*, (1958). N-terminal sequences of the isolated peptides were determined by Edman's phenyl*iso*thiocyanate procedure (Edman, 1970) and the subtractive method of Shearer *et al.* (1967). C-terminal residues and sequences were investigated in some cases by digestion with carboxypeptidases A and B or by hydrazinolysis (Akabori *et al.*, 1957). Amide groups in the peptides were detected by either direct Edman degradation or by estimation of the net charge of the peptides during electrophoresis at pH 6.5.

RESULTS AND DISCUSSION

We isolated two forms of phospholipase A_2 found in Japanese Mamushi venom. The isolated enzymes, A_2-I and A_2-II, each gave a single protein band in disc electrophoresis in polyacrylamide gel. The enzyme, A_2-I, had a molecular weight of 13,800 and was strongly basic as indicated by its isoelectric focusing point of pH 10.0. The enzyme, A_2-II, had the same molecular weight as enzyme

A_2-I. However, it was relatively acidic and its isoelectric point pH 4.0. Thus, the protein molecules of the two enzymes differed significantly in charge distribution.

The amino acid compositions of snake venom phospholipases A_2-I and A_2-II are shown in Table 1. The two isolated enzymes of Mamushi venom gave total residues of 126. However, clear differences between these enzymes were found in the number of glutamic acid and lysine residues. The differences seem to account for the difference in the isoelectric points. A common characteristic in all snake venom enzymes is that the half-cystine contents are extremely high, constituting more than 10% of the total amino acid residues. Tyrosine and glycine contents also appear to be relatively high, and overall proportions of amino acid residues in all species are rather similar. However, their molecular sizes differ greatly, the molecular weights of the enzymes from *Naja naja* and *Crotalus adamanteus* venoms being approximately twice those of the enzymes from other species. The similarities in their amino acid compositions strongly suggest that the enzymes of *N. naja* and *C. adamanteus* venoms represent dimeric forms, while those from other species are monomeric. To study the structure-function relationship of the phospholipase A_2 molecule, the whole amino acid sequence of acidic phospholipase A_2-II was determined.

In the native protein, no N-terminal amino acid reactive to phenyl*iso*thiocyanate was detected by Edman degradation. The C-terminal end was resistant to both carboxypeptidases A and B. However, qualitative analysis by the tritium labeling procedure revealed a single spot corresponding to that of cystine on paper chromatograms with two solvent systems. On hydrazinolysis after performic acid oxidation, the C-terminal cysteic acid was also identified.

The first cleavage of this protein with cyanogen bromide followed by Sephadex G-50 chromatography gave rise to three fragments, Br-III-A (heptapeptide), Br-III-C (tripeptide), and Br-I (Fig. 1).

The amino acid compositions of CNBr-peptide fragments are shown in Table 2. The large fragment, Br-I, was composed of a total of 113 amino acid residues, and all of the half-cystine residues of the starting material were recovered in this fragment, but no homoserine could be detected. The fragments, Br-III-A and Br-III-C, on the other hand, each contained one mole of homoserine, indicating that the two methionine residues found in the whole protein were recovered in these fragments. A sum of the total amino acid residues in the three fragments was approximately the same as the total of the whole protein.

The complete or partial amino acid sequences of CNBr-peptide fragments are shown in Table 3. No N-terminal end of Br-III-A was detectable. Therefore, Br-III-A was further degraded with α-chymotrypsin and by acid hydrolysis; seven peptides were obtained after separation by paper chromatography and paper electrophoresis. By Edman degradation and carboxypeptidase A digestion, the structure of Br-III-A was determined as shown in this table. The N-terminal

Table 1. Amino Acid Compositions[a] of Forms of Phospholipase A_2 Isolated from Snake Venoms, Bee Venoms, and Porcine Pancreas

Amino acid	*Agkistrodon halys blomhoffii*		*Naja naja*		*Crotalus adamanteus*		*Crotalus atrox*	*Laticauda semifasciata*	*Crotalus durissus terrificus*	Porcine pancreas	Bee venom
	A_2-I	A_2-II	Peak 1	Peak 2	Aα	Aβ					
Asp	14	17	42	40	30	30	34	11	9.0	23	23
Thr	6	5	8	8	13	13	16	6	6.0	7	16
Ser	5	5	10	9	13	13	16	7	6.0	10	14
Glu	6	12	15	15	24	24	28	9	8.2	7	9
Pro	5	5	8	6	16	16	18	5	4.6	6	7
Gly	10	13	19	17	24	24	30	10	10.4	6	15
Ala	5	8	23	22	15	15	18	8	5.8	8	6
½ Cys	14	16	19	22	30	30	28	12	10.6	14	12
Val	4	4	8	8	11	11	8	4	2.0	2	8
Met	3	2	0	0	2	2	4	1	1.6	2	4
Ile	7	7	8	8	11	11	14	3	4.4	5	6
Leu	5	3	10	10	11	11	14	5	5.6	7	13
Tyr	10	9	14	14	16	16	14	10	9.2	8	11
Phe	5	5	8	8	10	10	8	3	5.6	5	7
Lys	17	8	10	10	16	16	14	7	9.0	9	17
His	2	1	2	2	5	5	4	2	1.8	3	9
Arg	6	4	10	12	12	12	10	4	8.0	4	8
Trp	2	2	7	6	7	7	2	1	2.0	2	3
NH_3	5	8	32	24	16	16	–	–	–	–	–
Total	126	126	221	217	266	266	260	108	110	130	188

[a]Values are expressed as the assumed number of residues per mole of protein.

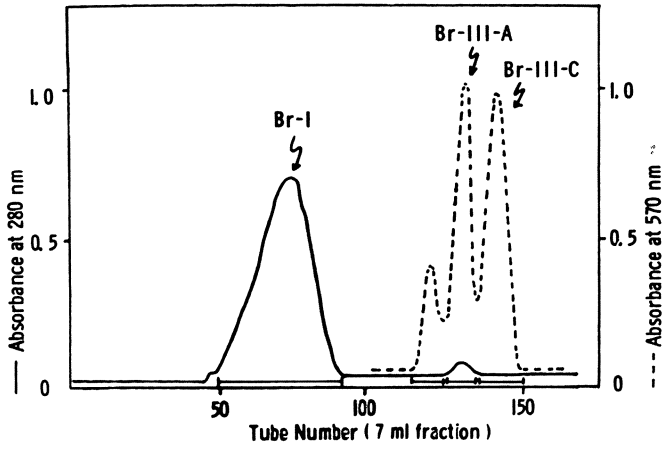

Fig. 1. Elution pattern of CNBr-peptides of snake venom phospholipase A_2-II from a Sephadex G-50 column. The protein (7.2 μmol) was dissolved in 4.5 ml of 70% formic acid and treated with 130 mg of CNBr (dissolved in 0.5 ml of 70% formic acid) for 24 hr at room temperature. After removal of excess reagents with a rotary evaporator and by freeze drying, the CNBr-degradation product was dissolved in 5 ml of 10% acetic acid and applied to a column of Sephadex G-50 (3.0 × 140 cm), equilibrated with the same solvent. The column was eluted with the same solvent at a flow rate of 50 ml/hr and 7-ml fractions were collected. The large fragment, Br-I, was detected by absorbance at 280 nm, and the other fragments, Br-III-A and Br-III-C, with ninhydrin reagent after alkaline hydrolysis.

sequence of Br-III-A was also confirmed with a synthetic peptide, Pyr-Phe-Glu, and the N-terminal pyroglutamic acid was established by mass spectrometry. The sequence of Br-III-C was established by Edman degradation and mass spectrometry. As for the large fragment, Br-I, direct Edman degradation provided the N-terminal sequences as shown in Table 3.

The native protein had no N-terminal amino acid reactive to phenyl*iso*thiocyanate nor had Br-I any homoserine residues. Thus, the total alignment of CNBr-peptide fragments must be as follows: Br-III-A→Br-III-C→Br-I.

The large fragment, Br-I, was reduced and carboxymethylated. The product was subsequently digested with TPCK-trypsin and the digest was fractionated on a column of Dowex 50-X2. Nine major peaks were obtained, and some of them were further purified by gel filtration on a Sephadex G-25 column, paper chromatography, and paper electrophoresis. Through these procedures, eight major and five minor peptides and free lysine were obtained. The sum of the amino acid residues of these tryptic peptides agreed well with the whole amino acid composition of the original fragment, Br-I. The sequences of these tryptic peptides are shown in Table 4. In these peptides, T-8-1 seemed to be connected to Br-III-C through T-7-1, which was free lysine, because the sequence of T-8-1 was identical to the N-terminal sequence of undegraded Br-I. On the other hand,

Table 2. Amino Acid Compositions[a] of CNBr-Peptides Obtained from A_2-II

Amino acid	Br-III-A	Br-III-C	Br-I	Br-I-CM	Total CNBr-peptides	A_2-II[f]
Asp	–	–	15.9 (16)	17.4 (17)	16–17	17
Thr	0.9 (1)	–	4.1 (4)	3.8(4)	5	5
Ser	–	0.8 (1)	4.1 (4)	4.0(4)	5	5
Glu	1.7 (2)	–	10.4 (10)	9.8(10)	12	12
Pro	–	–	5.2 (5)	4.7 (5)	5	5
Gly	–	–	12.7 (13)	13.2 (13)	13	13
Ala	–	–	7.7 (8)	7.9 (8)	8	8
½ Cys	–	–	14.8 (15)	13.5[b] (14)	14–15	16[g]
Val	–	–	4.1 (4)	3.7 (4)	4	4
Met	0.8 (1)[c]	0.3 (1)[c]	–	–	2	2
Ile	1.1 (1)	–	5.6 (6)	6.3 (6)	7	7
Leu	0.8 (1)	1.0 (1)	1.2 (1)	1.3 (1)	3	3
Tyr	–	–	7.9 (8)	7.6 (8)	8	9
Phe	1.0 (1)	–	3.6 (4)	3.7 (4)	5	5
Trp	–	–	[d] (2)[g]	1.6[e] (2)	2	2[e]
Lys	–	–	8.0 (8)	7.9 (8)	8	8
His	–	–	1.2 (1)	1.2 (1)	1	1
NH_3	0.8	–	9.5	8.6		
Arg	–	–	4.0 (4)	4.1 (4)	4	4
Total residues	7	3	113	113	123–124	126

[a] Hydrolysis was carried out *in vacuo* at 110°C in 5.7 N HCl for 24 hr. Values are expressed as molar ratios, using the values or average values for the reference amino acids underlined. Values for threonine and serine were extrapolated to zero time. Nearest or possible integers are shown in parentheses.
[b] Determined as carboxymethylcysteine.
[c] Determined as homoserine.
[d] Not determined due to destruction.
[e] Obtained from acid hydrolysis with 4 N methane-sulfonic acid for 24 hr.
[f] Obtained from acid hydrolysis with 5.7 N HCl for 24, 48, and 72 hr.
[g] Obtained from sequence data.

T-1-2 must be located in the C-terminal part of Br-I, since it contained the C-terminal cystine derivative as did the whole protein.

To align the tryptic fragments with overlapping sequence, thermolytic digestion of alkylated Br-I was performed and the digest was fractionated on a Sephadex G-25 column followed by purification on a Bio-Gel P-4 column, a Dowex 50-X2 column, and by paper chromatography and paper electrophoresis. The sequences of the purified thermolytic peptides are shown in Table 5. In these peptides, TL-4-7A, TL-7-2A TL-2/3-3B, and TL-2/3-4B-2 provided the overlaps of the tryptic peptides. Some parts of the sequences, undetermined in

Table 3. Partial Amino Acid Sequences[a] of CNBr-Peptides Obtained from A_2-II

Br-III-A	(1-7) :	Glu-Phe-Glu-Thr-Leu-Ile-Hse-OH
Br-III-C	(8-10) :	Ser-Leu-Hse-OH[b]
Yield, %		65 26
Br-I	(11-126) :	Lys-Ile-Ala-Gly-Arg----------Cys-OH
Yield, %		96 68 52 50 30
Br-I-CM	(11-126) :	Lys-Ile-Ala-Gly-Arg----------CmC-OH
Yield, %		60 67 84 42 34

[a]The amino acids identified by the direct Edman procedure are indicated by arrows (⟶) under which their over-all yield relative to the quantity of starting peptide is given.

[b]This sequence was determined also by the subtractive Edman degradation procedure.

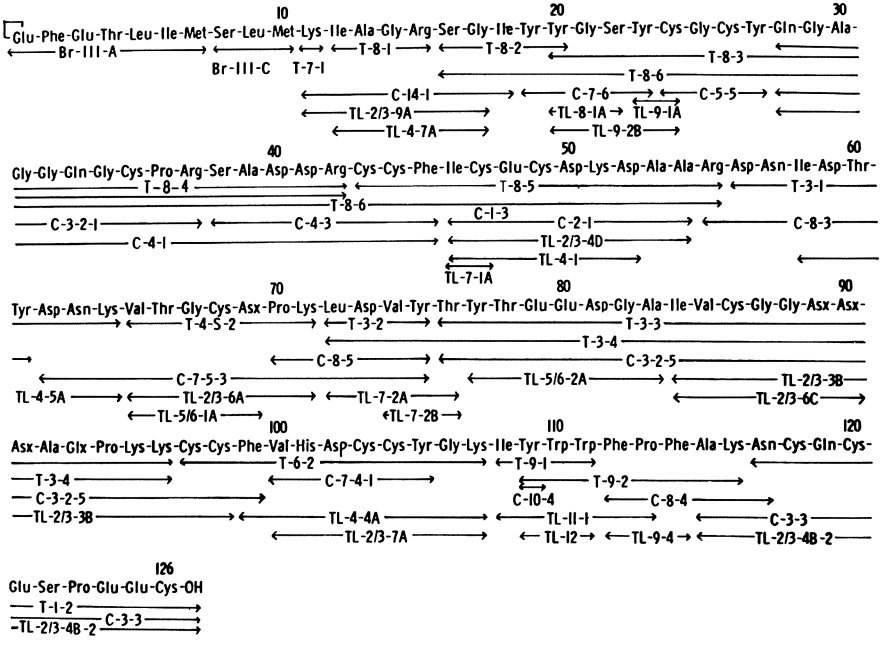

Fig. 2. Amino acid sequence of phospholipase A_2-II isolated from the venom *Agkistrodon halys blomhoffii*. The peptides obtained from digests with trypsin (T), α-chymotrypsin (C), and thermolysin (TL) are shown by arrows.

Table 4. Partial Amino Acid Sequences[a] of Tryptic Peptides Obtained from Br-I-CM

T-7-1 (11) : Lys-OH

T-8-1 (12-15) : Ile-Ala-Gly-Arg-OH
Yield, % 76 54 30

T-8-2 (16-20) : Ser-Gly-Ile-Tyr-Tyr-OH
Yield, % 63 83 60 37 37

T-8-3 (20-42) : Tyr-Gly-Ser-Tyr-CmC-Gly-(Asx$_2$, Ser$_1$, Glx$_2$, Pro$_1$, Gly$_4$, Ala$_2$, CmC$_2$, Tyr$_1$, Arg$_1$)-Arg-OH
Yield, % 82 56 46 52 16 30

T-8-4 (28-42) : Gln-Gly-Ala-Gly-Gly-Gln-Gly-CmC-Pro-(Arg$_1$, Ser$_1$, Ala$_1$, Asx$_2$)-Arg-OH
Yield, % 60 50 46 40 33 30 20 10 13

T-8-5 (43-55) : (Asx$_2$, Glx$_1$, Ala$_2$, CmC$_4$, Ile$_1$, Phe$_1$, Lys$_1$)-Arg-OH

T-3-1 (56-64) : Asp-Asn-Ile-Asp-Thr-Tyr-Asp-Asn-Lys-OH
Yield, % 72 68 97 48 36 46 35 35

T-4-S-2 (65-71) : Val-Thr-Gly-CmC-Asx-Pro-Lys-OH

T-3-2 (72-75) : Leu-Asp-Val-Tyr-OH
Yield, % 80 54 51 53

T-3-3 (76-96) : Thr-Tyr-Thr-Glu-Glu-Asp-Gly-(Asx$_3$, Glx$_1$, Pro$_1$, Gly$_2$, Ala$_2$, CmC$_1$, Val$_1$, Ile$_1$, Lys$_1$)-Lys-OH
Yield, % 65 70 75 80 60 30 15

T-6-2 (97-107) : CmC-CmC-Phe-Val-His-(Asx$_1$, Gly$_1$, CmC$_2$, Tyr$_1$)-Lys-OH
Yield, % 90 70 45 45 30

T-9-1 (108-111) . (Ile$_1$, Tyr$_1$, Trp$_2$)

T-9-2 (109-116) : Tyr-Trp-Trp-Phe-Pro-Phe-Ala-Lys-OH

T-1-2 (117-126) : Asn-CmC-Gln-CmC-Glu-(Ser$_1$, Pro$_1$)-Glu-Glu-CmC-OH
Yield, % 55 70 25 13 13

[a]Arrows in the sequences represent its establishment by the direct Edman procedure (⟶), the subtractive Edman procedure (⇢), amino acid analysis after carboxypeptidase A or B digestion (⟵) and dansylation after carboxypeptidase B digestion (⟷). The yield by the direct Edman procedure is expressed as the overall yield relative to the quantity of starting peptide.

Table 5. Partial Amino Acid Sequences[a] of Thermolytic Peptides Obtained from Br-I-CM

TL-2/3-9A	(11-17)	Lys-Ile-Ala-Gly-Arg-(Ser_1, Gly_1)-OH
Yield, %		37 31 10 8 7
TL-4-7A	(12-17)	Ile-Ala-Gly-Arg-(Ser_1, Gly_1)-OH
Yield, %		49 28 15 5
TL-8-1A	(20-22)	Tyr-Gly-Ser-OH
TL-9-1A	(23-24)	Tyr-CmC-OH
TL-9-2B	(20-24)	Tyr-Gly-Ser-(Tyr_1, CmC_1)-OH
TL-2/3-4D	(46-54)	Ile-CmC-Glu-CmC-Asp-Lys-Asp-Ala-Ala-OH
Yield, %		46 48 13 7 9
TL-4-1	(46-52)	Ile-CmC-Glu-CmC-Asp-Lys-Asp-OH
Yield, %		22 18 6 9 5
TL-7-1A	(46-47)	Ile-CmC-OH
TL-2/3-6A	(65-71)	Val-Thr-Gly-CmC-Asx-(Pro_1, Lys_1)-OH
TL-7-2A	(72-76)	Leu-Asp-Val-Tyr-Thr-OH
Yield, %		79 25 47 36 11
TL-5/6-2A	(77-83)	Tyr-Thr-Glu-Glu-Asp-Gly-Ala-OH
Yield, %		40 46 30 23 21 3
TL-2/3-3B	(84-98)	Ile-Val-(Asx_3, Glx_1, Pro_1, Ala_1, CmC_3, Gly_2, Lys_2)-OH
TL-2/3-6C	(84-90)	Ile-Val-CmC-Gly-Gly-Asx-Asx-OH
TL-4-4A	(99-107)	Phe-Val-His-(Asx_1, CmC_2, Tyr_1, Gly_1, Lys_1)-OH
TL-2/3-7A	(100-107)	Val-His-Asx-CmC-CmC-Tyr-(Gly_1, Lys_1)-OH
TL-11-1	(108-113)	Ile-(Tyr_1, Trp_2, Phe_1, Pro_1)-OH
TL-12	(109-111)	Tyr-Trp-Trp-OH
TL-2/3-4B-2	(115-126)	Ala-Lys-Asn-(CmC_2, Glx_4, Ser_1, Pro_1)-CmC-OH
Yield, %		24 11 3

[a]Arrows in the sequence represent its establishment by the direct Edman procedure (⟶), and the subtractive Edman procedure (⇢). The yield by the direct Edman procedure is expressed as the overall yield relative to the quantity of starting peptide.

the tryptic peptides, were established by TL-2/3-4D, TL-4-1, TL-2/3-7A, and TL-2/3-6C.

To examine the unknown alignments of tryptic peptides, α-chymotryptic digestion of alkylated Br-I was performed. The digest was fractionated on a column of Dowex 50-X2. Further purification of the peptides was performed

by paper chromatography and paper electrophoresis. The sequences of the purified chymotryptic peptides are shown in Table 6. In these peptides, C-7-6, C-4-3, C-8-3, C-7-5-3, C-8-5, and C-3-2-5 apparently constitute the overlaps of the tryptic peptides. The other peptides, C-4-3, C-5-5, C-3-2-1, and C-3-3 contributed to establish the undetermined sequences of the tryptic peptides.

The complete amino acid sequence of phospholipase A_2-II of *A. halys blomhoffii* venom was elucidated by aligning the tryptic peptides on the basis of the known compositions and the partially known sequences of the thermolytic and chymotryptic peptides (Fig. 2). All of the overlaps except for T-6-2 and T-9-2 through 108-Ile were proven. Thus, these two fragments could not be located anywhere other than the positions shown in Fig. 2.

When we accomplished the sequence studies on Mamushi venom phospholipase A_2-II, we received information regarding the complete amino acid sequences of snake venom enzymes from *Bitis gabonica* and *Naja melanoleuca* (Botes and Viljoen, 1974; Joubert, 1975). Fig. 3 shows the comparison of the sequences of the three venom enzymes from different species and of the hog pancreatic enzyme (De Haas *et al.*, 1970a,b). It is evident that a relatively high degree of homology exists among the amino terminal sequences of the four enzymes. Especially, a high degree of conservation of the same sequences from residues 20-25 and further the residues 41-44 can be found. This fact suggests

Table 6. Partial Amino Acid Sequences of α-Chymotryptic Peptides Obtained from Br-I-CM

C-14-1	(11-18) :	Lys-Ile-Ala-Gly-Arg-(Ser$_1$, Gly$_1$, Ile$_1$)-OH
Yield, %		62 28 22 32 20
C-7-6	(19-23) :	Tyr-Tyr-(Gly$_1$, Ser$_1$, Tyr$_1$)-OH
Yield, %		74 27
C-5-5	(24-27) :	CmC-Gly-CmC-Tyr-OH
C-3-2-1	(28-37) :	Gln-Gly-Ala-Gly-Gly-Gln-Gly-(CmC$_1$, Pro$_1$)-Arg-OH
Yield, %		80 90 87 11 12 7 4
C-4-3	(38-45) :	Ser-Ala-Asp-Asp-Arg-CmC-CmC-Phe-OH
Yield, %		50 30 23 45 13 20
C-8-3	(55-61) :	Arg-Asp-Asn-Ile-Asp-Thr-Tyr-OH
Yield, %		50 20 10 15 6
C-7-5-3	(62-75) :	Asp-Asn-Lys-(Val$_1$, Thr$_1$, Gly$_1$, CmC$_1$, Asx$_1$, Pro$_1$, Lys$_1$)-Leu-Asp-Val-Tyr-OH
Yield, %		8 29
C-8-5	(70-75) :	Pro-Lys-Leu-Asp-Val-Tyr-OH
Yield, %		70 76 80
C-3-2-5	(76-99)	(Thr$_2$, Tyr$_1$, Glx$_3$, Asx$_4$, Gly$_3$, Ala$_2$, Ile$_1$, Val$_1$, CmC$_3$, Pro$_1$, Lys$_2$)-Phe-OH
C-7-4-1	(100-105) :	Val-His-Asp-CmC-CmC-Tyr-OH
C-8-4	(112-117) :	Phe-Pro-Phe-Ala-Lys-Asn-OH

[a] Arrows in the sequence represent its establishment by the direct Edman procedure (→), Edman-dansylation (⇢) and carboxypeptidase A digestion followed by amino acid analysis (←).

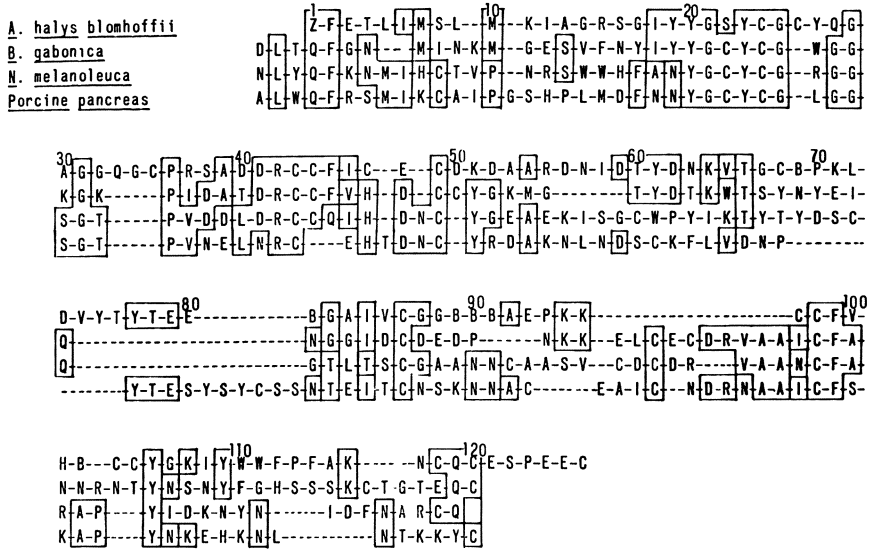

Fig. 3. Comparison of amino acid sequences of reptilian and mamalian phospholipase A_2. Boxes enclose the identical residues in the sequences of the three snake venom enzymes and the porcine pancreatic enzyme.

that the regions are structurally and functionally important for the enzyme molecules. The locations of their half-cystine residues are also similar; about 50% of total half-cystine residues occupy the same positions with some deletions and insertions of other amino acid residues. However, in spite of these similarities, the overall sequence homologies among four enzymes seem to be rather low. For example, a total homology of Japanese Mamushi venom with those of *B. gabonica* venom, *N. melanoleuca* venom, and hog pancreatic enzyme was 34%, 20%, and 22%, respectively. Moreover, the linear sequences of the reptilian and mammalian phospholipase A_2s differ greatly from that of the honey bee venom enzyme (Shipolini *et al.*, 1971). Such significant difference of the sequences suggests a rapid rate of molecular evolution of phospholipase A_2.

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Recent Results on the Structure of Scorpion and Snake Toxins

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INTRODUCTION

During the last fifteen years, a general method of purification was developed in our laboratory in order to isolate toxins from scorpion venoms (Miranda and Lissitzky, 1958; Miranda *et al.*, 1960; Miranda, 1964; Rochat *et al.*, 1967; Miranda *et al.*, 1970a). This method was found to be efficient for purification of snake neurotoxins (Miranda, 1964; Miranda *et al.*, 1970b; Kopeyan *et al.*, 1973). We report, in this paper, the results which have recently been obtained on both scorpion and snake toxins.

MATERIALS AND METHODS

Venoms. The venoms of *Androctonus australis Hector* and *Centruroides suffusus suffusus* were prepared in our laboratory (for details see Miranda *et al.*, 1970a). The venoms of *Androctonus mauretanicus*, *Buthus occitanus paris*, *Buthus occitanus tunetanus*, and *Leiurus quinquestriatus quinquestriatus* were obtained from F. G. Celo (Zweibrücken, Germany), and the venom of *Naja mossambica mossambica* from D. Muller (Johannesburg, South Africa).

Purification. Exhaustive technical details have already been published (Miranda *et al.*, 1970a, b; Rochat *et al.*, 1974).

Sequence Determination and Location of Disulfide Bonds. The methods used (reduction and S-methylation of half-cystine residues, amino acid hydrolysis, automatic and manual Edman degradation, enzymatic digestions, purification of peptides mainly by gel filtration, reversible adsorption on Sephadex, paper chromatography, and high-voltage paper electrophoresis) have been extensively described (Rochat *et al.*, 1970; Rochat *et al.*, 1970c; Rochat *et al.*, 1972; Rochat *et al.*, 1974; Kopeyan *et al.*, 1974).

RESULTS AND DISCUSSION

From the six scorpion venoms which have been studied so far in our laboratory, 23 different toxins that are active on mammals have been characterized. They were found to be pure according to such physicochemical criteria as equilibrium chromatography and starch gel or disk electrophoresis. Their amino acid compositions have been determined (Miranda *et al.*, 1970a; Martin, 1974; Rochat *et al.*, 1976; Garcia *et al.*, 1976; Rosso *et al.*, 1976).

These proteins are each composed of a single polypeptide chain crosslinked by four disulfide bridges. In addition to the sequence data which have previously been obtained on eight different scorpion toxins (Rochat *et al.*, 1970b; Rochat *et al.*, 1970c; Rochat *et al.*, 1972), Fig. 1 gives recent results concerning 10 additional toxins that are also active on mammals. A maximum of homology in the sequences is obtained when half-cystine residues are placed at identical sequence positions. This result is particularly obvious when considering the N-terminal part: residue no. 2 is basic (Lys or Arg); residue no. 3 is acidic (Asp or Glu) except in the case of Bop I (where Gly was found); residue nos. 4, 6, and 7 are always hydrophobic; Tyr is always found in position 5; Asn is found in position 11 in all toxins of African origin but Gly exists in the two American toxins; Tyr is generally constant in positions 15 (with the exceptions of Lqq V and Am II which contain Phe) and 26 (except when this position corresponds to a deletion as for AaH I, I′, and III).

It had previously been claimed that scorpion toxins formed a new set of homologous proteins divided into three groups according to similarities in structure (Rochat *et al.*, 1970b). Group I would comprise toxins AaH I, I′ and III; group 2 toxins AaH II, Am II, Bot III and III′, Lqq V; group 3 toxins Bot I, I′, I″, I‴ and II, Lqq III, Bop I and II. However, these last two toxins which obviously belong to the third group, when considering the first seven residues, show in positions 8 (for both) and 9 (for Bop II), residues that are found at the same positions in toxins of group 2. To these three groups, one has to now add group 4 which comprises both toxins I and III of *Centruroides suffusus suffusus*. In contrast to the specific toxicities of known snake neurotoxins, those of scorpion toxins differ considerably from one to another. Of particular interest is the comparison of the toxins AaH II, Bot III and Bot III′.

```
                    10        20        30        40        50        60        70
AaH I      KRDGYIVYPN-NCVYHCVPP-----CDGLCKKN-GGSSGSSCFLV-PSGLACWC-KDLPDNVPIKDTSRK-CT
AaH I'     KRDGYIVYPN-NCVYHCIPP-----CDGLCKKN-GGSSGSSCFLV-PSGLACWC-KDLPDNVPIKDTSRK-CT
AaH III    VRDGYIVNSK-NCVYHCVPP-----CDGLCKKN-GASSGSSC...

AaH II     VKDGYIVDDV-NCTYFCGR---NAYCNEECTKL-KGESG-YCQWASPYGNACYCYK-LPDHVRTKGPGR--CH
Bot III    VKDGYIVDDR-NCTYFCGR---NAYCNEEC...
Bot III'   LKDGYIVDDR-NCTYFCGT---NAYCNEECVKL-KGE...
Lqq V      LKDGYIVDDK-NCTFFCGR---NAYCNNEC...
Am  II     LKDGYIIDDL-NCTFFCGR---NAYCNDXC...

Bop II     GRDAYIADDX-NCAYXCAL---XXYCN...
Bop I      GRGVYIADIA-NCAY...
Bot I      GRDAYIAQPE-NCVYECAE---NSYCNDWC...
Bot I'     VRDAYIAQNY-NCVYTCFK---NEYCNDLCXXN-G...
Bot I''    GRDAYIAQPE-NCVYECAK...
Bot II     GRDAYIAQPE-NCVYECAK---NWYCND...
Lqq III    VRDAYIAKNY-NCVYECFR---DSYCNDLC...

Css I      -KEGYLVSKSTGCKYECLKLGDNDYCL...
Css II     -KEGYLVSKSTGCKYECLKLGDNDYCLRECKQQYGKSSGGYCYAF-----ACWC-(THLYEQAVVWPLPNKTCN)
```

AaH : Androctonus australis Hector ; Am : Androctonus mauretanicus ;
Bop : Buthus occitanus paris ; Bot : Buthus occitanus tunetanus ;
Css : Centruroïdes suffusus suffusus ; Lqq : Leiurus quinquestriatus quinquestriatus;
X : not determinated ; - : deletion.

Fig. 1. Amino acid sequences of scorpion toxins active on mammals.

Table 1. Amino Acid Composition of *Androctonus australis Hector* Toxin II and *Buthus occitanus tunetanus* Toxins III and III'

Amino acid	AaH II	Bot III	Bot III'
Aspartic acid	8	9	8
Threonine	3	3	3
Serine	2	2	2
Glutamic acid	4	4	5
Proline	3	3	1
Glycine	7	7	7
Alanine	3	3	3
Half-cystine	8	8	8
Valine	4	4	5
Methionine	0	0	0
Isoleucine	1	1	1
Leucine	2	1	3
Tyrosine	7	7	6
Phenylalanine	1	1	1
Lysine	5	5	4
Histidine	2	1	1
Arginine	3	4	5
Tryptophan	1	1	2
Total	64	64	65
References	Miranda *et al.*, 1970	Miranda *et al.*, 1970	Rochat *et al.*, 1976

Taking into account their amino acid compositions (see Table 1) and the sequences which have been established (Fig. 1), one can expect that the high homology found in their N-terminal parts will also exist in their C-terminal sequences. As it has been found that toxin AaH II is twice more active than toxin Bot III and fifty to sixty times more active than Bot III', the nature of the amino acid residues found in positions 10 and 19 might be of importance for the biological activity of these proteins. So, in the case of scorpion toxins, the determination of primary sequences might well give a better chance to obtain significant information on structure-activity relationships than in the case of *Elapidae* and *Hydrophidae* neurotoxins.

As these three proteins were found to show identical antigenic properties (Delori *et al.*, 1976), Bot III', which is the less potent, can be considered as an almost natural anatoxin of AaH II: this might well be of importance in antivenomous serotherapy.

The positions of the four disulfide bridges in toxin II of *A. a. Hector* have been determined (Kopeyan *et al.*, 1974). They were found to link the half-cystine residue nos. 12 and 63, 16 and 36, 22 and 46, and 26 and 48 (see Fig. 2). The main difficulty of this work originated in the high resistance of the native

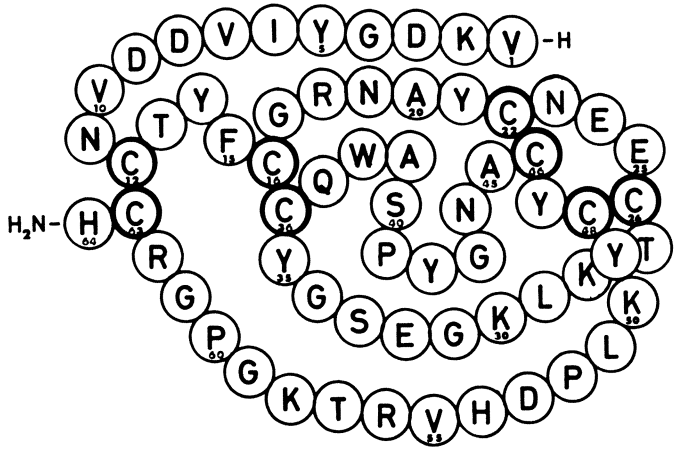

Fig. 2. Schematic diagram of covalent structure of toxin II of *Androctonus australis Hector*.

Table 2. Amino Acid Composition of Toxins from *Naja mossambica mossambica*, *Naja naja atra* (Cobrotoxin), and *Naja nigricollis*

Amino acid	*Naja mossambica mossambica* toxins			*Naja naja atra* cobrotoxin	*Naja nigricollis* toxins	
	I	II	III		I[a]	II
Aspartic acid	7	8	8	8	7	7
Threonine	8	8	7	8	8	8
Serine	3	4	3	4	2	2
Glutamic acid	7	6	5	7	6	5
Proline	4	4	4	2	5	5
Glycine	6	5	4	7	5	5
Alanine	–	–	1	–	–	–
Half-cystine	8	8	8	8	8	8
Valine	1	1	1	1	2	2
Methionine	–	–	1	–	–	1
Isoleucine	1	1	1	2	3	4
Leucine	2	2	2	1	2	1
Tyrosine	2	2	2	2	1	1
Phenylalanine	–	–	–	–	–	–
Lysine	4	4	5	3	6	6
Histidine	2	3	3	2	2	2
Arginine	7	6	6	6	3	3
Tryptophan	1	1	2	1	1	1
Total	63	63	63	62	61	61
References	Rochat *et al.*, 1974			Yang *et al.*, 1969	Kopeyan *et al.*, 1973	

[a]Toxin I of *N. nigricollis* corresponds to toxin α isolated by Karlsson *et al.*, 1966.

Fig. 3. N- and C-terminal sequences of *Naja mossambica mossambica* toxins (T-I, T-II, and T-III); C, cobrotoxin (Yang *et al.*, 1969); Nn I, toxin I of *Naja nigricollis* (Eaker and Porath, 1972; Kopeyan *et al.*, 1973); Nn II, toxin II of *Naja nigricollis* (Kopeyan *et al.*, 1973).

toxin to the proteolytic enzyme action. This is probably due to the compact conformation of the molecule (Chicheportiche and Lazdunski, 1970).

In the case of toxin I of *A. a. Hector*, two disulfide bridges were found in homologous positions when compared to toxin II: they link half-cystine residue nos. 12 and 62, 24 and 46 (Rochat *et al.*, 1970a). Thus it is likely that the half-cystine residues will be paired in the same manner in every scorpion toxin that is active on mammals. Therefore, the disulfide bonds must play a fundamental role in the structure-activity relationships of these miniproteins.

The last *Elapidae* venom which has been studied in our laboratory belongs to the snake known as *Naja nigricollis mossambica* Peters (Broadley, 1968a) or, according to the more recently proposed reclassification of this author (Broadley, 1968b), to *Naja mossambica mossambica*. Three neurotoxins have been purified and finally characterized by automatic sequencing and carboxypeptidase digestion (Rochat *et al.*, 1974). Their partial sequences are shown in Fig. 3 together with those of cobrotoxin of *Naja naja atra* and toxins I and II of *Naja nigricollis*.

When these six proteins are compared in the amino acid compositions (Table 2) and in the primary sequences, it becomes obvious that the three toxins of *Naja mossambica mossambica* are more similar to cobrotoxin than to the toxins I and II of *Naja nigricollis*. According to Broadley's proposed reclassification, *Naja nigricollis* is *Naja mossambica pallida* (Broadley, 1968b). Our results may show some taxonomical importance as it has been found that *Naja nigricollis* toxins I and II show amino acid sequences very close to those of toxins I and II of *Naja haje* (Kopeyan *et al.*, 1973).

SUMMARY

The application of a general method of purification to six different scorpion venoms led to the purification of 23 toxins active on mammals. Most of these proteins (18) were further characterized by amino acid sequence determination. The positions of the four disulfide bridges of one of them, toxin II of *A. a. Hector*, have been determined. From the venom of an *Elapidae* snake *N. m. mossambica*, three toxins have been characterized.

ACKNOWLEDGMENTS

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Chemical Synthesis of a Peptide with Cobrotoxin Activity

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INTRODUCTION

It is generally accepted that the solid-phase synthesis (SPS) developed by Merrifield can not at present be used for the confirmation of the primary structure proposed for a small protein by its synthesis. However, it is well recognized that SPS possesses very attractive and useful features such as speed, simplicity, automation, and so on. We intend collecting some information which might help to achieve clean synthesis of a small protein, perhaps by a conventional method or by solid-phase fragment synthesis. We plan to gain such information from the experimental results obtained during stepwise solid-phase synthesis of a polypeptide with the target sequence of a known small protein.

We have carried out stepwise solid-phase syntheses of a basic pancreatic trypsin inhibitor (Noda *et al.*, 1971) and ribonuclease T_1 (Izumiya *et al.*, 1972) with the intentions mentioned above. In this presentation we describe the features of solid-phase synthesis of cobrotoxin (CTxin) and its analogues.

CTxin was isolated from cobra venom, and its amino acid sequence of 62

residues was determined by Yang in 1969. The attempted analogues syntheses in this study are [29-Tyr]-CTxin (62 residues) in which 29-Trp in natural CTxin is replaced with Tyr, and [24-Ala]-CTxin (16–62) (47 residues) which is a fragment with 16-Gly as the N-terminus.

METHODS

Procedure in Solid-Phase Synthesis. Commercial 2% or 1% crosslinked polystyrene was chloromethylated in the usual manner, and resin containing 1.4- or 0.69-mmol/g Cl was obtained. This was converted to HCl·Asn-polymer with 0.22- or 0.34-mmol/g Asn. Two runs (Proc A and Proc B) of SPS in manual fashion were made for syntheses of Boc (*t*-butyloxycarbonyl)-peptide polymer of 62 or 47 amino acid residues. The schedule of a cycle for the incorporation of each Boc-amino acid was almost the same as described before (Noda *et al.*, 1971), with the exception of the use of Lys (2,4-Cl_2 Z) (2,4-dichlorobenzyloxycarbonyl) and Tyr (2,6-Cl_2 Bzl) (2,6-dichlorobenzyl). 1 N Hcl-AcOH was used as a cleaving reagent for Boc groups. After the incorporation of Trp in position 29, 1% β-mercaptoethanol was added to the 1 N HCl-AcOH reagent and also the acetic acid washing solvent, and the air in the reaction vessel was completely replaced by N_2 gas. Yields of the fully protected peptide-polymers are summarized in Table 1.

Isolation of SH-Peptide from Protected Peptide-Polymer. Cleavage of the polypeptides from the solid support together with the removal of all protecting groups was achieved by treatment with HF in the presence of anisole. After evaporation of HF and *in vacuo* drying, the residue was extracted with 5% acetic acid. The filtered solution was washed with ethyl acetate to remove

Table 1. Yield of Protected Peptide-Polymer from H-Asn-Polymer

	Proc A		Proc B	
	CTxin	CTxin	[29-Tyr]-CTxin	[24-Ala]-CTxin (16–62)
H-Asn-polymer used	2.4 g (0.5 mmol)	2.4 g (0.8 mmol)	1.2 g (0.4 mmol)	1.2 g (0.4 mmol)
Protected peptide-polymer obtained	4.4 g	5.2 g	2.9 g	2.8 g
Yield				
weight increase	39%	33%	39%	48%
Cl^- titration	–	40%	43%	54%

Table 2. Yield of Crude SH-Peptides

	Synthetic CTxin	
	Proc A	Proc B
Protected peptide-polymer used	500 mg	300 mg
Time with HF at 0°C	1 hr	1 hr, with Trp
Amount of crude-peptide	55 mg	52 mg
	36%	52%

anisole and lyophylized. Yields of SH-peptide in powder are summarized in Table 2.

Preparation of SS-Peptide from SH-Peptide. The crude SH-peptide (each 50 mg in Proc A and Proc B) was chromatographed on a column (2.6 × 50 cm) of Sephadex G-25 with 0.1 M acetic acid as an eluant. Each major component was oxidized as described in the literature (Aoyagi *et al.*, 1972). Yields of the oxidized SS-peptides were 21 mg for Proc A and 27 mg for Proc B.

RESULTS AND DISCUSSION

Treatment of Natural CTxin with HF. Since it was planned to treat the final, fully protected peptide-polymer in the SPS with HF, it was important to examine the stability of natural CTxin to HF treatment. The natural toxin (5 mg) was treated with HF (5 ml). After about 1 hr, the solution was evaporated and the residue was tested for CTxin activity on mice. The product treated with HF possessed the toxicity identical to that of the natural toxin. This preliminary experiment indicated that a synthetic, protected peptide-polymer can be treated safely with HF.

Properties of SS-Peptide in Proc A. The toxicity of SS-peptide in Proc A was measured by intraperitoneal injections of progressively diluted peptide solution into mice, natural CTxin being used as a reference compound. The results showed that the SS-peptide possessed a specific toxic activity corresponding to about 6% of that of natural CTxin.

Effects of the SS-peptide and natural CTxin on the response to acetylcholine was determined with isolated frog rectus abdominis. About 20 μg of the SS-peptide showed effect equivalent to 1 μg of CTxin.

Immunodiffusion tests with the peptides were carried out. The results indicated that the SS-peptide showed behavior similar to that of CTxin. This experiment suggests that a synthetic peptide may be purified by applying a specific immunochemical technique.

FEATURES OF THIS STUDY

Some results in Proc A have briefly reported (Aoyagi *et al.*, 1972). Many parts of our studies have apparently not yet been completed. A polypeptide corresponding to CTxin and two analogues synthesized by Proc B are presently under purification. Furthermore, it is questionable whether the SS-peptide with a specific activity of 6% of that possessed by CTxin contains molecules identical to those of natural CTxin. Nevertheless, our experiments may provide some useful information for future clean synthesis of CTxin and its analogues.

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Synthetic Studies of α-Bungarotoxin

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INTRODUCTION

Neurotoxic snake poisons have in recent years attracted the attention of widespread investigators as powerful tools for the study of neuromuscular transmission mechanisms. In view of this, an urgent problem was the relationship among the primary structure, conformational states, and biological properties of the neurotoxins, which together would serve as basis for understanding their mode of action at the molecular level. In our opinion, the progress achieved in modern peptide chemistry has paved the way for wide use of synthetic methods for this purpose, similar to their use in the structure-function studies of oligopeptidic hormones and antibiotics.

Accordingly we have undertaken total synthesis of α-bungarotoxin (Fig. 1), a 74-membered constituent of the venom of the Taiwanian krait, *Bungarus multicinctus* (Mebs *et al.*, 1971) used by several workers for isolation and study of the cholinergic receptor from the postsynaptic membrane (see, for instance, Miledi *et al.*, 1971; De Plazas and De Robertis, 1972; Martinez-Carrion and Raftery, 1973). The synthesis of α-bungarotoxin is planned to be utilized as basis for the preparation (1) of a series of analogues of modified amino acid composition and, (2) of labeled toxin derivatives with ^{2}H, ^{13}C, and ^{15}N, required for signal assignments in the NMR (nuclear magnetic resonance) spectra and in securing stereochemical information. This report describes the present state of these studies.

RESULTS AND DISCUSSION

There are two main approaches to the synthesis of peptides: the classical and Merrifield's solid support methods. Although the latter is being used with

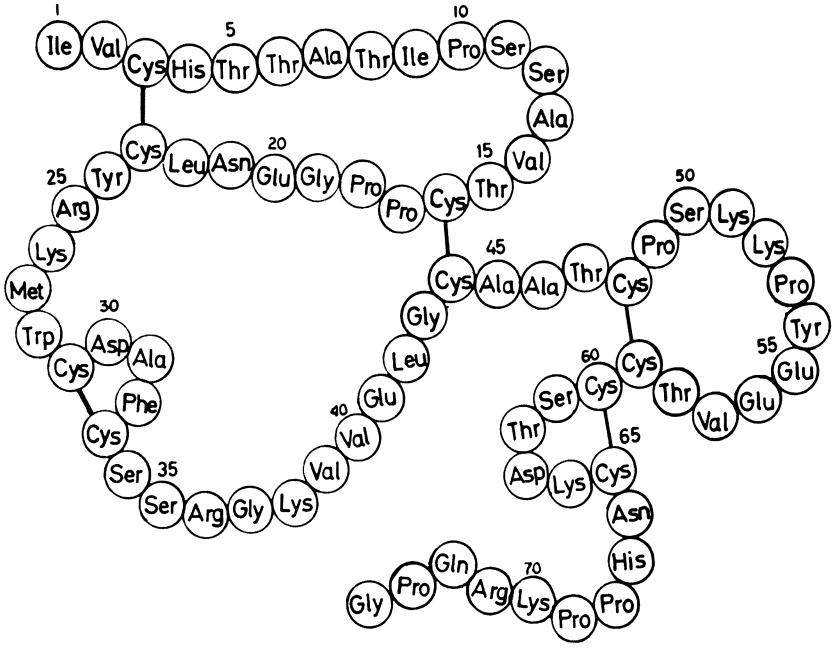

Fig. 1. Primary structure of α-bungarotoxin.

more and more confidence for the synthesis of large peptide molecules as years go by, its well-known shortcomings, due mainly to the difficulties in the elimination of the shortened, erroneous sequences and purity control of the end product, inclined us to select the classical route to α-bungarotoxin. Two variants are possible in such an approach. The first is to use minimum protective groups and consequently the azide procedure for condensing the peptide fragments. A characteristic feature of this method is the solubility of the long peptides in aqueous-organic media. This enables ion-exchange chromatography to be used for the isolation and purification of the intermediate peptides. However, the presence of numerous unprotected functional groups sharply increases the probability of side reactions, thereby lowering the yield of the desired product. We adopted another variant and synthesized fragments completely protected by hydrophobic groupings. Side reactions in the formation of the peptide bond should thus be reduced to a minimum and one should expect enhanced solubility of the intermediate peptides in organic solvents.

Our strategy of α-bungarotoxin synthesis included first the preparation of all short fragments, so selected that as far as possible their C-terminal residues would be glycine or proline (see below, Fig. 13). This is then to be followed by condensation of the fragments to obtain the exhaustively protected α-bungarotoxin from which the protective groups are removed and the resulting

reduced form of the toxin is oxidized by air. According to the data obtained with the toxin from the cobra *Naja naja atra* (Yang, 1967), one should expect spontaneous ring closure via appropriate disulfide bonds. The *t*-butyloxycarbonyl group (Boc) was predominantly used for protecting the α amino groups; for protecting the side chain functions use was made of the benzyl group (Bzl) in the case of serine and threonine, of the tosyl group (Tos) in the case of aspartic and glutamic acids, the 2,6-dichlorobenzyl group [Bzl(Cl_2)] for tyrosine, the benzyloxycarbonyl group (Z) for lysine, and the diphenylmethyl group (benzhydryl, Bzh) for asparagine and glutamine. As a rule, the synthesis was carried out with the C-terminal carboxyl free, making use of their salts with tertiary amines or sodium bicarbonate in organic or aqueous organic media. Such a set of protecting groups provided for their retention, under the conditions, of Boc removal during synthesis of the fragments, and for the possibility of their concurrent removal from the protected sequence 1-74 by treatment with liquid HF at 0°C for one hour. The Boc group was removed with a 25%-50% solution of CF_3COOH in methylene chloride, by boron trifluoride etherate in acetic acid or by mercaptoethanesulfonic acid in acetic acid.

Fragment 1-10 (Fig. 2) was prepared by stepwise build-up of the chain from the C-terminal proline residue by the N-hydroxysuccinimide ester (ONSu)

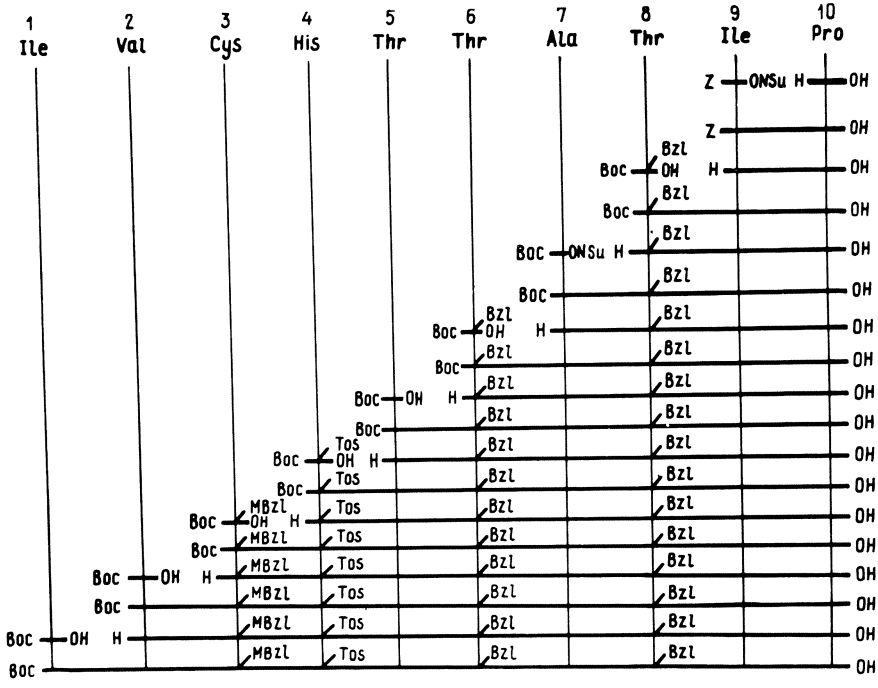

Fig. 2. Scheme for synthesis of the α-bungarotoxin protected (1-10) sequence.

method or by means of *N,N'*-dicyclohexylcarbodiimide (DCCI) in the presence of 1-hydroxybenzotriazole (HBT). Initially attempts were made to use the 2,4-dinitrophenyl group for protecting the histidine imidazole ring, but it soon had to be rejected owing to its enhanced mobility and the instability of peptides containing a 2,4-dinitrophenylhistidine residue. Figure 3 schematically represents the synthesis of the next fragment, the protected nonapeptide corresponding to the 11–19 sequence of the toxin. The Z-Pro-Pro prepared by the *p*-nitrophenyl ester (ONP) method was coupled with *t*-butyl glycinate by means of carbodiimide in the presence of HBT, the same method also being used in the succeeding stages.

Owing to the formation of an amide bond between fragments (20–27) and (28–32) the activation of an optically active residue (Met) was required. We selected for this reaction the azide method and accordingly synthesized the hydrazide of the protected heptapeptide (21–27) (Fig. 4). Bearing in mind the presence of the benzyl protecting group, we planned to incorporate the glutamic acid residue 20 after azide condensation of the two fragments. The hydrazide was synthesized by the consecutive addition of ϵ-benzyloxycarbonyllysine to methionine methyl ester (by the N-hydroxysuccinimide ester method) and of N^G-tosylarginine (by the mixed anhydride method with isobutyl chloroformate in dimethylformamide at -20 to -30°C). Following this, 2,6-dichlorobenzyltyrosine, methoxybenzylcysteine, and leucine were incorporated, again by the acti-

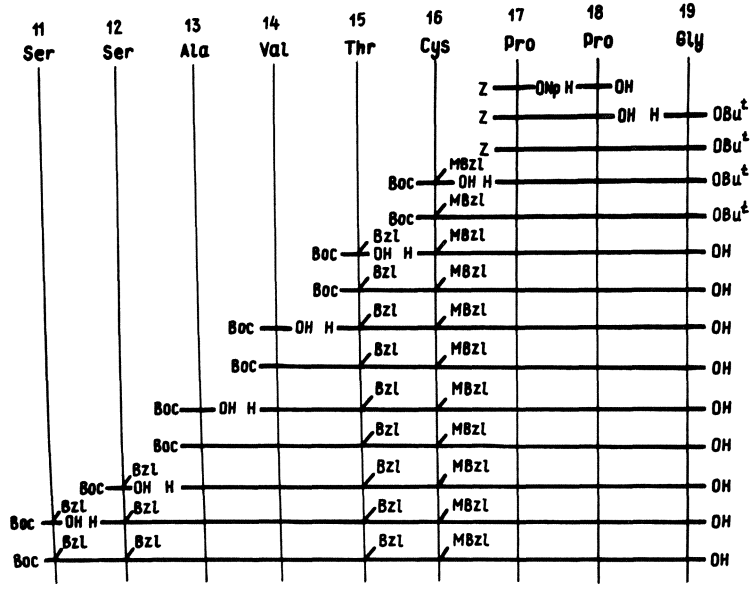

Fig. 3. Scheme of synthesis of the α-bungarotoxin protected (11–19) sequence.

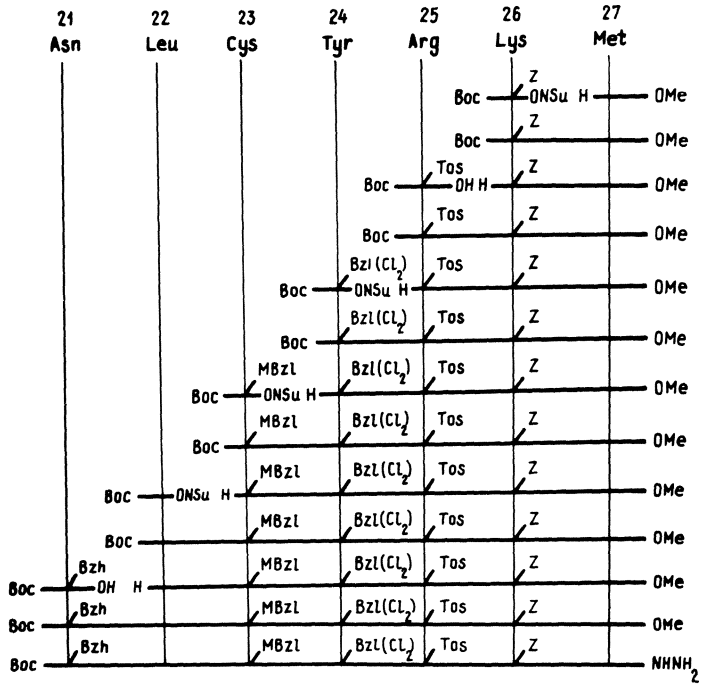

Fig. 4. Scheme for synthesis of the α-bungarotoxin protected (21–27) sequence.

vated ester method. Benzhydrylasparagine was incorporated by the DCCI/HBT method. Hydrazinolysis of the resultant heptapeptide was performed as usual (10-fold excess of hydrazine hydrate in dimethyl formamide, 25°C, 72 h).

The fragment (28–32) following was synthesized by stepwide upbuilding from benzyl phenylalanate (Fig. 5). Boc-Cys(MBzl)-Ser(Bzl)-Arg(Tos)-Gly (sequence 33–37, Fig. 6) was prepared issuing from *t*-butyl glycinate. Z-Arg(Tos)-Gly-OBu^t was synthesized by the mixed anhydride method, the Z-protective group being eliminated by hydrogenolysis. The protected tripeptide was prepared by the activated ester method. The C- and N-protecting groups were then both simultaneously removed. At the stage of formation of the protected tetrapeptide (34–37) during which the succinimide ester of Boc-benzylserine was allowed to react with the N-methylmorpholine salt of the C and N termini unprotected tripeptide, the formation in considerable amounts of a substance lacking a free carboxyl group was observed, whose structure has as yet not been elucidated. No side products were observed in the succeeding stage.

The fragment (38–43) (Fig. 7) was synthesized starting with glycine and stepwise upbuilding with the aid of the activated ester method (N-hydroxysuccinimide and *p*-nitrophenyl esters). The peptides (44–49) (Fig. 8) and (50–

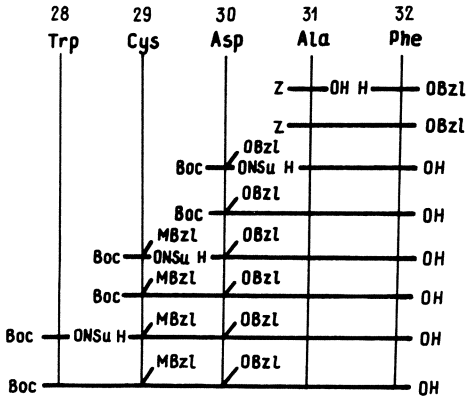

Fig. 5. Scheme for synthesis of the α-bungarotoxin protected (28–32) sequence.

53) (Fig. 9) were smoothly synthesized by the N-succinimide or carbodiimide methods. The protected undecapeptide (54–64) was synthesized by azide condensation of fragments (58–61) and (62–64) followed by chain elongation from the N-terminus with the aid of the N-hydroxysuccinimide ester method (Fig. 10).

The pentapeptide (65–69) was synthesized according to the scheme shown in Fig. 11. From nitrobenzyl prolinate and succinimide benzyloxycarbonylprolinate, a dipeptide was obtained, after which the N-protective group was eliminated and the resultant amino ester was made to react with Boc-tosylhistidine by the DCCI/HBT method. Benzhydrylasparagine and methoxybenzylcysteine were incorporated by the activated ester method.

Finally, the last fragment (70–74) was synthesized starting from benzyl glycinate (Fig. 12). The dipeptide was obtained by the carbodiimide method, the

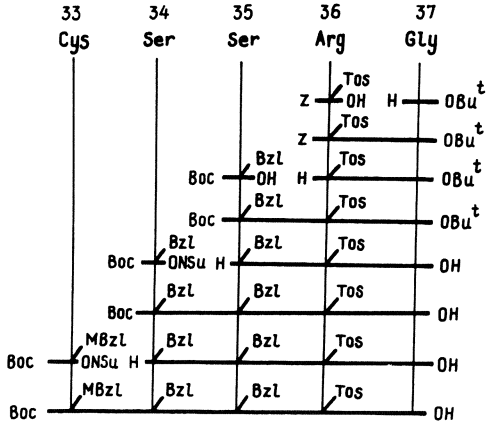

Fig. 6. Scheme for synthesis of the α-bungarotoxin protected (33–37) sequence.

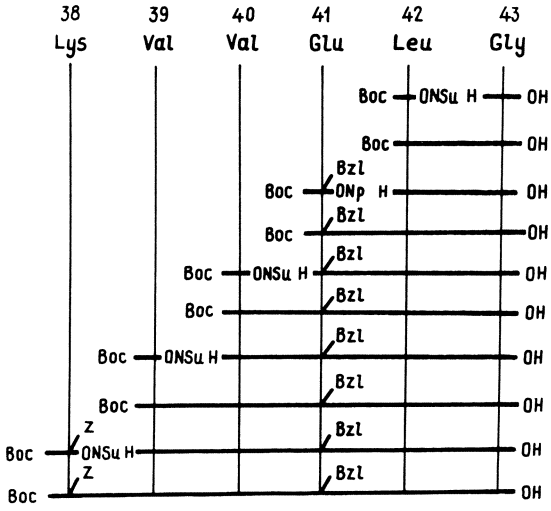

Fig. 7. Scheme for synthesis of the α-bungarotoxin protected (38–43) sequence.

tripeptide by the N-hydroxysuccinimide method, tosylarginine, and *o*-chlorobenzylcarbonyllysine were incorporated with the aid of isobutyl chloroformate.

Thus, all of the fragments covering the entire amino acid sequence of the toxin (Fig. 13) have been synthesized. They were purified by recrystallization or chromatography on silica gel; sometimes the unreacted amino acid component was removed by filtration through the H^+ form of a macroporous Amberlite XE-89 ion exchanger. The individuality of the synthesized peptides was con-

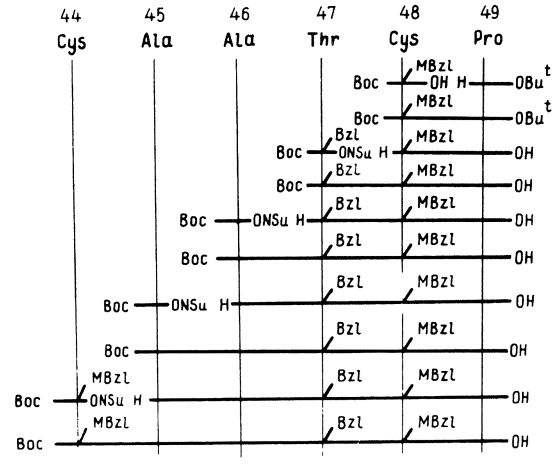

Fig. 8. Scheme for synthesis of the α-bungarotoxin protected (44–49) sequence.

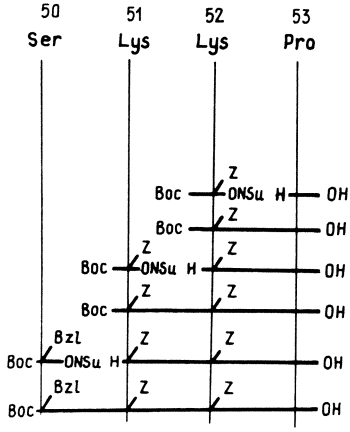

Fig. 9. Scheme for synthesis of the α-bungarotoxin protected (50–53) sequence.

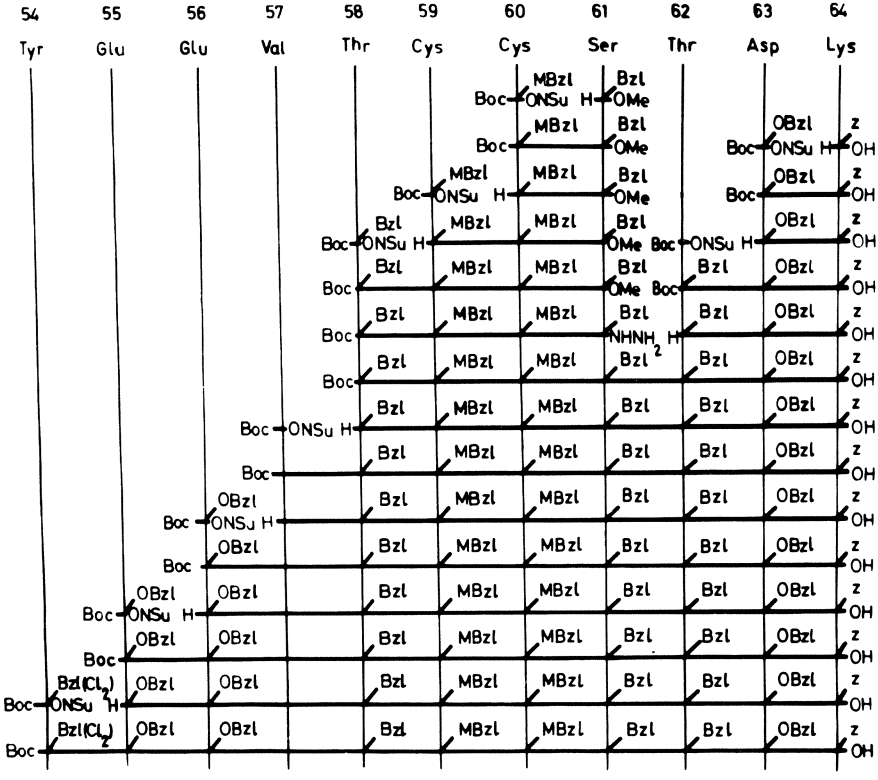

Fig. 10. Scheme for synthesis of the α-bungarotoxin protected (54–64) sequence.

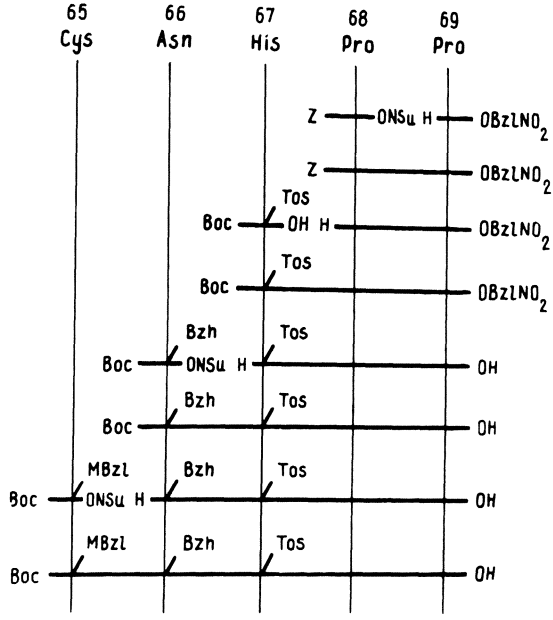

Fig. 11. Scheme for synthesis of the α-bungarotoxin protected (65–69) sequence.

trolled by thin-layer chromatography on silica gel (Merck and Eastman Kodak plates), by elemental analysis and quantitative amino acid analysis.

At present, work is in progress to join these fragments together. Model experiments showed that no racemization of the C-terminal residues of the peptides occurred on activation by the DCCI/HBT method. The experiments were carried out by synthesizing the LLL and LDL diastereomers of the tripeptide

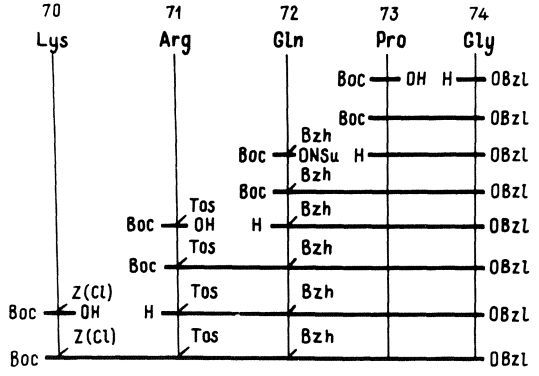

Fig. 12. Scheme for synthesis of the α-bungarotoxin protected (70–74) synthesis.

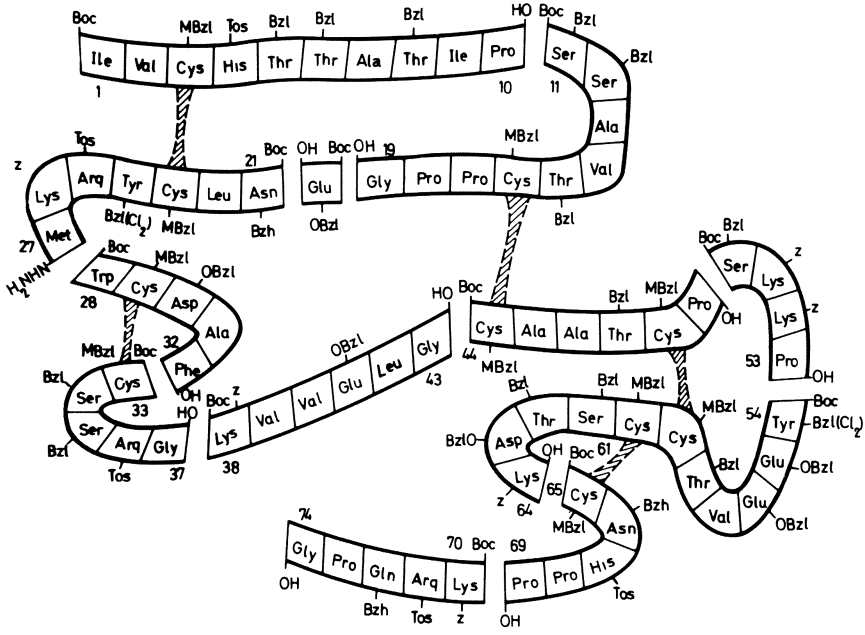

Fig. 13. Protected α-bungarotoxin fragments obtained by total synthesis. Hatched areas indicate disulfide bridges in the toxin.

Boc-Asp(OBzl)-Lys(Z)-Cys(MBzl)-OH and their NMR ^{13}C spectra were recorded as were the spectra of known mixtures of the two containing 5% and 1% of the LDL isomer. These were compared with the spectra of the tripeptide obtained by DCCI/HBT condensation according to the scheme 2 + 1. The results obtained (Fig. 14) showed that racemization, if any did occur, did not exceed 1%.

The DCCI/HBT condensation of the fragments (44–49) and (50–54) yielded the decapeptide (44–54), excellently recrystallizing from ethyl acetate. A similar procedure was used to prepare the decapeptide (28–37). The latter was condensed with the fragment (38–43) by the mixed anhydride method, yielding the protected hexadecapeptide (28–43) which was purified by gel filtration (Fig. 15).

The merits of the strategy used in the synthesis came quite vividly to the fore in the course of the study. The yields of the protected peptides at all stages of the condensation, except in the synthesis of the tetrapeptide (34–37), were very good; as a rule 60%–80% and in a number of cases even 90%. The blocking of the side chain functional groups reduced side reactions to a minimum, often the reactants were the only impurities in the crude product. All peptides were satisfactorily soluble in the usual organic solvents (dioxane, chloroform, ethyl acetate, dimethyl formamide, or tetramethyl urea). Despite the as yet incom-

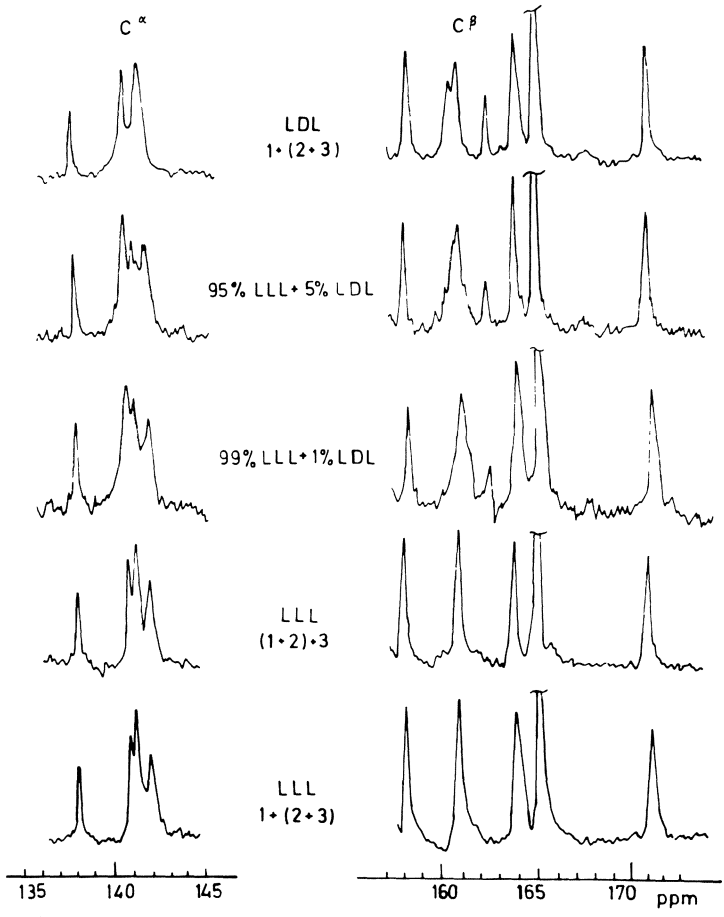

Fig. 14. C^{β} and C^{γ} signals in the ^{13}C NMR spectra of LLL and LDL diastereomers of Boc-Asp(OBzl)-Lys(Z)-Cys(MBzl)-OH and of their mixtures.
1 2 3

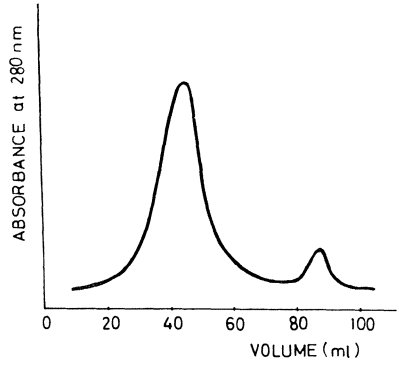

Fig. 15. Elution curve of the protected (28–43) fragment of α-bungarotoxin from an LH-20 column in tetramethyl urea.

plete synthesis of α-bungarotoxin, we strongly hold to the opinion that the methods and procedures described can be recommended for synthesis of large peptide molecules in solution.

ACKNOWLEDGMENTS

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Occurrence of Thr^6-Bradykinin and Its Analogous Peptide in the Venom of *Polistes rothneyi iwatai*

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INTRODUCTION

Since Mathias and Schachter (1958) demonstrated the presence of kininlike substances, named wasp kinin, in one of the European wasp (*Vespula vulgaris*) venoms, two other wasp kinins have been isolated and their structure proposed (Nakajima *et al.*, 1967; Yoshida and Pisano, 1972). One of these kinins is polistes kinin isolated from the venom of American wasp (*Polistes annularis*) and the other is vespula kinin from the venom of American yellow jacket (*Vespula maculifrons*). Both peptides contained the bradykinin sequence in their structures.

We would like to present the most recent findings which permit a proposal of the structures of other analogous peptides in the venom of *Polistes rothneyi iwatai*. This report deals with the separation, isolation, and chemical characterization of these materials.

MATERIALS AND METHODS

Venom sacs and lancets of 364 wasps (*Polistes rothneyi*) were homogenized in 20 ml of absolute methanol. The homogenate was centrifuged (2000 rpm,

5 min) and the precipitate was again extracted and centrifuged three times, each with 5 ml of methanol. All of the supernatants were combined, evaporated under reduced pressure below 4°C and used as the starting material.

Determination of Oxytocic Activity. Rat uteri were suspended in an 8-ml muscle chamber with de'Jalon's solution and the assays were carried out by comparing the contraction heights with synthetic bradykinin as standard.

Dansylation and Dansyl-Edman Procedure. Dansylation of the peptide (1-10 nmol) was performed by the procedure reported previously (Gray, 1967; Tamura *et al.*, 1973). The Dansyl-Edman procedure was performed according to Gray (1967). After hydrolysis of the dansylated peptide with 6 N hydrochloric acid at 90°C for 16 hr, the N-terminal amino acid was identified by thin-layer chromatography of silica gel H with two solvent systems of *n*-butanol-acetic acid-water (4:1:5) and isopropanol-methyl acetate-28% ammonia (9:7:4). All of the reagents used for dansylation and Edman degradation were redistilled or recrystallized and kept under N_2 gas in a refrigerator.

Enzymatic Degradation of the Peptides. The enzyme solution was prepared as follows. Each of TPCK-trypsin and γ-chymotrypsin (Worthington Biochemical Co.) was diluted to 100 μg/ml with 0.1 N triethylamine-bicarbonate buffer (pH 8.0). The peptide of about 10 nmol was dissolved in 100 μl of 0.1 N triethylamine-bicarbonate buffer (pH 8.0), to which 10 μl of the enzyme solution was added. The mixture was incubated at 37°C for 4 hr. After incubation, the mixture was lyophilized. The residue was dissolved in 20-50 μl of methanol and an aliquot (about 2 μl) was chromatographed on a thin layer of Silica gel H.

RESULTS AND DISCUSSION

The processes of separation and isolation of active principles are summarized in Fig. 1. The starting material was dissolved in 2 ml of dilute formic acid (pH 3), adsorbed and chromatographed on an SE-Sephadex column by linear-gradient elution with water to 0.5 N ammonium formate (pH 6.5). The activity on rat uterus contraction appeared in four peaks, P-1, P-2, P-3, and P-4 (Fig. 2). Fraction P-2 was further separated into two peaks by gel-permeation chromatography with Sephadex G-10 (Fig. 3). The first peak, P-2-1, was eluted in the void volume of the column, while the second one, P-2-2, appeared to have been adsorbed on the column.

The rat uterus-contracting activity of P-2-1 was lost by chymotrypsin but not by trypsin treatment. This fraction was finally purified by SE-Sephadex column chromatography by flat elution with 0.2 N ammonium formate (pH 6.5). After dansylation, the active principle showed a single fluorescent band in the thin-layer chromatography on Silica gel H.

Chemical Characterization of the Active Principle in P-2-1. The active principle (equivalent to 10 μg of bradykinin in activity) was hydrolyzed with

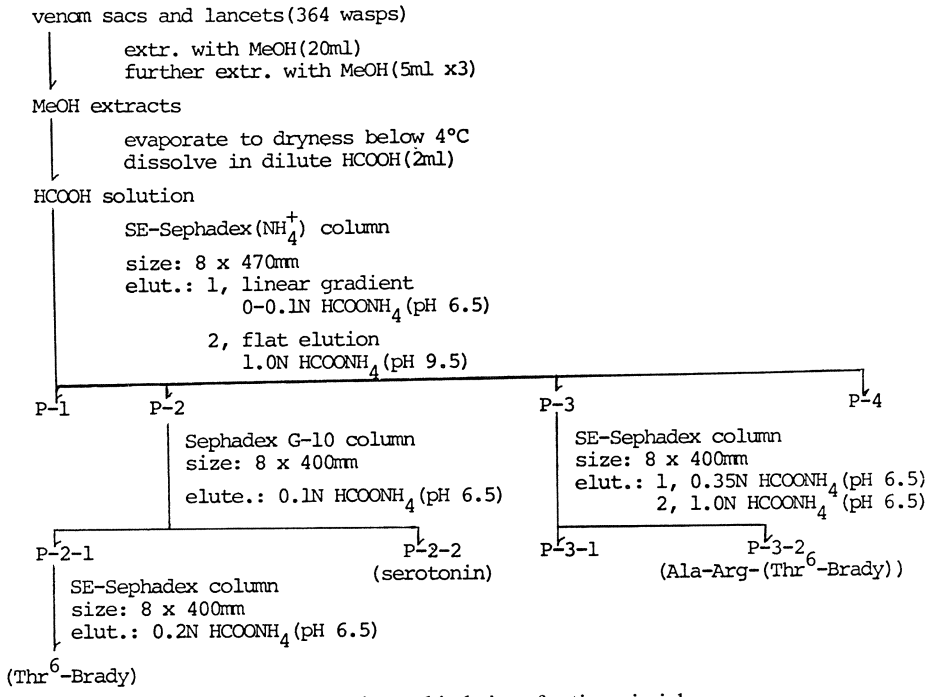

Fig. 1. Separation and isolation of active principles.

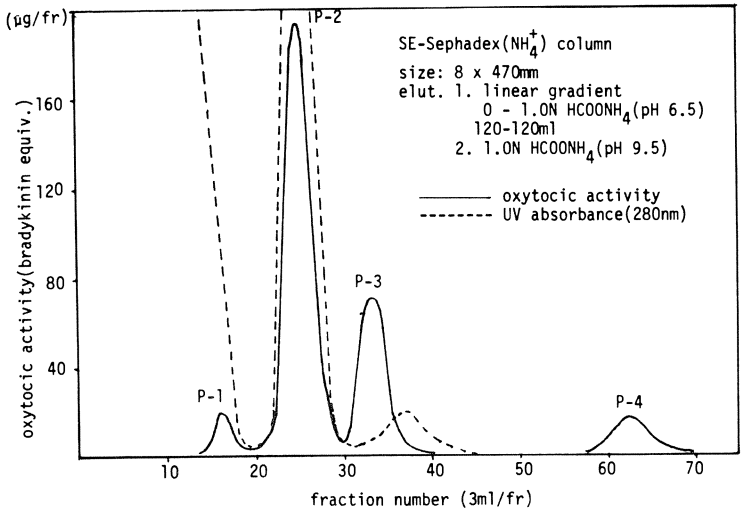

Fig. 2. SE-Sephadex chromatogram of the oxytocic substances in wasp (*Polistes rothneyi*) venom.

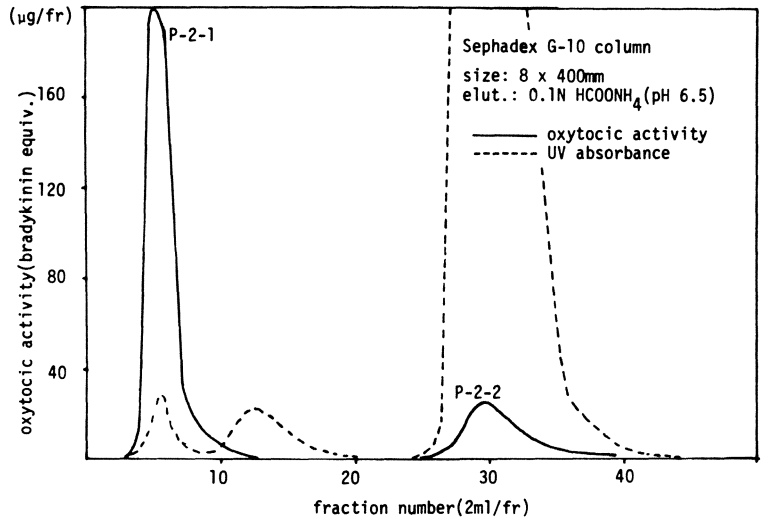

Fig. 3. Sephadex G-10 chromatogram of the oxytocic substances in fraction P-2.

6 N hydrochloric acid at 110°C for 24 hr. The hydrolysate was assayed by an amino acid analyzer (JEOL 5-AH). Amino acid composition of the peptide in P-2-1 was $Arg_{1.97}$, $Thr_{1.04}$, $Pro_{2.84}$, $Gly_{1.00}$, $Phe_{1.77}$, which was similar to that of bradykinin except that one mole of serine in the composition of bradykinin was replaced by threonine. Dansyl procedure showed dansyl-arginine as the N-terminal amino acid, which was also the same to that of bradykinin. The dansylated peptide was cleft by chymotrypsin and produced the same fluorescent fragment as that derived from dansyl-bradykinin, which was dansyl-Arg-Pro-Gly-Phe. These results indicate the following structure for this peptide:

$$Arg^1\text{-}Pro^2\text{-}Pro^3\text{-}Gly^4\text{-}Phe^5\text{-}Thr^6\text{-}Pro^7\text{-}Phe^8\text{-}Arg^9$$
(Thr^6-bradykinin)

Chemical Characterization of the Active Principle in P-2-2. This active principle was not susceptible to trypsin nor chymotrypsin digestion. The principle showed a maximum absorption at 275 nm and a shoulder at 295 like the typical UV spectrum of serotonin. In thin-layer chromatography on Silica gel H, the dansyl derivative of this principle showed overlapping to dansylated serotonin with a solvent system of benzene-dioxane-acetic acid (90:25:4). The active principle in P-2-2 was identified as serotonin.

Chemical Characterization of the Active Principle in P-3. The active principle in P-3 was a mixture of some materials, and separated further into two peaks by SE-Sephadex chromatography. The first peak was not yet purified, but the latter peak was observed as a single fluorescent band by thin-layer chromatog-

raphy after dansylation. Biological activity of this fraction was lost by chymotrypsin, but not by trypsin digestion. These behaviors were similar to those of P-2-1. The dansyl derivative, however, was cleft by either chymotryptic or tryptic treatment. This indicates that the active site of this principle was not destroyed by trypsin digestion. The amino acid composition of this principle was as follows: $Arg_{3.06}$, $Thr_{1.01}$, $Pro_{3.21}$, $Gly_{1.19}$, $Ala_{1.00}$, $Phe_{2.16}$. This composition showed that the peptide contained one mole each of argining and alanine in addition to the amino acid composition of Thr^6-bradykinin. The N-terminal amino acid of the peptide was alanine. The dansylated peptide was split by trypsin digestion to form dansyl-Ala-Arg and Thr^6-bradykinin. Dansyl-Edman procedure showed the Ala-Arg-Arg-Pro-Prolyl sequence at the N-terminal position of the peptide. From these findings, the structure of this peptide was deduced as follows:

Ala-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg
Ala-Arg-(Thr^6-bradykinin)

The venom of *P. rothneyi* contained at least six active principles; the main active substances were serotonin and Thr^6-bradykinin. The amounts of these active substances contained by the venom from one wasp were approximately 1.2 μg of serotonin, 0.7 μg of Thr^6-bradykinin, and 0.13 μg of Ala-Arg-(Thr^6-bradykinin) (Table 1). On the other three minor active principles, we did not succeed in isolating the pure forms and more wasps will be necessary to elucidate the structures of these materials.

Thr^6-bradykinin was already synthesized by Schröder and Hempel (1964) prior to the findings in the biological field, and its biological activity was reported to be equivalent to that of bradykinin. Occurrence of the The^6-bradykinin was first mentioned by Dunn and Parks (1970) as plasma kinin in the turtle, which was released from turtle plasma by glass activation. Identification of the same peptide in a free form in an invertebrate indicates the wide spread of this peptide in nature.

The chromatographic behaviors of Thr^6-bradykinin were quite similar to

Table 1. Active Principles in *Polistes rothneyi* Venom

Serotonin	1.2 μg/wasp
Thr^6-bradykinin	0.7 μg/wasp
Ala-Arg-(Thr^6-bradykinin)	0.13 μg/wasp
Unidentified materials	
P-1	0.05 μg/wasp[a]
P-3-1	0.23 μg/wasp[a]
P-4	0.06 μg/wasp[a]

[a]Oxytocic activity equivalent to bradykinin.

Wasp Kinins

Pyr-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Nakajima et al (1967)
Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Yoshida and Pisano (1972)
Ala-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg	
Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg	

Amphibian Kinins

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Anastasi et al (1965)
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ile-Tyr(SO_3H)	Anastasi et al (1966)
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Val-Ala-Pro-Ala-Ser	Nakajima (1968)
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Gly-Lys-Phe-His	Yasuhara et al (1973)

Fig. 4. Bradykinin analogues of nonmammalian origin.

those of bradykinin. The difference of the seryl and the threonyl residue in the bradykinin sequence was not distinguishable by ion-exchange column, paper, or thin-layer chromatography. It is hardly possible to identify either one of these by any pharmacological or chromatographical method. Further study will be necessary to differentiate these two peptides.

Figure 4 displays bradykinin and all of its analogues ever isolated from non-mammalian origins. It is common among the wasp peptides that the additional parts to the bradykinin sequence are located at the N-terminal position of the peptides. In contrast, in bradykinin analogues in the amphibian skin, the additional peptides are located at the C-terminal of the sequence. These structural relationships between the peptides in wasp venom and those in frog skin arouse great interest in comparative studies on kinin formation including formation of plasma kinin. The future work could possibly reveal the physiological significance of kinins distributed in nature.

SUMMARY

Six kinds of active principles which contract the isolated rat uterus, guinea pig ileum, and are hypotensive, have been demonstrated in the venom of the Japanese wasp (*P. r. iwatai*). Three major principles were purified by chromatography and isolated in pure forms. The elucidation of chemical characteristics of these principles resulted in the presence of serotonin, Thr^6-bradykinin and Ala-Arg-(Thr^6-bradykinin). The amounts of these substances contained per one wasp, were approximately 1.2 μg (serotonin), 0.7 μg (Thr^6-bradykinin), and 0.13 μg [Ala-Arg-(Thr^6-bradykinin)], respectively.

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Vespula Kinins: New Carbohydrate-Containing Bradykinin Analogues

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INTRODUCTION

As a part of our studies on naturally occurring vasoactive peptides, we have reported the structures of two new peptides, Ranatensin Pyr-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2 (Nakajima *et al.*, 1970) and Polistes kinin Pyr-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly-Bradykinin (Nakajima *et al.*, 1967).

We now report on two new glycopeptides, Vespula kinins, isolated from the venom sacs of the yellow jacket *Vespula (Dolichovespula) maculifrons*.

MATERIALS AND METHODS

Extraction. Live insects brought to the laboratory were stored up to 4 days at 5°C before being killed by immersion in methanol. Venom sacs from 1300 insects were removed by simply pulling the lancet. Sacs were stored six months at −10°C prior to extraction. Five milliliters of 1% acetic acid was added to the 1300 sacs and the mixture was homogenized at room temperature in a

Potter-Elvehgem homogenizer. The residue obtained after centrifugation was similarly extracted five more times and the extracts were combined.

Bioassay. The smooth muscle contracting activity was determined with an isolated rat uterus preparation with bradykinin as a standard. Rat arterial blood pressure was measured through a cannula inserted in the femoral artery of 250-300-g Sprague-Dawley rats, anesthetized with sodium pentobarbital (30-40 mg/kg, ip). Pressure was recorded with a Statham P23DB transducer. Samples were administered through a cannula in the femoral vein.

Chromatography. SP-Sephadex C-25 (Pharmacia) was used to prepare two columns, one 0.9 × 55 cm and the other 0.9 × 15 cm. Conditions under which the large column was used are given in Fig. 2. Peptides adsorbed onto the small column were eluted with a linear gradient made with 60 ml each of 0.3 M ammonium formate, pH 6.5, and 1.0 M ammonium formate, pH 6.5. Active fractions were freeze dried to remove the buffer. It was often necessary to repeat freeze drying several times (by adding distilled water to the dried sample) to effectively remove ammonium formate.

Droplet countercurrent chromatography (Tanimura *et al.*, 1970) was performed with an all-teflon unit (Yoshida *et al.*, 1971, 1976) consisting of 200 columns 20 cm long made of 14-gauge tubings. The columns were connected with 24-gauge tubings.

Enzymatic Hydrolysis. The enzymes used were all purchased from Worthington Biochemical Corp.; these were trypsin-TPCK 185 U/mg, δ-chymotrypsin 45 U/mg, carboxypeptidase A-DFP 35 U/mg, carboxypeptidase B-DFP 95 U/mg, and leucine aminopeptidase 100 U/mg. The usual incubation conditions were that 1 μg of the enzyme was incubated at 25°C with 1-10 nmols of peptides for 2 hr in 100 μl of TEA-CO_2 buffer, pH 8.5.

Amino Acid Analysis. A norleucine internal standard was added to peptides prior to hydrolysis in 5.7 N HCl at 110°C for 24 hr. Amino acids were analyzed on a Beckman model 120-C analyzer using single column modification and range card for high sensitivity.

Carbohydrate Analysis. Internal standards of xylose and glucosamine were added to the samples prior to hydrolysis in 2.5 N trifluoroacetic acid at 100°C for 6 hr or in 5.7 N HCl at 110°C for 24 hr. The hydrolysates were analyzed by the methods of Tamura *et al.* (1968) and Imanari *et al.* (1969). Galactosamine was also determined with the Beckman amino acid analyzer.

Sequence Analysis. Amino acid sequences were determined by the dansyl-Edman method (Gray, 1967) with the following modifications: PTC and buffer were removed by directing a nitrogen stream for 20 min into the tube heated at 70°C instead of the use of heat and vacuum; *n*-butylchloride was used instead of *n*-butylacetate, and the aqueous phase was frozen before removal of *n*-butylchloride. Analysis of dansyl peptides was also employed (Tamura *et al.*, 1973), as well as the subtractive Edman method and direct analysis of the PTH amino

acids was performed by gas chromatography (Pisano and Bronzert, 1969). Dansyl amino acids were identified with a polyamide sheet (Woods and Wang, 1967).

RESULTS

Purification. The acetic acid extract from 1300 venom sacs tested on the rat uterus contained an activity equivalent to 1.1 mg of bradykinin. Two major peaks of activity, P-1 and P-2, were observed when the freeze dried material, 250 mg, was fractionated on a 0.9 X 55 cm column of SP-Sephadex (Fig. 2). Fractions 126–132 (P-1) and 133–143 (P-2) were combined and freeze dried. Each of P-1 and P-2 was dissolved in 100 μl of the lower (aqueous) phase and purified by droplet countercurrent chromatography (Fig. 3). Pure P-1 was obtained by this step, but pure P-2 was obtained after rechromatography on a 0.9 X 15 cm SP-Sephadex column (Fig. 1).

Characteristics of P-1 and P-2. Both P-1 and P-2 contracted rat uterus and guinea pig ileum, relaxed rat duodenum, and lowered rat blood pressure. Neither P-1 nor P-2 was inactivated by trypsin, carboxypeptidase A, or leucine amino-

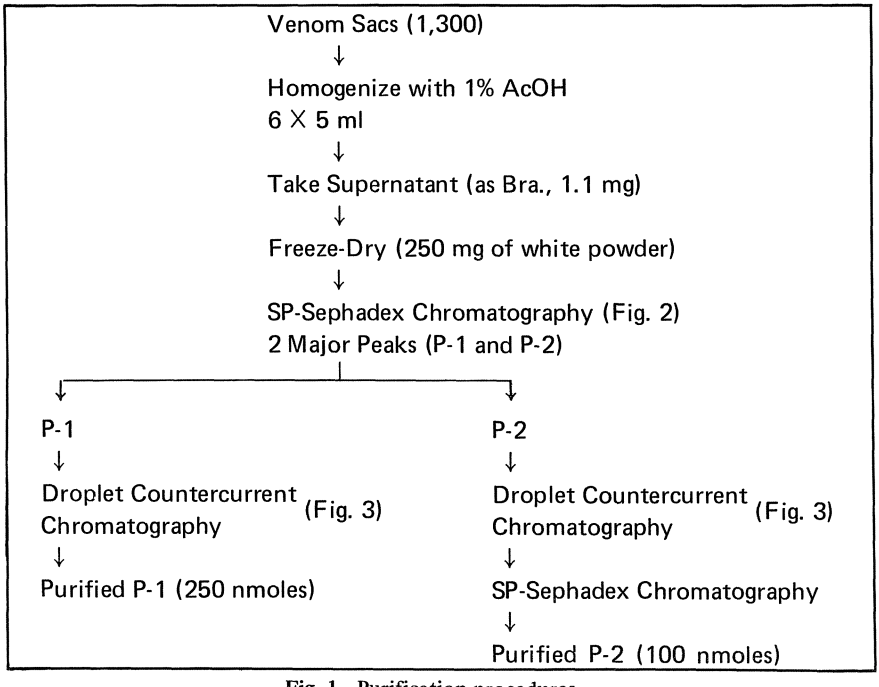


Fig. 1. Purification procedures.

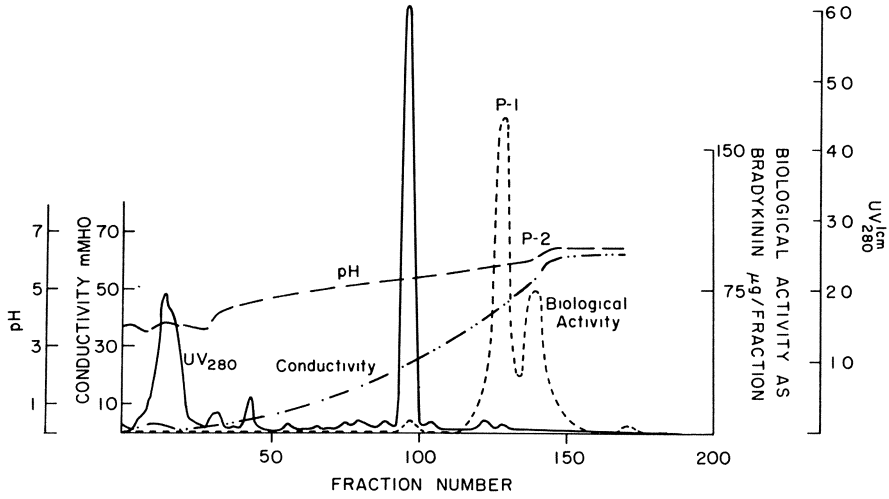


Fig. 2. SP-Sephadex chromatography of Vespula kinins. Column, 0.9 X 55 cm, equilibrated with initial buffer; elution, linear gradient elution made by a 4-chamber unit 100 ml of 0.01 M ammonium formate, pH 3.6, 100 ml of 0.05 M ammonium formate, pH 5.0, 100 ml of 0.20 M ammonium formate, pH 5.0, 100 ml of 1.00 M ammonium formate, pH 6.6; flow rate, 20 ml/hr; fraction volume, 3.3 ml.

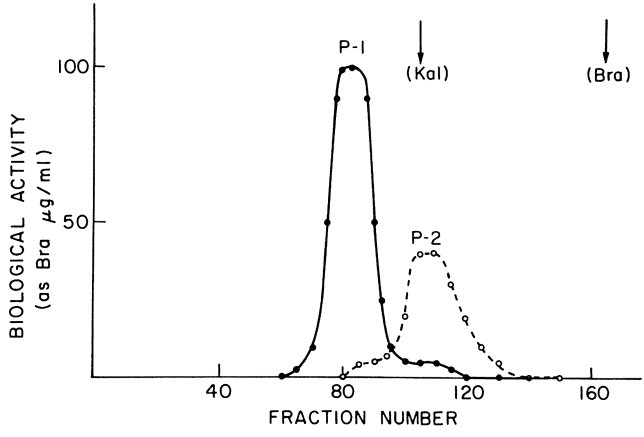


Fig. 3. Droplet countercurrent chromatography. Column, see Materials and Methods; solvent, *sec*-BuOH-trifluoroacetic acid-H_2O (120:1:160), moving phase was aqueous (lower) phase; flowrate, 2.4 ml/hr; fraction volume, 0.6 ml; elution volumes of representative peptides, kallidin (Kal) and bradykinin (Bra), are also shown.

Table 1. Amino Acid and Carbohydrate Compositions

	Arg	Thr	Ser	Pro	Gly	Ala	Phe	Galactose	Galactosamine
P-1	5	3	1	3	2	1	2	3	4-5
P-2	5	2	1	3	2	0	2	3	4-5

peptidase, but they were inactivated by chymotrypsin and carboxypeptidase B. The amino acid and carbohydrate contents are shown in Table 1.

Amino Acid Sequence of P-1. Seven cycles of the dansyl-Edman procedure gave partial sequence of P-1 as Thr-Ala-Thr-Thr-Arg-Arg-Arg- and yielded a decapeptide with N-terminal Gly having the following compositions: Arg 2, Ser 1, Pro 3, Gly 2, and Phe 2 with no galactose or galactosamine. This peptide was indistinguishable from authentic Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Gly-Bradykinin) when analyzed by thin-layer chromatography (Tamura *et al.*, 1973). The decapeptide and Gly-Bradykinin also gave the same peptide degradation products when treated with chymotrypsin and carboxypeptidase A as evidenced by the similar mobilities of the dansylated fragments on thin layer chromatograms and the same N-terminal amino acids. Thus the decapeptide was identified as Gly-Bradykinin. Gly-Bradykinin was obtained also by trypsin digestion of P-1. The amino acid sequence deduced for the native peptide was Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg.

Carbohydrate Assignment. Only dansyl threonine and no dansyl galactosamine was detected in acid hydrolysate of dansylated native peptide, indicating that the peptide contained N-blocked galactosamine such as N-acetylgalactosamine. Since no carbohydrate was detected in Gly-Bradykinin, isolated from a tryptic peptide of P-1 or by seven cycles of Edman degradation of P-1, it was concluded that carbohydrate could be linked through an O-glycosidic bond to threonine at positions 1, 3 and 4.

Four cycles of Edman degradation followed by analysis of PTH amino acids by gas chromatography and carbohydrate analysis of the aqueous layer for a cleavage product not adsorbed to SP-Sephadex (H^+) (Fig. 4 and Table 2) indicated that threonine at positions 3 and 4 contained carbohydrates (Fig. 5). Removal of carbohydrates from the native peptide by β-elimination reaction (Tanaka and Pigman, 1965) should theoretically lead to the loss of two threonines and no loss of serine. Apparently the β-elimination reaction was incomplete because the molar ratio for threonine dropped from 2.80 to 1.62 (Table 2).

The significant levels of galactosamine and galactose recovered in the fraction not adsorbed onto SP-Sephadex (H^+) after three and four cycles of Edman

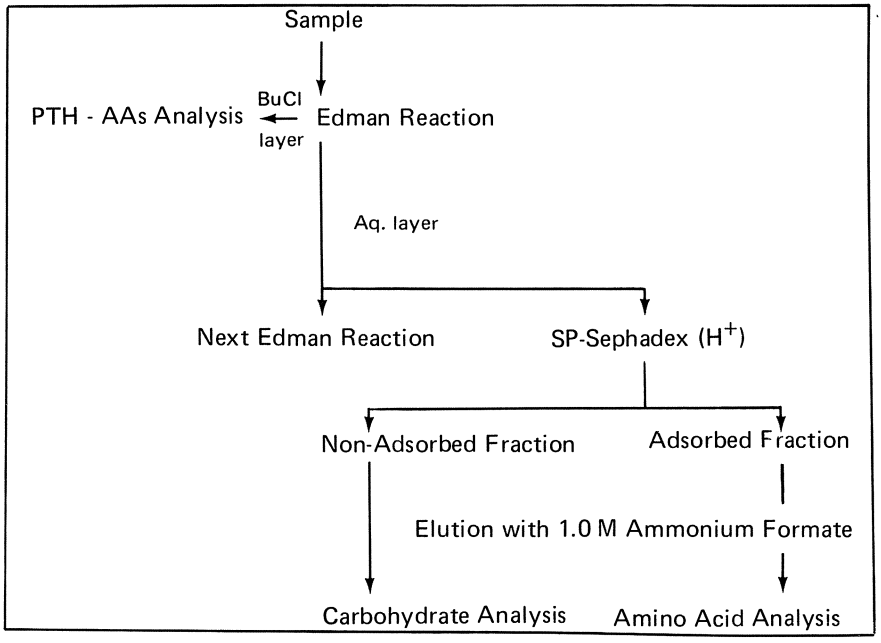


Fig. 4. Scheme for carbohydrate assignment.

degradation (Fig. 4 and Table 2) and the accounting of the four to five residues of galactosamines and three residues of galactose of the native peptide clearly pointed to the location of the carbohydrates at the threonine residues at positions 3 and 4.

PTH-Thr and PTH-Ala were identified by gas chromatography after the first and the second Edman degradations, respectively, but PTH-Thr was not identified after the third or fourth cycles.

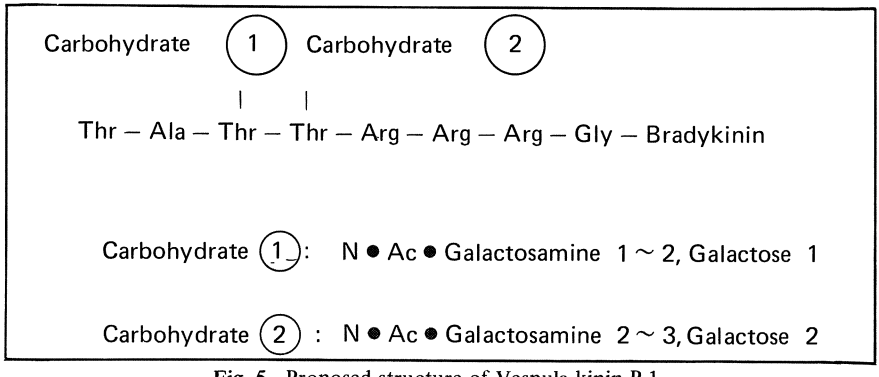


Fig. 5. Proposed structure of Vespula kinin P-1.

Table 2. Carbohydrate Assignment

	Fraction adsorbed to SP-Sephadex									Fraction not adsorbed to SP-Sephadex		PTH-AA
	Thr	Ser	Pro	Gly	Ala	Phe	Arg	Galacto-samine[a]	Galactose[b]	Galacto-samine[b]	Galactose[b]	
Native peptide	2.80	0.95	3.04	2.20	1.00	2.00	4.89	4.8	2.6	–	–	–
After 1 cycle Edman reaction	1.98	0.97	2.89	2.02	1.00	2.00	5.00	4.6	–	Trace	Not detected	Thr
After 2 cycle Edman reaction	1.95	0.99	3.03	2.11	0.22	2.00	5.07	4.3	–	Trace	Not detected	Ala (Thr: trace)
After 3 cycle Edman reaction	1.24	0.98	3.14	2.05	0.16	2.00	5.05	3.5	–	1.6	0.8	(Ala: trace)
After 4 cycle Edman reaction	0.60	0.96	2.95	2.16	0.17	2.00	4.60	1.2	–	3.0	1.8	Not detected
After β-elimination reaction	1.62	1.06	3.04	2.50	1.13	2.00	4.81	2.4	–	1.1	1.0	–

[a]Hydrolysis 5.7 N HCl 110°C 24 hr. analysis by amino acid analyzer.
[b]Hydrolysis 2.5 N TFA 110°C 6 hr. analysis by gel chromatography.

Amino Acid Sequence of P-2. Analysis of P-2 by the procedures given above including bioassay, amino acid and carbohydrate analyses, N-terminal amino acid analysis, and the formation of Gly-Bradykinin by trypsin digestion, indicated the amino acid sequence of P-2 as Thr-Thr-Arg-Arg-Arg-Gly-Bradykinin. P-2 also contained as much galactose and galactosamine as did P-1 and they were presumably attached to both threonine residues.

DISCUSSIONS

Vespula kinins may be the first reported naturally occurring vasoactive glycopeptides. The peptide most similar to Vespula kinins is Polistes kinin. Polistes kinin shares all the properties with bradykinin, i.e., it cotracts rat uters and guinea pig ileum, relaxes rat duodenum, lowers rat blood pressure, increases the vascular permeability, and produces pains. Unlike bradykinin, Polistes kinin is not inactivated by the lung and is a strong histamine releaser from mast cells.

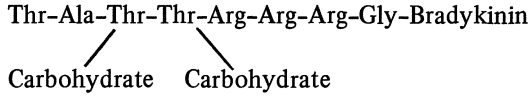
Vespula kinins may be uniquely active because of their greater basicitv and carbohydrate content. It is probable that these peptides contribute to the pain, inflammation, and swelling associated with yellow jacket stings.

SUMMARY

By the rat uterus bioassay, an activity equivalent to that of 1.1 mg of bradykinin was found in a dilute acetic acid extract of 1300 venom sacs of *V. maculifrons*.

Two major activities, P-1 and P-2, were separated on an SP-Sephadex column, and each of them was further purified by droplet countercurrent chromatography. Pure P-1 was obtained by this step: pure P-2 after rechromatography on an SP-Sephadex column.

Sequence determination of P-1 by the DNS-Edman method and carbohydrate analysis of PTH-amino acids indicated the following structure:



The other major activity, P-2, was also a carbohydrate-containing bradykinin analogue very similar to P-1.

Vespula kinins P-1 and P-2 are the first reported vasoactive glycopeptides. The high content of Arg and Thr are other notable features.

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Structure and Function of Ricin D

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Ricin D, one of the three toxic proteins present in castor bean (*Ricinus communis L.*), was purified by gel filtration on Sephadex G-75 followed by DEAE-cellulose chromatography at pH 8.5 and crystallized in the presence of 10^{-6} M cupric ion (Ishiguro *et al.*, 1971a). Terminal analysis of ricin D, giving Ile and Ala at N-termini and Ser and Phe at C-termini, suggested that ricin D consists of two peptide chains (subunits) (Ishiguro *et al.*, 1971b). After performic acid oxidation, these peptide chains, designated as Ile chain and Ala chain denoting their N-terminal amino acids, were separated by DEAE-cellulose chromatography in the presence of 8 M urea at pH 7.0 (Funatsu *et al.*, 1971a). Their amino acid compositions (Table 1) indicated that the two chains completely differed in structure. Both chains contain oligosaccharide consisting of glucosamine (G) and mannose (M). Two glycopeptides (Asx-G_2M_6-Asx-Gly-Thr and G_2M_7-Asx-Asx-Thr-Glx-Pro) were isolated from Ala chain and one (Ile-G_2M_4-Asx-Phe) from Ile chain after pronase digestion of each chain (Funatsu *et al.*, 1971a).

To find how many disulfide bonds are involved in the linkages between the two chains, reductive cleavage of disulfide bonds in ricin D with β-mercaptoethanol in the absence or presence of different concentrations of urea and 6 M guanidine-HCl was carried out. Analysis for CM-cysteine obtained by carboxymethylation after reduction at pH 8.6 revealed that a single disulfide bond was reduced in the absence of urea, about four bonds in 2-4 M, five bonds in 6-8 M urea solutions, and all six bonds only in the presence of 6 M guanidine-HCl (Funatsu *et al.*, 1971a). Ricin D, reduced in the absence of urea and carboxymethylated (R_0CM-ricin D), was found to migrate in polyacrylamide gel electrophoresis at pH 8.3 in three bands, one of which corresponded to that of native ricin D.

Table 1. Amino Acid Compositions of Ricin D and Its Subunits

	Numbers of residues		
	Ricin D	Ile chain	Ala chain
Aspartic acid	59	24	35
Threonine	35	16	19
Serine	37	17	19
Glutamic acid	51	27	21
Proline	26	14	13
Glycine	36	16	18
Alanine	37	22	15
Half-cystine	12	2	10
Valine	29	13	13
Methionine	6	3	3
Isoleucine	35	20	16
Leucine	42	21	23
Tyrosine	20	13	8
Phenylalanine	18	12	5
Lysine	9	2	7
Histidine	6	3	3
Arginine	32	18	14
Tryptophan	8	2	6
Total	498	245	248
Mannose	17	4	13
Glucosamine	6	2	4
Molecular weight	59,038	28,443	30,573

After removal of native ricin D by gelfiltration on Sephadex G-75 in deionized water, R_0CM-Ile and R_0CM-Ala chains were separated by either DEAE-cellulose chromatography at pH 8.5 (Funatsu *et al.*, 1971a) or affinity chromatography on Sepharose 4B with 5 mM phosphate buffer pH 7.1, containing 0.2 M NaCl at 7°C. The finding of one CM-cysteine in each chain proved that Ile and Ala chains were linked together by a single disulfide bond. After tryptic digestion of R_0CM-Ile chain, the CM-cysteine-containing peptide was isolated by Dowex 1 X 2 column chromatography followed by paper chromatography and then its sequence was established to be CM·Cys-Ala-Pro-Pro-Pro-Ser-Phe-Gln-Ser.

As to the optical rotation of the separated R_0CM chains, as shown in Table 2, a marked difference was observed in b_0 value. The helical contents of ricin D, R_0CM-Ile and R_0CM-Ala chains were calculated to be approximately 10.5%, 0.3%, and 25.0%, respectively, whereas the values of a_0 were almost the same (Funatsu *et al.*, 1971b). Although these results suggest that no gross alteration in the tertiary structure occurred by the cleavage of the disulfide bond connecting the two chains, the evidences that ricin D resistant to tryptic or peptic attack

became digestible, and its toxicity to the mouse decreased remarkably (Table 2) by cleavage of this interchain disulfide bond, indicate that this bond plays an important role both in formation of the rigid conformation of ricin D and in eliciting the high toxicity to the mouse.

One way to elucidate the relationship between Ile and Ala chains in eliciting the toxic action was to examine such other functions as inhibition of the *in vivo* growth of ascites tumor cells (Lin *et al.*, 1970), suppression of cell protein synthesis (Lin *et al.*, 1971; Olsnes and Pihl, 1972a,b), and the cytoagglutinating action (Nicolson *et al.*, 1974) of the separated chains. For this purpose, Sarcoma 180 ascites tumor (SA) cells were used. After intraperitoneal injection with the SA cells treated with each chain, the mice were weighed. The growth of SA cells was inhibited only by the R_0CM-Ile chain, suggesting that the toxic principle of ricin D may be the Ile chain. On the other hand, the cytoagglutinating activity was found only in R_0CM-Ala chain and its activity was twofold that of ricin D (Table 2). This indicates that the cytoagglutinating action of ricin D is exclusively associated with the Ala chain but binding of the Ile chain does not contribute to this action. On the basis of the finding that the cytoagglutinating action of *R. communis* lectin RCAII, corresponding to ricin, is due to the binding of the lectin molecule to galactoselike residues in complex cellular carbohydrates (Nicolson *et al.*, 1974), it was obvious that Ala chain moiety in ricin D would bind to galactoselike residues of the membrane constituents of SA cells. Moreover, this was supported by the facts that its cytoagglutinating action was inhibited by an addition of galactose or lactose and only the R_0CM-Ala chain retained on Sepharose.

Table 2. Properties of Ricin D and Its Subunits

	Ricin D	R_0CM-Ile chain	R_0CM-Ala chain
$s_{20,w}$ ($\times 10^{13}$)	4.64	3.45	3.52
Molecular weight (by Archibald method)	60,000	30,400	30,600
Isoelectric point (by ampholine electrophoresis)	7.34	7.42	5.17
Optical rotation			
a_0 (deg)	−138	−134	−133
b_0 (deg)	−66	−2	−158
Helical content assumed, %	10.5	0.3	25.0
Toxicity			
LD_{48} μgN/g mouse	0.001	0.029	0.30
%	100	3.5	0.33
Cytoagglutinating activity, minimum concentration, μg/ml			
Human red blood cells, 4%	140	–	75
Sarcoma ascites tumor cells, 1.7×10^7/ml	10	>100	4

The inhibitory effect of R_0CM chains on uptake of L-leucine-^{3}H into SA cells was also investigated principally according to the method of Lin *et al.* (1971). Sa cells, pretreated for 30 min with each chain, were incubated with L-leucine-^{3}H for 60 min at 37°C and then collected by centrifugation. After washing three times, the radioactivity incorporated into the cell was counted. As a result, ricin D, R_0CM-Ile and R_0CM-Ala chains inhibited approximately 75%, 13%, and 37% of the amino acid incorporation by SA cells, respectively. Lactose reduced the inhibition of the incorporation by ricin D and the R_0CM-Ala chain to about 30% and 11%, respectively, but did not affect the inhibition by the R_0CM-Ile chain. Since the inhibitory effect of the R_0CM-Ala chain on the amino acid incorporation in the cell-free system was remarkably lower than that of ricin D or the R_0CM-Ile chain (Onozaki, unpublished), the higher inhibitory action of the Ala chain toward amino acid incorporation into SA cells suggests that the Ala chain alters the conformation of the cell membrane after binding to galactoselike residues of the cell surface. From these results, it became clear that Ile and Ala chains of ricin D corresponded to the A and B chains of ricin obtained by Olsnes and Pihl (1973).

Consequently, the two separated chains of ricin D possess different functions toward SA cells. The lethal action of ricin D is due to the inhibition of protein synthesis in the cell by the Ile chain, and the Ala chain carries the Ile chain effectively on the surface of the cell, alters the property of the membrane, and accelerates the penetration of the Ile chain into the cell. With regard to the penetration of the Ile chain, following structures of Ile chain seem to be favorable: (1) the disulfide bond between the two chains is located near the C-terminus of the Ile chain and is exposed on the surface of the ricin D molecule; (2) the Ile chain contains no intramolecular disulfide bond; and (3) a continuous sequence of hydrophobic amino acids is distributed frequently in the Ile chain.

To find which amino acid residues in the Ile chain contribute to its toxic action, the properties of some modified ricin D were investigated. Although it has already been shown that acetylation of free amino groups in ricin D decreases its toxicity, maleylation of ricin D caused a more remarkable decrease in its toxicity and cytoagglutinating activity than acetylation, due probably to the introduction of negative charges. However, it was not certain how free amino groups in the Ile chain contribute to its toxic action. It was necessary, therefore, to determine the toxicity of selectively maleylated ricin D, in which only free amino groups in the Ile chain were maleylated (m-Ile-ricin D). Maleylation of ricin D was carried out at pH 9.0 and 2°C for 2 hr according to the method of Butler *et al.* (1969) by use of approximately 90-mol maleic anhydride per free amino group of ricin D. As shown in Fig. 1, a major product lost the affinity to Sepharose 4B, and its toxicity and cytoagglutinating activity were about 1.1% and less than 5% those of ricin D, respectively. The degree of maleylation was spectrophotometrically determined to be 11.6, indicating that all eleven amino

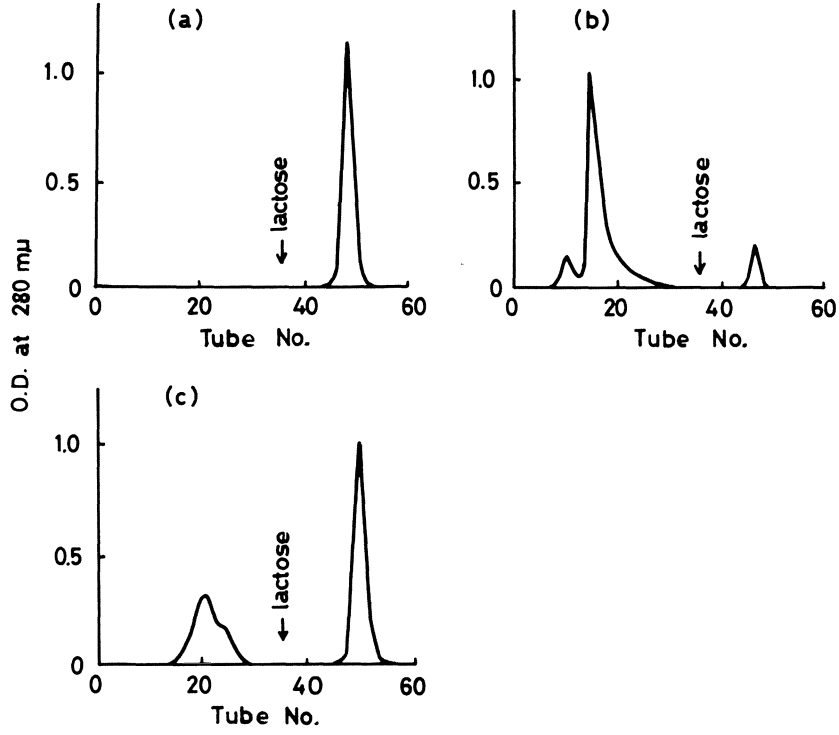

Fig. 1. Affinity chromatography of native and maleylated ricin D. Native and maleylated ricin D were applied to Sepharose 4B columns previously equilibrated with 5mM phosphate buffer, pH 7.1, containing 0.2 M NaCl and eluted with the same buffer. The adsorbed ricin D and its derivatives were eluted by adding 0.1 M lactose in the same buffer. Operation temperature was 7°C. (a) native ricin D, (b) maleylated ricin D, major fraction was referred to as maleyl-ricin D, and (c) the product obtained by reduction-reoxidation of the mixture of ricin D and maleyl-ricin D.

groups of ricin D were maleylated. m-Ile-ricin D was prepared by replacing maleyl-Ala chain in maleyl-ricin D with an unmodified Ala chain. Namely, a mixture of maleyl-ricin D and ricin D was treated with 1% β-mercaptoethanol at pH 8.6 at 30°C for 2 hr, then reoxidized by stirring at 7°C for 20 hr after removal of β-mercaptoethanol through a Bio-Gel P-10 column. The resulting products were separated by affinity chromatography on Sepharose into two fractions; unadsorbed components containing the maleyl-Ala chain as its component, and adsorbed components containing an unmodified Ala chain. m-Ile-ricin D contained in the adsorbed fraction was easily separated from unmodified ricin D in the same fraction by DEAE-cellulose chromatography at pH 8.5 (Fig. 2). m-Ile-ricin D, in which all three free amino groups of only the Ile chain

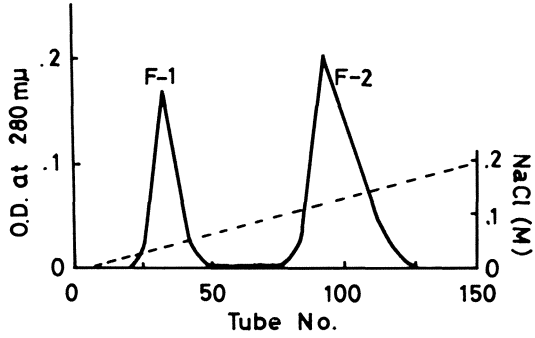

Fig. 2. Separation of native ricin D and the selectively maleylated ricin D. The adsorbed fraction in Fig. 1c was applied to a DEAE-cellulose column previously equilibrated with 5 mM Tris-HCl buffer, pH 8.5, and eluted by linearly increasing concentration of NaCl from 0 to 0.2 M in the same buffer. The first fraction was native ricin D and the second one a maleyl-Ile chain containing ricin D.

were maleylated, restored the full cytoagglutinating activity, whereas its toxicity was about 15% that of ricin D and about 14-fold that of maleyl-ricin D, clearly showing the contribution of free amino groups of the Ile chain and Ala chain to the toxic action of ricin D.

Iodination of ricin D also, decreased remarkably its toxicity. Ricin D was iodinated with KI_3 in a molar ratio of 1 : 20 at pH 7.0 and 4°C and excess iodine was removed with $Na_2S_2O_3$. The iodinated tyrosine residues were spectrophotometrically counted to be approximately five, of which three were in the Ala chain and the other two in the Ile chain. The toxicity of iodinated ricin D was markedly reduced (to about 2% that of ricin D), while the remaining cytoagglutinating activity was about 13% that of ricin D and the affinity to Sepharose was still held. These results suggest that at least five tyrosine residues are exposed on the surface of ricin D molecule and either one or two tyrosine residues in the Ile chain might be essential for the toxic action of ricin D.

We know many cases in which proteolytic degradation of biologically active proteins, especially of enzymes, furnishes important information on the relationship between their structures and functions. Although ricin D is not hydrolyzed with trypsin or pepsin as described above, it was found that nagarse, a protease from *B. subtilis*, could hydrolyze ricin D. Ricin D was digested at pH 8.0 and 30°C with 1/200 (wt./wt.) of nagarse, and the N-terminal amino acids of the products precipitable in 66% ethanol, were analyzed by the DNP method. As shown in Fig. 3, the amount of Ile decreased with digestion time and a new N-terminal Glu increased in proportion to the decrease of Ile, while that of Ala did not decrease. Partially degraded ricin D was purified by DEAE-cellulose chromatography at pH 8.5 and referred to as ricin N. Ricin N had Ala and Gln at N-termini and a slightly smaller S_{20w} value than that of ricin D. Both toxicity

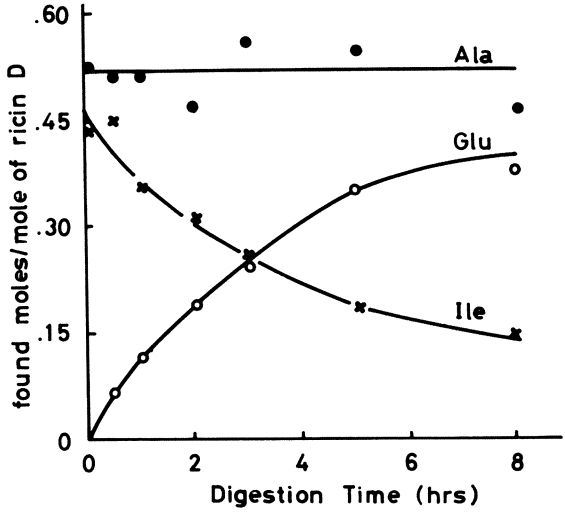

Fig. 3. N-terminal amino acids of partially degraded ricin D. Ricin D was digested at pH 8.0 and 30°C with 1/200 (wt./wt.) of nagarse and the N-terminal amino acids of partially degraded ricin D were analyzed by the DNP method.

and cytoagglutinating activity of ricin N were identical to those of ricin D, indicating that the N-terminal region of the Ile chain in ricin D is not necessary for the toxic action. Since N-terminal sequence Gln-Glu-(Ser)- · · · in the Ile chain of ricin N was not found in the N-terminal sequence of the Ile chain of ricin D: Ile-Phe-Pro-Lys-Gln-Tyr-Pro-Ile-Ile-G_2M_4-Asn-Phe-Thr-Thr-Ala-Gly-Ala-Thr-Val-Gln-Ser-Tyr-Thr-Asn-Phe-Ile-Arg- · · · , it can be concluded that at least 26 amino acid residues, including one lysine residue, and oligosaccharide moiety are released by this limited hydrolysis with nagarse. Cyanogen bromide cleavage of the Ile chain revealed that another lysine residue which might be essential for the toxic action of ricin D is located in a one-third portion of C-terminal of the Ile chain.

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The Amino Acid Sequence of the Staphylococcal Enterotoxins

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INTRODUCTION

The staphylococci produce many biologically active substances among which are the staphylococcal enterotoxins, the causative agents of staphylococcal food poisoning. The ingestion of these substances by humans produces a variety of symptoms, the most common being vomiting and diarrhea in 2-6 hr. Although this illness is not a reportable disease in the United States, a large percentage of food-borne illnesses that are reported are caused by the ingestion of staphylococcal enterotoxins.

Several enterotoxins (enterotoxins A-E) have been identified on the basis of their reaction with specific antibodies (Bergdoll, 1972). The only satisfactory methods for the detection of the enterotoxins are dependent upon the use of the antibodies specific to each of the enterotoxins, which makes the detection of enterotoxin a time-consuming task. One possibility for improving this situation is the development of a common antibody based on some common structure of the enterotoxins. To determine whether such a structure exists it is necessary to elucidate the detailed structure, such as the amino acid sequence of several of the enterotoxins. The sequence of enterotoxin B has been determined (Huang and Bergdoll, 1970) and currently the sequence of enterotoxin A is under investigation. In this paper the preliminary findings from the enterotoxin A sequence studies are compared to those reported for enterotoxin B.

MATERIALS AND METHODS

The enterotoxin A used for these studies was purified by the method of Schantz *et al.* (1972).

The methods used in the sequencing are essentially those previously reported for the sequencing of enterotoxin B (Huang and Bergdoll, 1970).

RESULTS AND DISCUSSION

A comparison of the amino acid residues in enterotoxins A and B are given in Table 1. The residues found in the cyanogen bromide peptides agree very well with those reported for enterotoxin A (Schantz *et al.*, 1972) with the exception of tyrosine. Tyrosine residues are easily destroyed by acid hydrolysis,

Table 1. Amino Acid Residues in Enterotoxins A and B

Amino acid residue	Enterotoxin		
	A[a]	A[b]	B[c]
Lys	26	25	33
His	7	7	5
Arg	7	7	5
Asp	37	38	44
Thr	17	17	13
Ser	12	10	14
Glu	27	27	20
Pro	4	4	6
Gly	14	15	9
Ala	7	8	5
½ Cys	2	2	2
Val	12	14	16
Met	2	2	8
Ile	10	10	9
Leu	27	25	16
Tyr	14[d]	19	21
Phe	8	8	12
Trp	2	2	1
Total	235[d]	240	239

[a] Cyanogen bromide peptide composition.
[b] Schantz *et al.*, 1972.
[c] Huang and Bergodoll, 1970.
[d] Not complete.

hence, only after the sequence of all of the tryptic peptides from the cyanogen peptides is completed can they be accounted for.

There is a large number of basic amino acid residues in both enterotoxins with lysine residues predominating. There is, also, a large number of aspartic and glutamic acid residues in both, the total number being the same with some difference in the ratio of the two types of residues between the enterotoxins. There are differences in the numbers of the neutral amino acid residues in the two enterotoxins, but this is probably of no great significance.

One difference in the two enterotoxins that should be pointed out is in the number of methionine residues, 2 and 8 for A and B, respectively. The only significance that this appears to have is in employing the cyanogen bromide procedure, which splits the molecule at the methionine residues, in the sequence studies.

One major difference in the two enterotoxins is that in enterotoxin A the N-terminal residue has not as yet been identified. Originally this was thought to be alanine (Bergdoll, 1970) as analysis of the enterotoxin A first purified indicated it to be. The first scheme developed for purification of enterotoxin A yielded two fractions containing enterotoxin A, one being more basic than the other. The enterotoxin purified from the more basic fraction contained alanine as the N-terminal residue while the enterotoxin purified from a mixture of the two fractions gave a very low recovery of alanine as the N-terminal residue. No second residue was identifiable. The purified enterotoxin obtained by Schantz *et al.* (1972) with a mutant (Friedman and Howard, 1971) of the strain originally used for the purification gave no indication of alanine as the N-terminal residue. No attempts to repeat the original work have been made, but may be necessary to completely resolve this mystery. There is some indication that a proline residue is adjacent to the blocked N-terminus.

The C-terminal portion of enterotoxin B contains three terminal residues of lysine while enterotoxin A contains serine as the C-terminal residue, as shown below (It is observed that several of the residues in the C-terminal portion are identical or very similar if the third lysine residue of enterotoxin B is lined up with the C-terminal serine of enterotoxin A. Whether this part of the molecule is of significance is not known):

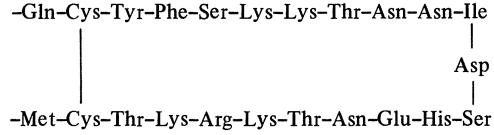
-Arg-Asp-Asn-Lys-Thr(Ser)-Ile-Asp-Ser-(Thr)-Glu-Asn-Met-His-Ile-Asp-
Ile-Tyr-Leu-Tyr-Thr-Ser (COOH)

Enterotoxin A

-Asn-Asn-Asp-Lys-Met- -Val-Asp-Ser- -Lys-Asp-Val-Lys-Ile-
Glu-Val-Tyr-Leu-Thr-Thr-Lys-Lys-Lys (COOH)

Enterotoxin B

One major similarity in the enterotoxins is the two half-cystine residues which may be a part of the most important sequence of the molecule. In enterotoxin B the number of residues between the two half-cystines is nineteen and this part has been labeled the cystine loop since the two half-cystines are joined in the native toxin as shown below:



Only a part of the cystine loop in enterotoxin A has been revealed so far, the parts involving the two half-cystine residues. These appear to be quite important as one of the half-cystine residues is part of a seven amino acid sequence that is identical to this part of the enterotoxin B chain as shown below:

-Thr-Ala-Cys-Met-Tyr-Gly-Gly-Val-Thr-Leu-His-Asp-Asn-Asn-Arg-Leu-
Thr-Glu-Glu-Lys-

Enterotoxin A

-Lys-Thr-Cys-Met-Tyr-Gly-Gly-Val-Thr-Glu-His-Gly-Asn-Asn-Glu-Leu-
Asp-Lys-Tyr-Tyr-

Enterotoxin B

It is proposed that this part of the molecule composes the active site since the activity of all of the enterotoxins appears to be identical. This will only be proved when the amino acid sequence of other enterotoxins is revealed.

There are similarities in the amino acid sequence of the two enterotoxins which involves the other half-cystine residue as shown below:

-Val-Asp-Leu-Gly-Ala-Tyr-Tyr-Gly-Tyr-Gln-Cys-(Thr,Asx,Pro,Gly,Gly,Ala)-Lys-

Enterotoxin A

-Val-Asp-Val-Phe-Gly-Ala-Asn-Tyr-Tyr-Gln-Cys-Tyr-Phe-Ser-Lys-Lys-Thr-Asn-

Enterotoxin B

It is proposed that this part of the molecule may be the major antigenic site since the sequence of the two enterotoxins is somewhat similar in this area but not identical, with each having three tyrosine residues in close proximity. One other point of interest is that there are no lysine residues within close proximity to either of these areas and according to Spero *et al.* (1971) lysine residues are not involved in either the toxic or antigenic sites.

Information available about the enterotoxins shows that they are a closely related group of proteins with a common toxic action. We believe that the site responsible for the toxic action is a common one in all of the enterotoxins and

is not involved in the antigenicity of the toxins. Further studies on the chemistry of enterotoxins should be very helpful in relating structure to antigenicity.

SUMMARY

The amino acid sequence of enterotoxin B and preliminary information on enterotoxin A showed a seven residue sequence including the half-cystine residue at position 112 (enterotoxin B) to be identical in the two enterotoxins. This portion of the molecule is proposed as the site responsible for the biological activity. The sequence around or near the other half-cystine residue (position 92 in enterotoxin B) contains three tyrosine residues in each of the enterotoxins but not in identical order. This area is proposed as the major antigenic site. There are similarities in the C-terminal portion of the molecules but this is not thought to be of importance. One major difference in the enterotoxins is that the N-terminus of enterotoxin A is blocked.

ACKNOWLEDGMENTS

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Production, Isolation, Chemistry, and Biological Properties of *Penicillium roqueforti* Toxin

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INTRODUCTION

Penicillium roqueforti has been used in food manufacturing since 500 A.D. (Matheson, 1921). Recently *P. roqueforti* from silage and milled rice in Japan was reported to produce toxic metabolites (Kanota, 1970; Kurata *et al.*, 1968). In Wisconsin, *P. roqueforti* was found as the principal microorganism isolated from ground moldy mixed grains and corn silage associated with cases of bovine abortion and placental retention (Still *et al.*, 1972). Both the *P. roqueforti* isolate from toxic moldy feeds and *P. roqueforti* NRRL 849 were found to produce a chloroform extractable toxin which was lethal to rats and mice by oral or i.p. (intraperitoneal) administration. The toxin was first isolated in pure form and partially characterized by Wei *et al.* (1973). More recently, the structure of the toxin has been fully elucidated (Fig. 1) (Wei *et al.*, 1975) and some of its biochemical effects have been studied. The present paper contains a brief review of research carried out in our laboratories on PR (*P. roqueforti*) toxin and includes some recent advances.

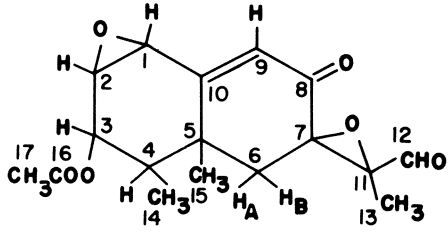

Fig. 1. Structure of PR toxin (I), $C_{17}H_{20}O_6$, M.W. 320.

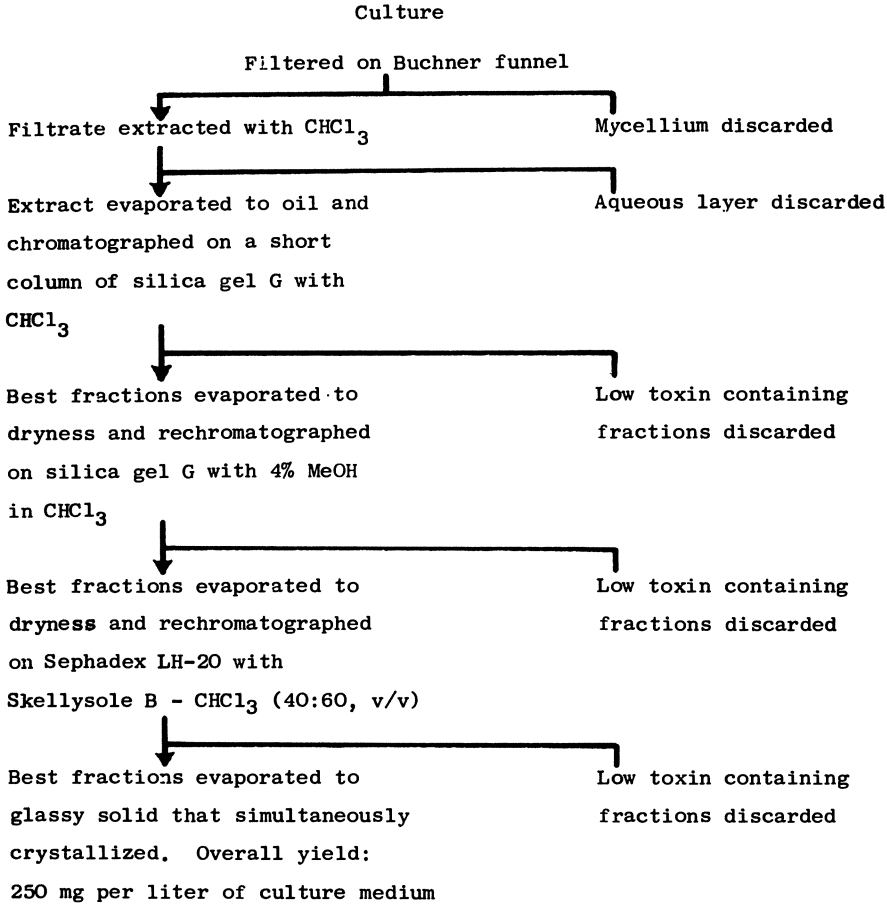

Fig. 2. Scheme for isolation and purification of PR toxin.

MATERIALS AND METHODS

Production and Isolation of PR Toxin. A semisynthetic medium containing 150 g sucrose and 20 g yeast extract (Difco) per liter was used for the mold culture. One-half liter Fernbach flasks containing 150 ml of medium per flask were autoclaved, inoculated with an aqueous spore suspension of *P. roqueforti*, and statically incubated at 24°C. Aliquots of the culture broth were extracted with chloroform and examined for toxin production each day of incubation by thin-layer chromatography. The cultures were harvested at the peak of toxin production, which usually came around the 13th day of incubation. Isolation and further purification of the toxin was carried out as represented schematically in Fig. 2.

Chemical Studies. Detailed methods for the chemical transformations of PR toxin to the related compounds II-VII (Fig. 3) have been described elsewhere (Wei *et al.*, 1973, 1975). Infrared spectra were recorded on a Beckman IR-5 infrared spectrophotometer, and ultraviolet spectra on a Beckman DB spectrophotometer.

***In Vivo* Uptake of ^{3}H-Valine in Rats.** Male rats, of the Sprague-Dawley strain, were i.p. administered 0.2-1.2 mg of PR toxin per 100 g body weight. After 110 min, or 10 minutes before sacrifice, 50 μCi/100 g body weight of L-(G-^{3}H)-valine was injected by the same route. The livers were removed and homogenized in ice cold 10% trichloroacetic acid. Protein was fractionated according to the method of Feng *et al.* (1972) for radioactivity measurements.

Synthesis of Macromolecules in Tumor Cells. Studies on the synthesis of DNA, RNA, and protein in Ehrlich ascites tumor cells were conducted according to the method of Ueno (1970).

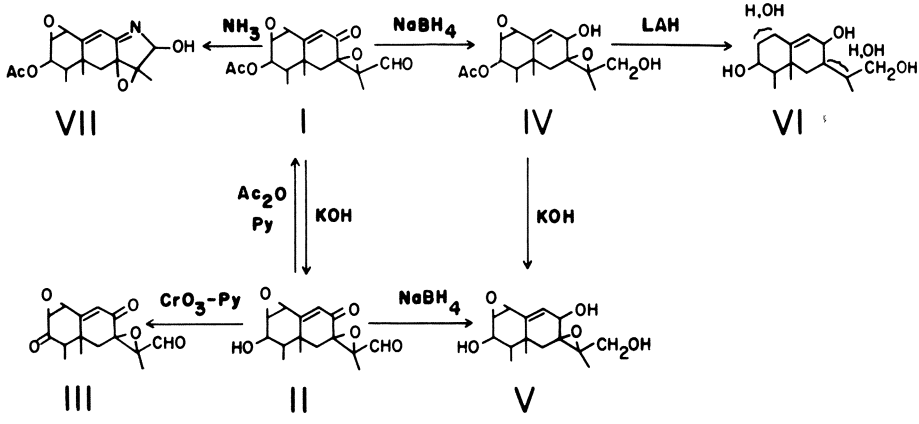

Fig. 3. Chemical transformation and derivatives of PR toxin.

RESULTS AND DISCUSSION

Production and Isolation of PR Toxin. Representative results of PR toxin production, as measured by thin-layer chromatography of the chloroform extracts vs time of culture harvest, are shown in Fig. 4. PR toxin appeared only between the 9th and 13th days of incubation. During the early stages, two major spots (A at higher and B at lower rf values) (Fig. 4) were closely associated with the toxin. The lower rf value compound was easily removed from the toxin by chromatographing on a short column of silica gel G developed with chloroform. The higher rf value compounds were separated from PR toxin by chromatographing on Sephadex LH-20 with Skellysolve B–chloroform (40:60, vol./vol.). At the 13th day of incubation, in this particular batch, PR toxin was the major component. Cultures harvested at such an optimal time not only gave higher

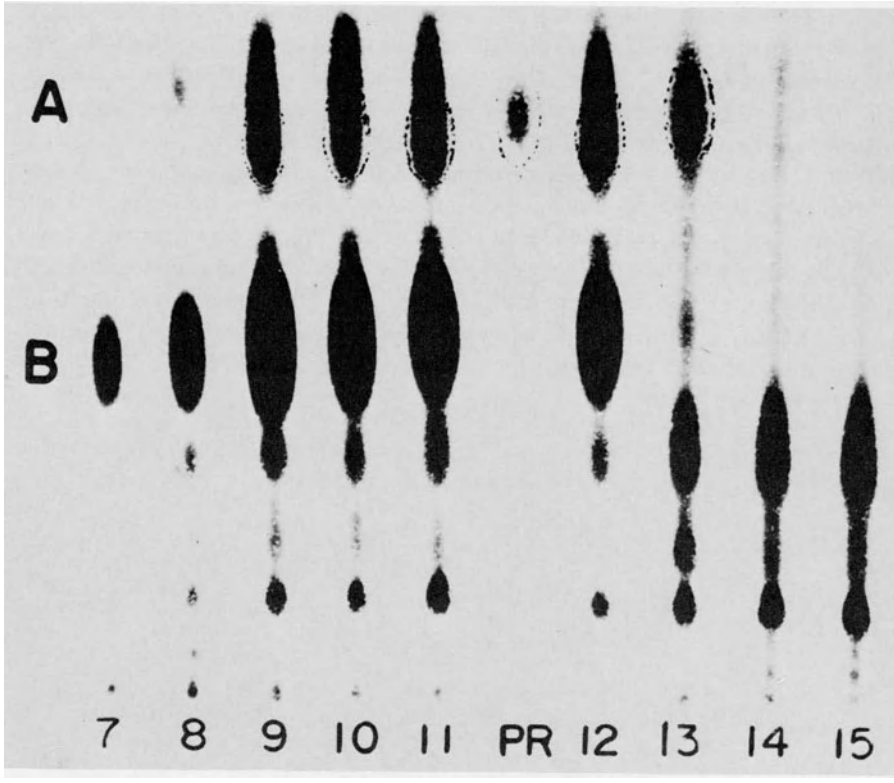

Fig. 4. Silica gel G thin-layer chromatograms of chloroform extract of *Penicillium roqueforti* culture broth. Numbers indicate the day of harvest. PR is PR toxin; A and B are impurities of unknown composition. The plate was developed with MeOH–$CHCl_3$ (4:96, vol./vol.), colored by charring with H_2SO_4.

yields of the toxin, but the further purification process was also much easier. Both compounds A and B were not toxic to mice. Their chemical natures are under investigation.

Chemistry of PR Toxin. Pure PR toxin is a colorless crystalline substance, m.p. 155–157°C, $[\alpha]_D^{25°}$ + 290 (c 1.34 in $CHCl_3$), and a molecular weight of 320. Elemental analyses indicated the molecular composition $C_{17}H_{20}O_6$. The UV spectrum showed a conjugated ketone chromophore in a ring system [λ_{Max}^{EtOH} 249 nm (ϵ, 15,278)].

The infrared spectrum contained no hydroxyl absorption, but exhibited three carbonyl bands at 1735, 1720, and 1680 cm^{-1}, indicating the presence of an ester and of conjugated and saturated carbonyl functions, respectively. From the PMR spectrum, an acetoxy group, an aldehyde function, an isolated ethylenic hydrogen, a disubstituted oxirane system, and a secondary and two tertiary methyl groups could be identified directly. These results, combined with NMR decoupling experiments, and extensive spectral data of the various chemical transformation products identified in Fig. 3, then led to assignment of the structure depicted in Fig. 1 to PR toxin. A full discussion of the structural evidence, together with detailed spectral data on all derivatives and including the 100 MHz PMR, 22.63 MHz ^{13}C NMR, and mass spectrum of PR toxin, has been published elsewhere (Wei *et al.*, 1975).

When exposed to UV light in ethanolic solution, PR toxin rapidly turned yellow, the 249-nm peak collapsed to a broad band around 245 nm, and a new peak simultaneously appeared at 400 nm (Fig. 5). The IR spectra of the toxins isolated from the cultures of *P. roqueforti* NRRL 849 and from the toxic moldy feed are shown in Fig. 6. The spectra are identical with each other.

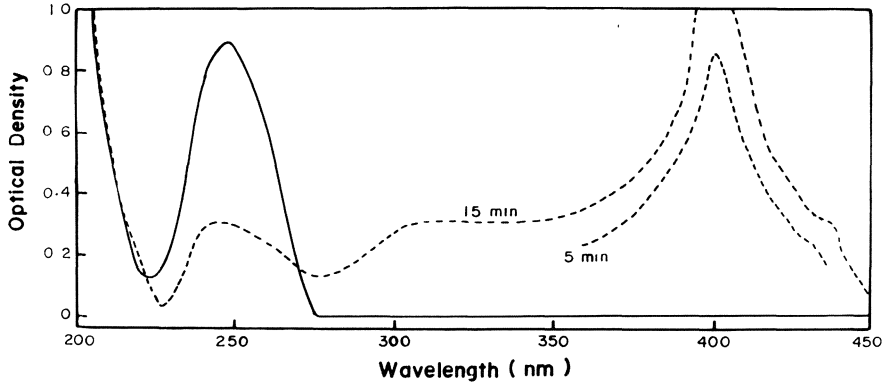

Fig. 5. Ultraviolet absorption spectrum of PR toxin (solid line) and of its UV radiation (dashed line) product. The toxin was dissolved in ethanol (15 μg/ml) and irradiated at a distance of 4 cm by Mineralight UVS 11, Ultra-Violet Products, Inc. San Gabriel, California.

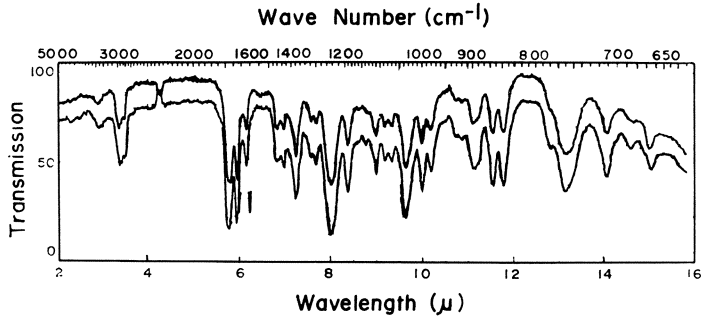

Fig. 6. IR spectra of PR toxin. The samples were dissolved in $CHCl_3$ and deposited on demountable cells as thin films by solvent evaporation. Top curve: sample from the culture isolated from moldy grain. Bottom curve: product from *Penicillium roqueforti* NRRL 849.

Toxicity and Biochemical Effects. The i.p. LD_{50} levels of PR toxin in weanling rats and mice were 11 and 5.8 mg/kg, respectively. The oral LD_{50} values were about 10 times larger. Within 10 min after an i.p. or oral lethal dose, the animals lost the ability to support their weight or developed breathing difficulties. The blood pressure and heart rate of the animals were decreased. Death usually occurred in a few hours to a few days.

A single injection of PR toxin strongly inhibits *in vivo* protein synthesis in the livers of rats. A straight line dose-response curve was obtained when the concentration of toxin was plotted against percentage of inhibition (Fig. 7).

The rates of DNA, RNA, and protein syntheses in *in vitro* Ehrlich ascites tumor cells (Table 1) were inhibited by 60% at a PR toxin concentration of 3.12×10^{-5} M, and by 97% at a concentration of 1.56×10^{-4} M. PR alcohol

Table 1. Inhibition of DNA, RNA, and Protein Syntheses in Ehrlich Ascites Tumor Cells

Compound	Concentration of toxins, M	Inhibition, %		
		DNA	RNA	Protein
PR toxin, I	0	0	0	0
	3.12×10^{-5}	76.6	78.3	63.2
	1.56×10^{-4}	97.7	98.9	98.6
PR alcohol, II	3.60×10^{-5}	79.0	64.0	58.5
	1.80×10^{-4}	98.6	98.3	98.4
PR imine, VII	3.14×10^{-5}	10.7	14.4	21.8
	1.57×10^{-4}	74.3	66.8	63.9
Tetrahydro-PR toxin, IV	3.09×10^{-5}	4.3	14.5	0
	1.55×10^{-4}	25.7	39.1	0

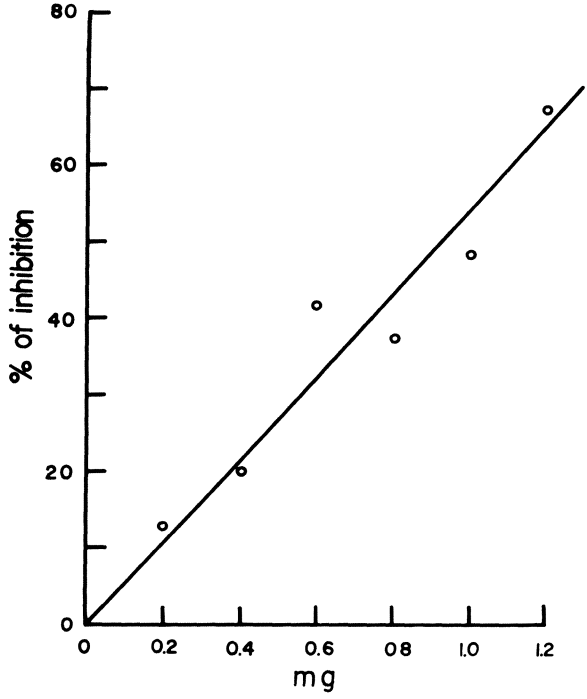

Fig. 7. Inhibition of protein synthesis in rat livers. Each value represents the mean from 4 rats injected with PR toxin (mg/100-g body weight).

(II) (Fig. 2), a deacetylation product, gave a similar inhibitory action to the parent toxin. The activity was apparently reduced when the aldehyde and ketone functions were altered by ammonia to form a five-membered ring compound, PR imine, VII. Reduction of the aldehyde and ketone groups to the corresponding alcohols resulted in loss of most of the activity. The mechanism of these inhibitory actions has not been established.

SUMMARY

P. roqueforti NRRL 849 grown on 2% yeast extract-15% sucrose broth were found to produce a new mycotoxin. The toxin has been isolated into pure form from the medium by chloroform extraction and column chromatography. Based on the results of analytical data, specific chemical transformations, and UV, IR, MS, and NMR spectroscopy, a structure for this toxin is proposed. The toxin has been shown to have a strong inhibitory effect on the biosynthesis of DNA, RNA, and protein.

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Structure of a Toxic Phospholipid in the Northern Blenny Roe

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INTRODUCTION

The roe of northern blenny or Japanese prickleback *Stichaeus grigorjewi* (Herzenstein) contains a toxic phospholipid inducing the severe gastrointestinal disorders in warm blooded animals. We designated it as dinogunellin and demonstrated that it has two moles of ammonia and one mole each of adenine, aspartic acid, glycerol, and phosphorus. We obtained also an unknown nitrogenous compound (compound A) in the crystalline state by mild base hydrolysis (Hatano and Hashimoto, 1974).

In the present study, we found that compound A possesses all of the nitrogen and phosphorus atoms of dinogunellin and represents the part other than monoacylated glycerol. When hydrolyzed with snake venom, it gave adenosine, phosphate, and a substance (compound B) considered to be 2-aminosuccinamide. Compound A was thus postulated to consist of the 5′-adenylic acid and 2-amino-

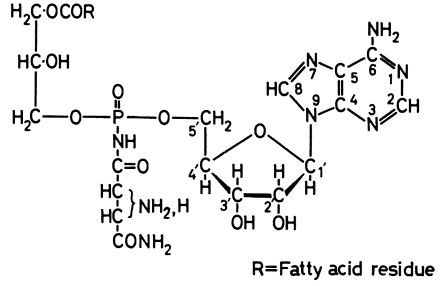

Fig. 1. Structure of dinogunellin.

succinamide moieties. In addition, the IR (infrared) spectrum and stability in hydrolysis suggested that compound B is linked to 5′-adenylic acid through a nitrogen–phosphorus (N—P) bond. Since compound A gave N-dinitrophenylaspartic acid in dinitrophenylation followed by acid hydrolysis, the 2-aminosuccinamide moiety was considered to have a free amino group and consequently to unite at one of its two amide groups with the phosphorus atom of 5′-adenylic acid. Supported by the NMR spectrum of compound A and other evidences given in our previous paper, we postulated compound A to be adenosine-5′-phosphor-*N*-[(2-(or 3)-amino-3-carbamoyl)-propionyl] amidate and dinogunellin to have the structure, in which 1-monoacylated glycerol is linked at the 3-position to the 5′-adenylic acid moiety of compound A through a phosphate ester linkage, as shown in Fig. 1. The position of an amino group in the 2-aminosuccinamide moiety is still obscure.

MATERIALS AND METHODS

For preparation of compound A, approximately 700 mg of dinogunellin was hydrolyzed by the method of Dawson (1960) and treated with Amberlite IRC-50 (H^+ form), as previously reported (Hatano and Hashimoto, 1974). After shaking with 2-butanol-chloroform, 1:2, to remove fatty acids, the aqueous layer was condensed to 10 ml, put onto a column (1.9 X 87 cm) of Sephadex G-10, and eluted with water. The eluate was collected in 5-ml portions and checked by UV absorption at 260 nm. The fractions (nos. 21–35) containing compound A were condensed to approximately 10 ml and left to stand at 0°C. The resulting crystals were recrystallized three times from hot water in needles. The yield was 10.5% in average in four batches of preparation.

Determination of UV and IR spectra, thin-layer chromatography, and analyses for amino acids, amide-nitrogen, adenine, and phosphorus were all carried

out as reported previously, unless otherwise stated. Molecular weight was estimated in water with a Hitachi vapor pressure osmometer (model 115).

Ribose was identified and estimated by hydrolyzing a 3.4-mg portion of compound A in 1 N HCl at 110°C for 1 hr. The hydrolysates were passed through a column (0.8 X 3 cm) of charcoal to remove adenine. The eluate with water was neutralized with Amberlite IR-4B (HCO_3^- form) and used for both estimation by the orcinol-HCl method (Mejbaum, 1938) and identification by thin-layer chromatography. To detect adenosine, a 1.0-mg portion of compound A was heated in 1 ml of 20% ammonia at 180°C for 3 hr in a sealed tube. The reaction mixture was condensed to remove ammonia, applied to a column (0.8 X 15 cm) of Dowex 1-X2 (Cl^- form), and eluted with a solvent system, 0.2 N ammonium hydroxide-0.02 N HCl-0.01 N sodium borate (Seki *et al.*, 1969). The elution was monitored by UV absorption at 260 nm, and the substance appearing as a peak was subjected to thin-layer chromatography.

To check for the presence of free amino groups, compound A (0.8 mg) was treated with 1-fluoro-2,4-dinitrobenzene as usual. The dinitrophenylated compound was then hydrolyzed in 6 N HCl at 105°C for 16 hr and N-dinitrophenyl-amino acid was examined by thin-layer chromatography on Avicel SF plates with 1.5 M phosphate buffer (pH 6.0) or phenol-water, 4:1. Spots were observed under a UV lamp.

For measurement of NMR spectrum, compound A (20 mg) was dissolved in 0.4 ml of d_6-dimethylsulfoxide (d_6-DMSO) or a mixture of d_6-DMSO and deuterium oxide (D_2O), and measured at 100 MHz by a Varian HA-100 high-resolution NMR spectrometer by using tetramethylsilane (TMS) as an internal standard.

Compound A was hydrolyzed with the venom of *Habu*, *Trimeresurus flavoviridis*, and that of rattlesnake, *Crotalus adamanteus*. A 58-mg portion was dissolved in a mixture of 4.2-ml 1.0 M glycine-NaOH buffer (pH 8.6), 4.2-ml 0.1 M magnesium chloride, 50-mg *C. adamanteus* venom, and 43.6-ml water, and the mixture was kept at 37°C for 3 hr. The hydrolysis products were filtered with a Zeineh microcondenser and the filtrate was lyophilized. The residue was dissolved in 2.0 ml of water, adjusted to pH 3.5 with acetic acid and applied to a column of charcoal (Wako Pure Chemicals), previously washed with 250 ml of water. The column was then washed with 200 ml of water and eluted with 500-ml 2% ammonia in 50% ethanol. The eluate was dried up and to this was added nearly 1 ml of water and one drop of 60% perchloric acid. The precipitate (approximately 10 mg) was recrystallized once from water and twice from methanol into needles (compound B). The yield was about 3 mg. A small portion of compound A was similarly hydrolyzed with snake venom and the hydrolysis products were examined by paper chromatography on Whatman no. 1 filter paper with 1-butanol-acetic acid-water, 5:2:3, as solvent.

RESULTS

Properties of Compound A. Compound A is soluble in hot water, slightly soluble in cold water, but not soluble in most fat solvents. It decomposes at 234-236°C. Analysis Found: C 36.61%, H 4.84%, N 23.71%, P 6.69% and molecular weight 459. Calculated for $C_{14}H_{21}N_8PO_8$: C 36.53%, H 4.60%, N 24.34%, P 6.73% and molecular weight 460. The UV spectrum shows an absorption maximum at 257 nm in 0.01 N HCl (ϵ 14,000) and at 259.5 nm both in water (ϵ 13,900) and in 0.01 N NaOH (ϵ 14,400). It is positive to ninhydrin, biuret-phosphomolybdotungstic acid, periodate-benzidine, and molybdate-perchloric acid reagents, but negative to Dragendorff reagent. It is very dull to ferric chloride-sulfosalicylic acid reagent. The ratios of adenine, ribose, aspartic acid, and amide-nitrogen to phosphorus were 0.9, 1.0, 1.0, and 1.8, respectively. On hydrolysis with 20% ammonia, a base identical with the authentic specimen of adenosine, both in elution pattern from a Dowex 1-X2 column and in thin-layer chromatography, was recognized. When compound A was hydrolyzed with the snake venom and examined by paper chromatography, adenosine was detected at rf 0.54, phosphate at rf 0.34, and compound B at rf 0.23. There was no difference in the mode of action between *T. flavoviridis* and *C. adamanteus* venoms, and the same patterns of degradation products were observed. In dinitrophenylation followed by acid hydrolysis, N-dinitrophenylaspartic acid was recognized at rf 0.78 when developed with 1.5 M phosphate buffer and at rf 0.53 with phenol-water, 4:1. Compound A does not form ammonium salt. These results suggest that the 2-aminosuccinamide moiety has a free amino group.

The IR and NMR Spectra of Compound A. The IR spectra of compound A and dinogunellin are given in Figs. 2 and 3, and the NMR spectrum of the former in Fig. 4. In the IR spectrum, compound A reveals absorption bands at 1680 cm^{-1}, assignable to C=O of $-CONH_2$ and at 1300 and 1245 cm^{-1}, assignable to phosphate. Absorption bands at 1130-1090 cm^{-1} may be assignable to C—N bond of phosphoramidate and those at 1040-820 cm^{-1} to N—P bond of phos-

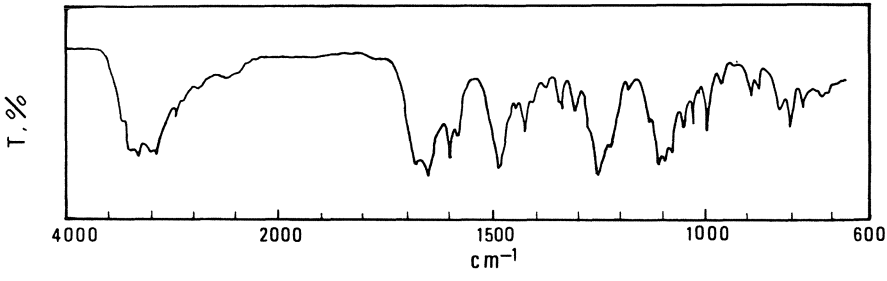

Fig. 2. IR spectrum of compound A in KBr.

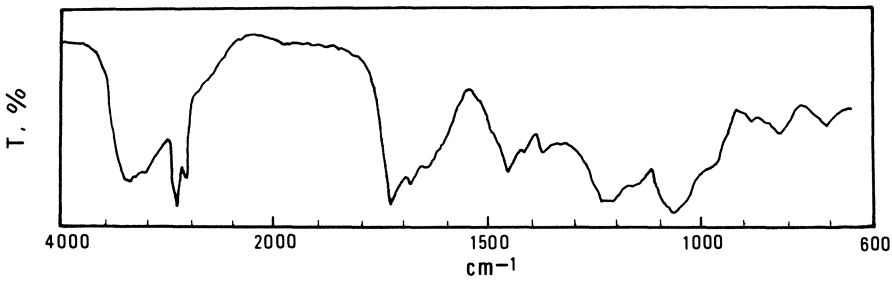

Fig. 3. IR spectrum of dinogunellin in KBr.

phoramidate (Chittenden and Thomas, 1966). The IR spectrum of dinogunellin is similar to that of compound A and reveals absorption bands at 3330-3100 cm^{-1} (N—H and O—H), at 3030-2950 cm^{-1} ($-CH_2$ and $-CH_3$) and at 1735 cm^{-1} (ester C=O), which indicate the presence of fatty acid moiety.

The NMR spectrum of compound A is very close to that of adenosine. The following signals are recognized; singlets at 8.10 (1H, H_2) and 8.52 (1H, H_8), doublet at 5.93 (1H, J = 5Hz, $H_{1'}$) and multiplets at 2.75 (2H, 2-aminosuccinamide, $-CH_2-$), 4.00 (2H, $2H_{5'}$), 4.22 (2H, $H_{3'}$, and $H_{4'}$), 4.54 (1H, $H_{2'}$), 5.50 (2H, 2'—OH and 3'—OH), 7.20 (4H, 6—NH_2 and 2-aminosuccinamide, $-NH_2$) and 7.84 (2H, 2-aminosuccinamide, $-CONH_2$). The resonances at 5.50, 7.20, and 7.84 disappeared in D_2O. The doublet at 5.93 was spin-spin coupled with the multiplet 4.54.

Properties of Compound B. Compound B was obtained as perchlorate from the hydrolysis products with snake venom. It showed fairly good agreement in

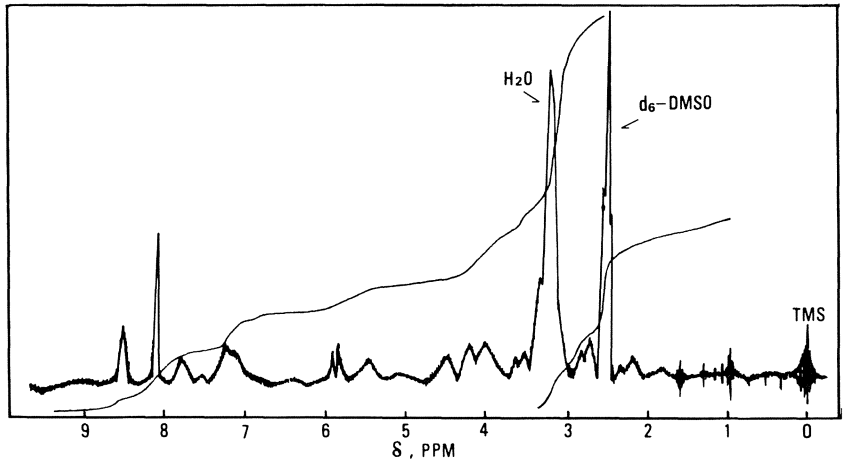

Fig. 4. NMR spectrum of compound A in d_6-DMSO.

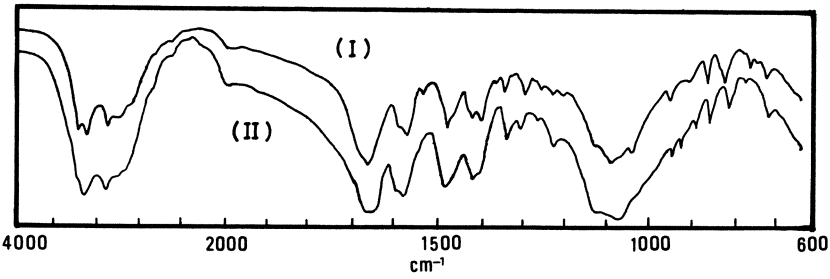

Fig. 5. IR spectra of compound B (I) and 2-aminosuccinamide perchlorate (II) in KBr.

thin-layer chromatography and IR spectrum with the authentic specimen of 2-aminosuccinamide perchlorate kindly supplied by Dr. H. Hirano, who synthesized it. On Avicel SF plates developed with 1-butanol-acetic acid-water, 5:2:3, compound B gave the main spot at rf 0.25 which coincided well with that of 2-aminosuccinamide perchlorate. A faint spot of adenosine was also recognized even after repeated recrystallization. The IR spectra of Compound B and authentic 2-aminosuccinamide perchlorate are shown in Fig. 5. Except for a few insignificant peaks at around 1300 and 750 cm^{-1}, both compounds show a good agreement.

DISCUSSION

Dinogunellin was found to be a unique phospholipid having both the adenosine moiety and a nitrogen-phosphorus bond. The UV spectrum of compound A indicated the presence of 9 substituted adenine and the positive reaction to periodate-benzidine reagent the presence of 2′- and 3′-OH of ribose. The hydrolysis products, especially those obtained with the snake venoms, suggested that dinogunellin contains the 5′-adenylic acid moiety. This was unequivocally supported by the NMR spectrum.

It was reported that the N—P bond is fairly stable to base but labile to acid (Moffatt and Khorana, 1961), and hydrolyzed with the rattlesnake (*C. adamanteus*) venom (Chambers and Moffatt, 1958). Degradation products obtained from dinogunellin or compound A were not contradictory to these reports.

The origin of two moles of ammonia and one mole of aspartic acid is reasonably ascribable to the 2-aminosuccinamide moiety. Since compound A afforded N-dinitrophenylaspartic acid, the 2-aminosuccinamide moiety is considered to have a free amino group. Consequently, one of its two amide groups should participate in the N—P linkage. Thus, the structure of compound A was assumed to be adenosine-5′-phosphor-*N*-[(2-(or 3)-amino-3-carbamoyl)-propionyl] amidate. This structure is not contradictory to the fact that compound A does not

form ammonium salt. It was previously reported that dinogunellin is of the lyso type having a free hydroxyl group at the 2 position of glycerol and affords glycerophosphate on hydrolysis. Dinogunellin is thus postulated to have the structure as shown in Fig. 1, although the final confirmation of structure should be done by synthesis.

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Ambrosic Acid, a New Irritant Principle from the Pollen of the Ragweed *Ambrosia arthemisiifolia*

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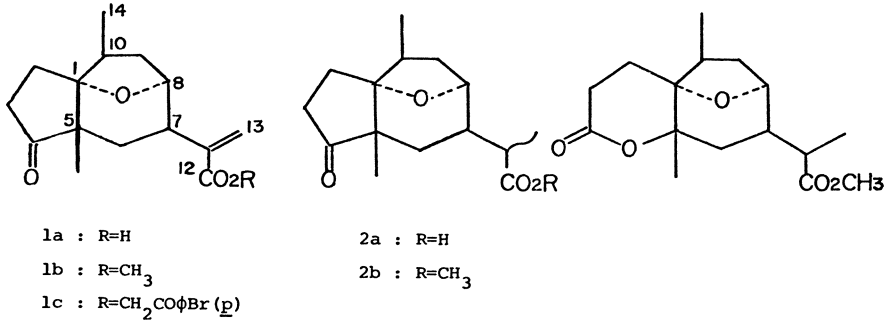
In connection with our studies on the physiologically active substance in the genus of *Gaillardia*, *Arthemisia* and their relatives in *Compositae*, we have recently investigated a poisonous principle in the common ragweed, *Ambrosia arthemisiifolia* (*Butakusa*). Since this plant was introduced into Japan from North America almost one century ago, it has made a wide distribution over the Japanese islands and been notorious for a cause of pollen allergy (hay fever) and also as a poisonous weed in cattle feed.

The sesquiterpene constituents thus far isolated from several populations of the same plant were found to be pseudoguaianolides such as coronopilin (Herz and Högenauer, 1961), psilostackyne (Bianchi *et al.*, 1968), pervin, cumanin and dihydrocumanin (Porter and Mabry, 1969), and germacranolides such as dihydroparthenolide (Bianchi *et al.*, 1968), arthemisiifolin, and isabelin (Porter *et al.*, 1970). In our own investigation, with few collections of this plant in the environs of Tokyo, none of the known sesquiterpene lactones described above could be found, but a new modified pseudoguaianoid named ambrosic acid was isolated, in pure form, most especially from the pollen. The present paper is concerned



1a : R=H
1b : R=CH_3
1c : R=$CH_2CO\phi Br$ (p)

2a : R=H
2b : R=CH_3

Fig. 1. The derivatives of ambrosic acid.

with the isolation and the structural elucidation of ambrosic acid formulated as 1a (Fig. 1).

The fresh pollen collected from the flowers, air dried chipped leaves, and stems were submitted to individual percolation with hot chloroform. The acidic part of each extraction furnished the identical crystalline substance in 0.21%, 0.08%, and 0.02%, respectively. $C_{15}H_{20}O_4$ (M^+ 264); m.p. 211-213°C; $[\alpha]_D$ + 77.0°C ($CHCl_3$); ν_{max}^{KBr} (cm^{-1}) 3380, 1701 (COOH), 1732 (cyclopentanone), 1622, 880 (C=CH_2); δ($CDCl_3$) 1.07s (>C—CH_3), 1.22d 6.2 (>CH—CH_3), 3.00m (>CH—C(=CH_2)COOH), 4.42m (>CH—O—C≥), 5.68 d 1.0, 6.44 d 1.0 (>C=CH_2), 10.22s (—COOH). The methyl ester (1b) was readily formed with CH_2N_2 or MeOH-H_2SO_4 as usual. $C_{16}H_{22}O_4$ (M^+ 278); m.p. 88-90°C; $[\alpha]_D$ + 64.4°C (EtOH); λ_{max}^{EtOH} (nm) (ϵ) 201 (9000), 286 (30); ν_{max}^{KBr} (cm^{-1}) 1748 (cyclopentanone), 1702, 1622 ($\Delta^{\alpha,\beta}$—COOMe); δ($CDCl_3$) 1.10s (≥C—CH_3), 1.26 d 6.5 (>CH—CH_3), 3.80s (—$COOCH_3$), 2.98 m (>CH—C(=CH_2)COOMe), 4.37 m (≥C—O—CH=), 5.56t 1.2, 6.29d 1.0 (>CH—C(=CH_2)COOMe). Catalytic hydrogenation 5% Pd-C or $NaBH_4$ reduction of 1b in MeOH gave in a moderate yield methyl dihydroambrosiate (2b): $C_{16}H_{24}O_4$ (M^+ 280); m.p. 130-132°C; $[\alpha]_D$ + 130°C (EtOH); λ_{max}^{EtOH} (nm) (ϵ) 287 (35); ν_{max}^{KBr} (cm^{-1}) 1745 (cyclopentanone), 1732 (ester); δ($CDCl_3$) 0.97 s (≡C—CH_3), 1.10 d 6.0 (>CH—CH_3), 1.20 d 6.0 (>CH—CH_3), 3.72s (—$COOCH_3$), 4.33 m (≥C—O—CH=). The corresponding dihydro acid (2a) was obtained on hydrolysis of 2a or dehydrogenation of 1a with 5% Pd-C in a reasonable yield: $C_{15}H_{22}O_4$ (M^+ 266); b.p.$_{1.5}$ 155-165°C (bath temperature). Ozonolysis of 1a in MeOH yielded bisnorambrosic acid (3a) with liberation of CH_2O being detectable by formol dimedon: $C_{13}H_{18}O_4$ (M^+ 238); m.p. 113-115°C [methyl ester (3b): $C_{14}H_{20}O_4$ (M^+ 252); m.p. 59-61°C].

The α, α-disubstituted cyclopentanone moiety in 1a is recognized by the positive Zimmermann test shown with 1, 2, and 3, and the parent peak at *m/e* 282 for deuterated compound of 2b, indicating an uptake of two deuterium

atoms in the molecule. Another substantial support was provided by *m*-chloroperbenzoic acid oxidation of 2b resulting in formation of δ-lactone (4) [$C_{16}H_{24}O_5$ (M^+ 296); m.p. 168–170°C]. The tertiary methyl signal of 4 shifts to a lower field than that of 2b, which should make it obvious only to enlarge the partial structure from the cyclopentanone to a pseudoguaian-4-one. Dehydrogenation of the diol (5a) [diacetate (5b): $C_{19}H_{30}O_5$ (M^+ 338); $b.p._{.0.45}$ 200–210°C (bath temperature)] prepared by treatment of 1b with $LiAlH_4$ in hot tetrahydrofuran, afforded in a poor yield chamazulene (trinitrobenzene adduct: m.p. 130–131°C), which was coincident with the authentic sample in every respect. The fourth oxygen atom of the molecule should be involved in an ether linkage survived on the $LiAlH_4$ reduction of 1b.

The above-mentioned facts should permit us to accommodate pseudoguaian-4-one-7-methacrylic acid including one possible ether bridge located most probably between C_1 and C_8 in the structure of ambrosic acid (1a). The positive rotatory dispersion Cotton effects of 1b (a = +21.1) and 2b (a = +21.0) in an MeOH solution were found to be very comparable with that of coronopilin (a = +61.5) suggesting *trans* ring fusion of the pseudoguaian skeletion of 1a (5β-CH_3/1α-OR). The final proof of the whole structure of 1a, including the exact location of the ether bridge, was obtained from proton nuclear magnetic resonance (^{1}H NMR) spin-decoupling experiments (100 MHz, $CDCl_3$) with 1b, except for the configuration of the C_{10}-β-methyl group. The multiplet signal appearing at 4.37 (H_8) changed to a doublet (J = 6 Hz) when irradiated at 3.22 (H_7), and to a singlet on simultaneous irradiation at 2.98 (H_7) and 2.22 (H_{9e}). On the other hand, the multiplet of H_7 became a sextet (J = 3.6 Hz) and a quartet on irradiation of H_{13} and H_8, respectively, sharpened considerably on double irradiation of H_{13} and H_8, and changed to a doublet (J = 6 Hz) when irradiated at both 4.37 (H_8) and ~2.22 (H_{6e}). The doublet of C_{14}-methyl protons appeared at 1.26 changed to a singlet on irradiation at ~2.22 (H_{10}), and the complex of H_{9a} and H_{6a} was contracted on simultaneous irradiation of H_7 and H_8. The following ^{13}C NMR data with 1b ($CDCl_3$) also support the structure of 1a mentioned above: δ ppm (I^{rel}) 14.92 (139) (C_{15}), 20.99 (129) (C_{14}), 26.82 (154) (C_6),* 27.91 (185) (C_9),* 33.61 (190) (C_2),* 33.98 (180) (C_3),* 39.07 (184) (C_{10}), 40.53 (166) (C_{16}), 51.81 (97) (C_7), 53.03 (74) (C_5), 74.14 (173) (C_8), 89.55 (56) (C_1), 124.50 (154) (C_{13}), 140.15 (60) (C_{11}), 167.21 (26) (C_{12}), 216.72 (40) (C_4) (where the asterisks represent tentative assignment.

The structure of ambrosic acid represented by 1a is thus established on the above-described chemical and spectral evidences (Inayama *et al.*, 1974).

An ambiguity remains, however, in the configuration of the C_{10}-β-methyl group in 1a, which was merely supported by the Cotton effect in ORD spectrum of methyl ambrosiate (1b). Owing to scarcity of the sample for further studies on the complete structural elucidation, we were obliged to decide to carry out

x-ray crystallographic analysis of the *p*-bromophenacyl ester (1c) in order to settle the stereochemistry specifically mentioned above and also to establish the absolute configuration of the confirmed structure of ambrosic acid (Inayama *et al.*, 1974).

p-Bromophenacyl ambrosiate was prepared by the usual method as one of the most suitable crystals for our x-ray analysis: $C_{23}H_{25}O_5Br$ (M^+ 461); m.p. 119-121°C; ν_{max}^{KBr} (cm^{-1}) 1744 (cyclopentanone), 1725, 1624 ($\Delta^{\alpha,\beta}$—COOR), 1700, 1586 (CO—Aryl); δ ($CDCl_3$) 1.13 s (⩾C—CH_3), 1.28 d 6.5 (>CH—CH_3), 3.02 m (>CH—C(=CH_2)COOR), 4.51 m (>CH—O—C⩽), 5.64 d 1, 6.42 d 1 (⩾C(=CH_2)COOR), 5.36 s (—$COOCH_2CO$—), 7.68 q 8.8 (aromatic H). Recrystallization from *n*-hexane afforded colorless prisms elongating along the c axis. The crystal belongs to the monoclinic system with lattice parameters and space group, a = 19.303 ± 0.02 Å, b = 7.982 ± 0.01 Å, c = 6.781 ± 0.01 Å, β = 89.34 ± 0.1°, V = 1044.7 Å^3 and $P2_1$. The density is calculated to be D_x = 1.46 g · cm^{-3} on an assumption that two molecules are contained in the unit cell.

Intensities were measured with a Rigaku Denki four-circle x-ray diffractometer with Ni-filtered CuK_α radiation. A total of 1186 independent structure factors are obtained which correspond to about 80% of the theoretically possible ones within 2θ of 120°. In order to measure the anomalous dispersion effect of the bromine atom, Zr-filtered MoK_α radiation was used, and the intensity data for 741 pairs of *hkl* and $hk\bar{l}$ reflexions are obtained which constitute the Friedel pairs.

The absolute structure was determined from the phases calculated by the anomalous dispersion method. The electron density map thus obtained showed

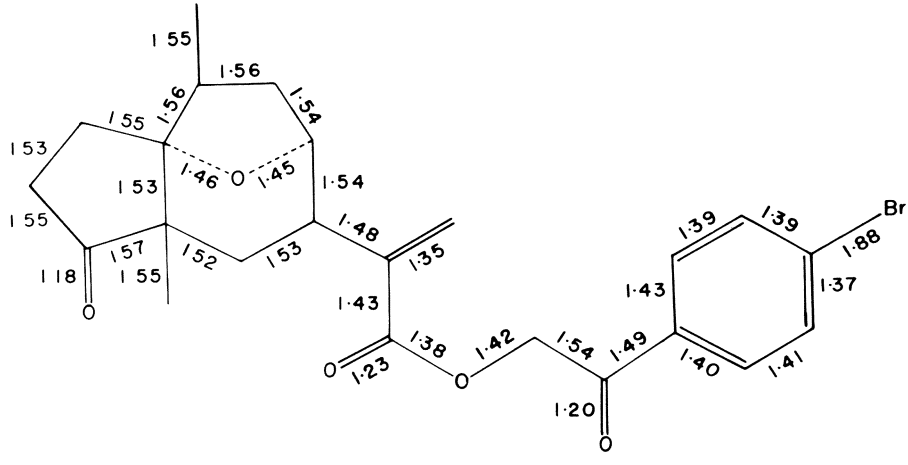


Fig. 2. The bond lengths of the molecule of *p*-bromophenacyl ambrosiate.

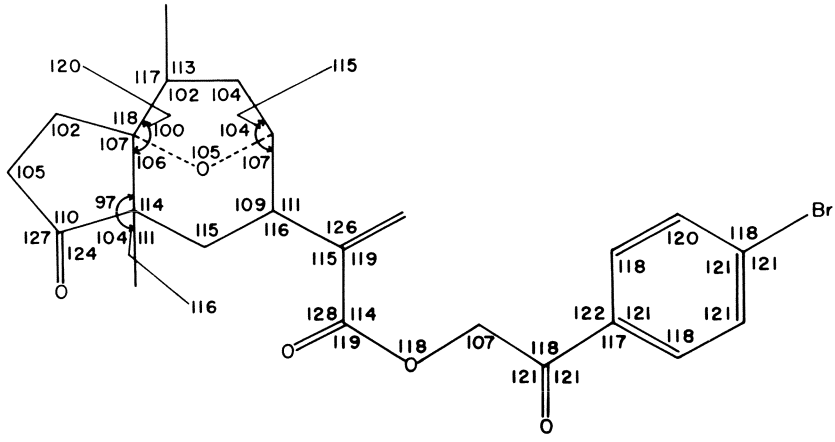


Fig. 3. The bond angles of the molecule of *p*-bromophenacyl ambrosiate.

the location of all 28 carbon and oxygen atoms. The refinement of the atomic parameters was carried out by the method of least squares based on the 1186 structure factors measured by CuK_α radiation. The R value was reduced to 0.073 allowing for the anisotropic thermal vibrations for each atom. The bond lengths and angles are shown in Figs. 2 and 3, respectively (standard deviations: 0.02 Å and 1°).

The absolute stereochemistry of the molecule has now been established as follows: C_5-βCH_3/C_1-αOR (*trans*), C_5-βCH_3/C_{10}-βCH_3 (*cis*), C_5-βCH_3/C_7-$\beta C(CH_2)COOR$ (*cis*), C_7-αH/C_8-βH (*trans*). The seven membered ring of the pseudoguaiane skeleton of ambrosic acid is forced to take a boat confor-

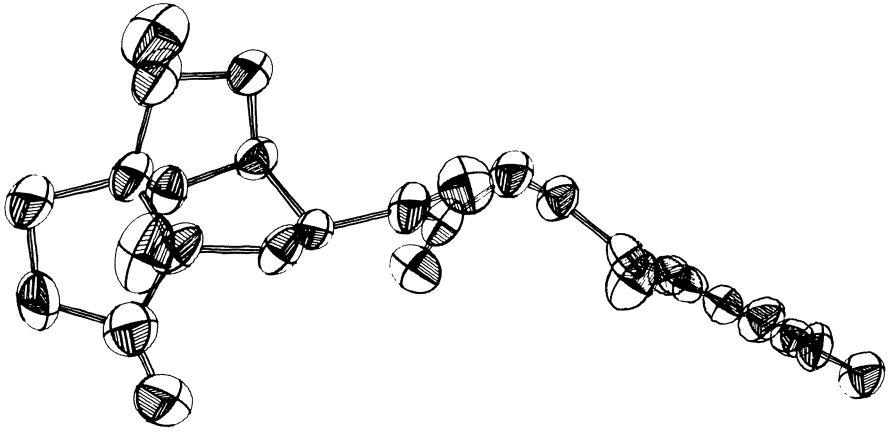


Fig. 4. The electron density map of the molecule of ambrosic acid.

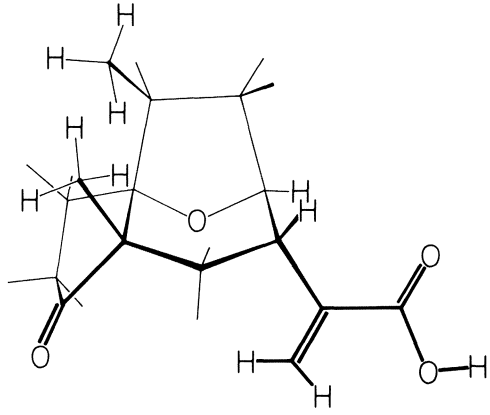


Fig. 5. The absolute stereostructure of ambrosic acid.

mation due to formation of the ether linkage between C_1 and C_8. It is of interest to note that the distance between the two β-methyl groups at C_5 and C_{10} is 3.23 Å slightly closer than usual, but comparable with those found between the 1, 3 diaxial methyl groups in certain fused cyclohexane ring systems (Akiyama *et al.*, 1970; Bjamer *et al.*, 1968) (Fig. 4).

In conclusion, the absolute structure of ambrosic acid (1a) is completely established by the foregoing chemical studies (Inayama *et al.*, 1974a) and x-ray analysis (Inayama *et al.*, 1974b) (Fig. 5).

Finally, a biogenetic consideration and an antigen reactivity test of ambrosic acid will be provided. As shown in a possible biogenetic scheme for the sesquiterpene lactones of *Gaillardia pulchella* (Fig. 6) (Inayama *et al.*, 1973), appropriate cyclization of a hypothetical *cis*-$\Delta^{1,10}$-germacranolide (A) such as arthemisiifolin and isabelin occurring in the same plant would favor leading through a guaianolide (B) to the pseudoguaianolide intermediate (C) similar to pervin, cumanin, and dihydrocumanin isolated from the same natural source. Certain biological cleavage of the lactone followed by protonation to the double bond and the concerted ether ring formation in C (route a) should make it obvious that a rise to the taget ambrosic acid (1a) is given. It is also of interest to suggest that transformation of C to pervin would probably proceed through

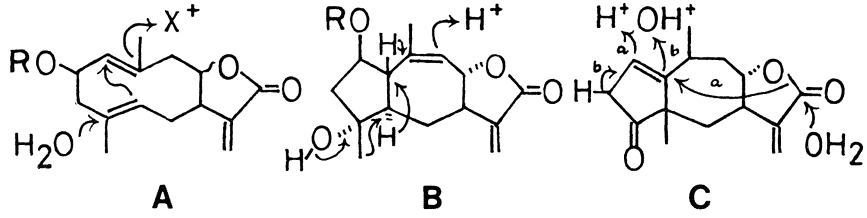


Fig. 6. A possible biogenetic scheme for ambrosic acid and pervin.

such an oxidative manner followed by hydrogenation as shown in route b *in vivo*.

In addition, we could find no antigen reacivity, at least with the antiserum prepared with aqueous ragweed pollen extraction, which was evidenced by no change of the precipitine lines resulted from several double immunodiffusion experiments. Further attempts to confirm *in vivo* sensitization and antigen reactivity using the acid and/or the haptenic sample is now in progress.

SUMMARY

A new irritant lipophilic sesquiterpenoid named ambrosic acid was isolated from the pollen of *A. arthemisiifolia*. Its absolute structure was completely established by chemical, spectral and x-ray crystallographic studies. A biogenetic scheme of the principle was proposed and a preliminary experiment for the antigen reactivity was described.

ACKNOWLEDGMENTS

The experiment was performed by Mr. S. Kawai of Torii Pharmaceutical Co., and aided by a grant from Hōansha (Japan) to whom our thanks are due.

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Activity of Selected Sea Snake Venoms on the Isolated Nerve–Diaphragm Preparation

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INTRODUCTION

The venom of sea snakes, family *Hydrophiidae,* is among the most potent of all known snake toxins. Although various signs and symptoms accompany sea snake envenomation, paralysis and respiratory depression are the most prominent. Crude sea snake venom and purified toxins characteristically exhibit neuromuscular activity. Purified neurotoxins have been isolated from *Laticauda semifasciata* (Tamiya and Arai, 1966), *Laticauda laticaudata* and *Laticauda colubrina* (Sato *et al.*, 1969), *Lapemis hardwickii* (Tu and Hong, 1971), *Enhydrina schistosa* (Karlsson *et al.*, 1972), and *Hydrophis cyanocinctus* (Liu *et al.*, 1973). Recently, during an expedition of the Research Vessel Alpha Helix to Ashmore Reef, venom was collected from several species which are more rarely encountered. A preliminary report indicated the neurotoxic activity of crude venom from each of *Astrotia stokesii*, *Aipysurus laevis*, *Hydrophis elegans*, and *Hydrophis beleheri* collected during that expedition (Barber *et al.*, 1974). Our present report includes additional data regarding the crude venoms of *A. stokesii*, *A. laevis*, and *H. elegans.*

MATERIALS AND METHODS

Crude venom from each of *A. Laevis, A. stokessi*, and *H. elegans* was obtained by using a pipette inserted directly over the fangs of the snake. The venom was freeze-dried and stored under refrigeration. Prior to use the freeze-dried venom was reconstituted in isotonic saline or distilled water.

Male Swiss Webster mice weighing 18.5-22 g were used for LD_{50} determinations. The LD_{50} values were calculated by the method of Litchfield and Wilcoxon (1949) after intramuscular injection of different doses of the venom, using five mice per dose.

The effects of the crude venoms on neuromuscular transmission were studied with the isolated guinea pig phrenic nerve-diaphragm muscle preparation similar to that described by Bulbring (1946). A preparation holder was specially constructed; it consisted of an L-shaped plastic tube containing two separate sets of platinum electrodes, one to stimulate the muscle directly and the other to stimulate the nerve.

Guinea pigs weighing 100-150 g were sacrificed by giving a sharp blow to the head. The chest was opened immediately and, to avoid excess handling of the nerve itself, a ligature was tied up the phrenic nerve at the neck. The nerve was then carefully dissected down to the diaphragm. A wedge of the diaphragm, approximately 1.5 cm wide and 2-3 cm long, was cut from the left side parallel to the muscle fibers, leaving a portion of a rib for easy attaching to the electrode holder.

A thread was attached from the top of the strip of the diaphragm to a Statham Universal Transducing Cell, model UC3, which was connected to the recorder, a Hewlett Packard oscillographic recorder, model 7402A, via a Statham bridge amplifier, model SC1100.

Alternate electrical pulses to the nerve and the muscle were generated with a Grass S44 stimulator in connection with a Grass S5 stimulator. In all cases the duration of the pulse was 0.25 msec and the pulse rate was 1/40 sec. For nerve stimulation, 10 V were used and 120 V for muscle stimulation.

The entire preparation was kept in a constant 37°C temperature bath, which had a volume of 60 ml, and was aerated with a mixture of 95% O_2 and 5% CO_2. Modified Krebs solution as suggested by Jenden (1955), containing a trace of insulin as suggested by Taylor (personal communication), was used to bathe the preparation. Venom, and, on occasion, prostigmine were added directly to the solution in the bath.

The effects of crude venoms were also studied with the denervated sensitized guinea pig diaphragm by the procedure described by Miledi (1962). The phrenic nerve of the guinea pig was severed and the diaphragm allowed to degenerate *in vivo* for 10 days resulting in generalized sensitization of the entire diaphragm to acetylcholine. The denervated diaphragm was then excised,

placed in the bath and the muscle response to additon of venoms or acetylcholine was observed.

RESULTS AND DISCUSSION

The following LD_{50} values were obtained for the crude venom which had been freeze dried and stored under refrigeration for approximately 10 months: *A. stokesii*, 250 μg/kg; *A. laevis*, 90 μg/kg; *H. elegans*, 200 μg/kg. After the injection of any venom, the activity of the mice remained normal for about 1 hr, followed by a period of inactivity. Depth and rate of respiration increased followed by paralysis of the hind legs. Death appeared to be caused by respiratory failure. Violent muscle spasms and convulsions also occurred for a brief period in animals injected with *A. laevis* venom.

The responses of the isolated nerve-diaphragm preparation to the venom are shown in Figs. 1-6. The general response was as follows. After the addition of venom, there was a lag period of 20-60 min. During this period, there was no change, or a small increase, in the height of the nerve-elicited contraction. Next a gradual geometrical decrease in the responses to nerve stimulation occurred and the response to direct stimulation of the muscle remained unchanged. The venom was then washed away from the bath. The height of the nerve-elicited contraction continued to drop for some time and then began a slow gradual increase. Recovery of the nerve ranged from nearly 100% to 40%. Electrical stimulation of the nerve was next stopped to provide a rest period. Nerve stimulation was resumed after the rest period and a second addition of the same venom was made. As can be seen there was no lag period in the muscle response following this second addition and nerve stimulation elicited a much more rapid and potent decrease in contraction (Fig. 1). Doubling the rest period resulted in a slight reestablishment of the lag period.

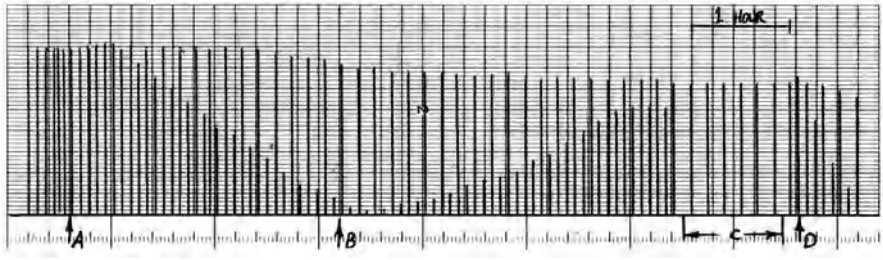

Fig. 1. Tracing of response of isolated nerve-diaphragm preparation to alternate electrical stimulation of nerve then muscle, following addition of *A. stokesii* crude venom. (A) addition of 0.5-μg/ml *A. stokesii* venom; (B) wash; (C) rest period; no nerve stimulation; (D) addition of 0.5-μg/ml *A. stokesii* venom.

The reductions in the nerve-elicited contractions caused by the three venoms are compared in Fig. 2. Different potencies of the crude venoms can be seen in this figure, reflecting the relative lethalities shown in the LD_{50} values. *A. laevis* venom has the most pronounced effect of the three and *H. elegans* venom the least effect. Figures 3-5 illustrate the dose-response effect of the venoms on the nerve-elicited muscle contraction.

The addition of prostigmine (Fig. 6) gave an immediate antagonistic effect, reducing the venom block to nerve stimulation of the muscle. Similar results were attained with all three crude venoms.

The response of the denervated, sensitized diaphragm preparation is shown in Fig. 7. Initial addition of acetylcholine elicited a contraction and gradual relaxation of the diaphragm muscle. The preparation was then washed free of acetylcholine and the venom was added to the bath. After 2 hr of incubation, acetylcholine was again added. As shown (Fig. 7), the second addition of acetylcholine, following incubation of the diaphragm with venom, elicited no muscle response. A partial muscle response to acetylcholine can again be elicited about 2 hr following the washing of the venom from the preparation.

The responses of the isolated nerve-muscle preparation to the venoms indicate that they may cause paralysis by affecting the myoneural junction. Initial action of the venom is slow. The 20-60 min lag time was first thought to be due to slow diffusion of venom to the endplate receptor sites. However, as

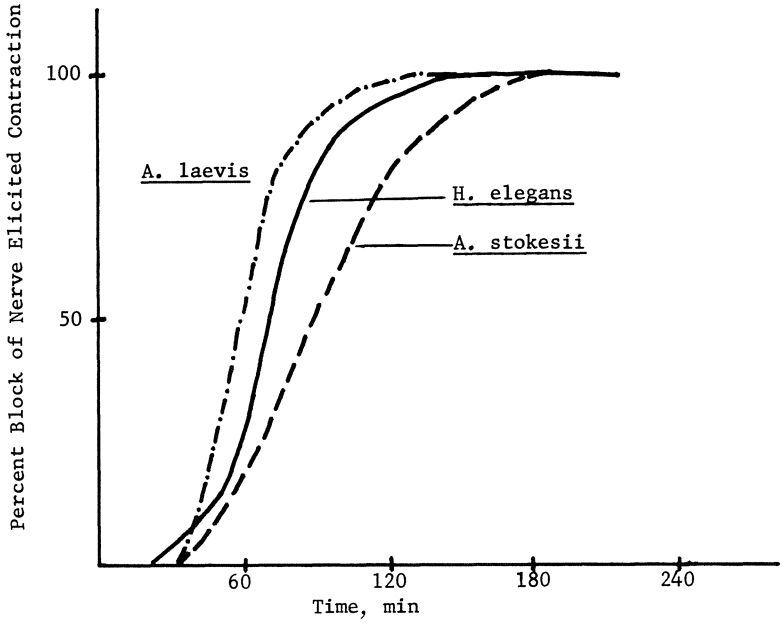

Fig. 2. Comparison of responses of the isolated guinea pig nerve-diaphragm preparation to *A. laevis, H. elegans,* and *A. stokesii* crude venoms, 0.5 μg/ml.

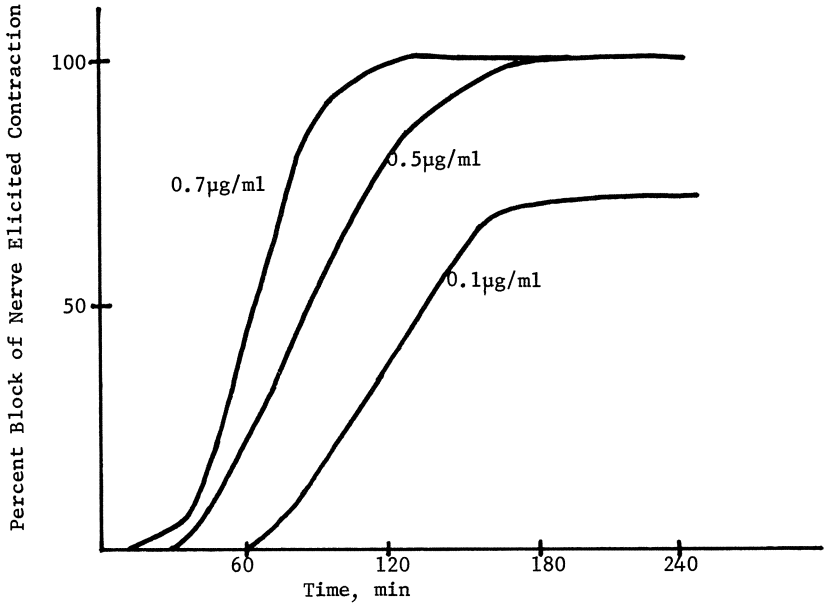

Fig. 3. Dose-response curves following addition of *A. stokesii* venom to the isolated guinea pig nerve-diaphragm preparation.

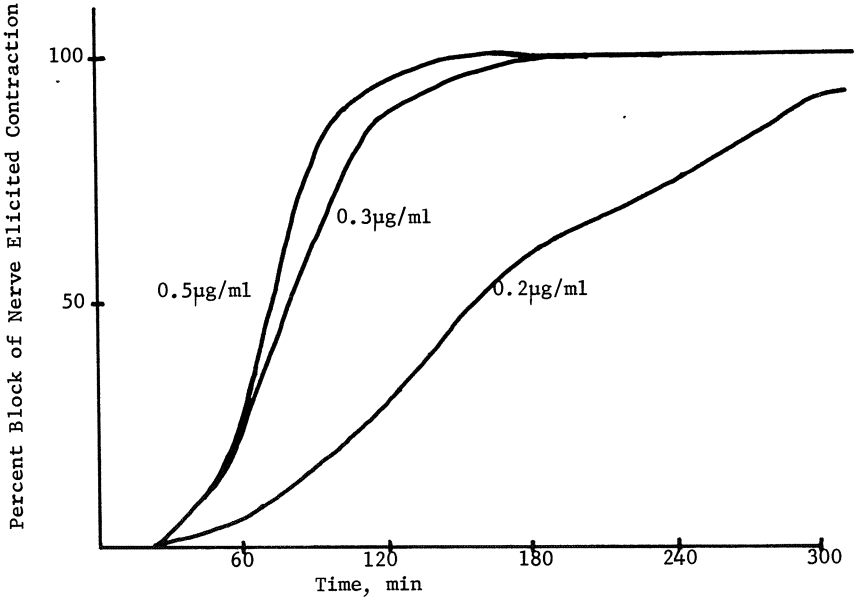

Fig. 4. Dose-response curves following addition of *H. elegans* venom to the isolated guinea pig nerve-diaphragm preparation.

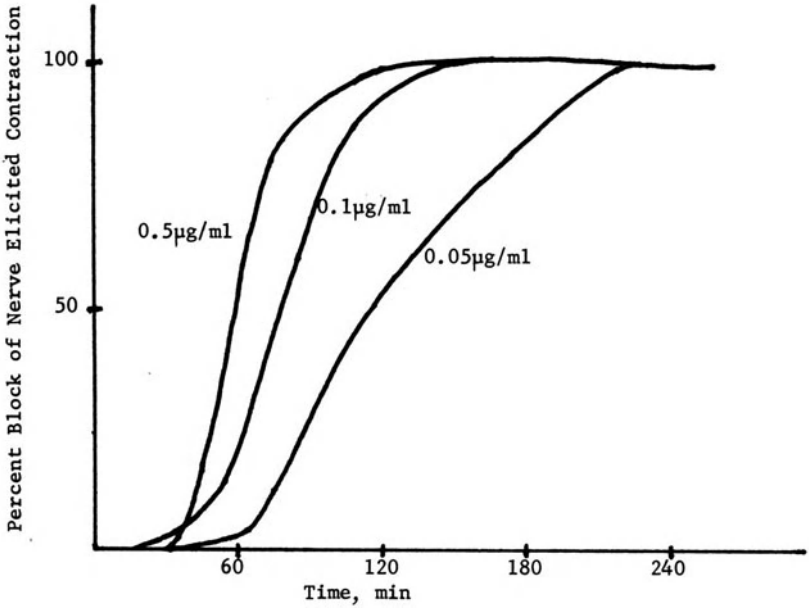

Fig. 5. Dose-response curves following addition of *A. laevis* venom to the isolated guinea pig nerve-diaphragm preparation.

shown in Fig. 2, the second addition of venom following the recovery period resulted in fairly rapid response. The implication is that either there exist non-active sites at the endplate receptors which bind much of the venom initially and, therefore, do not interfere with the second addition, or there are similar nonactive sites in the tissue itself. This theory for alternate binding sites in tissue has previously been postulated for other drugs (Triggle, 1968).

The specific mode of action of the crude venoms of *A. stokesii*, *A. laevis*, and *H. elegans* appears to be a blockade of neuromuscular transmission at the

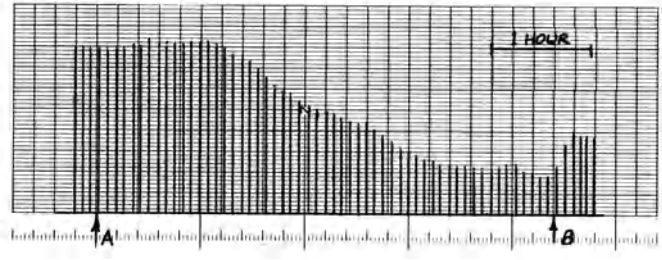

Fig. 6. Tracing of response of the isolated nerve-diaphragm preparation to alternate electrical stimulation of nerve then muscle following addition of *A. stokesii* venom (A) and prostigmine (B).

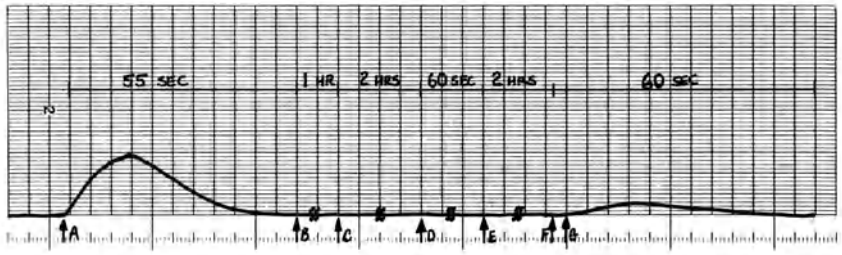

Fig. 7. Tracing of response of the denervated sensitized guinea pig diaphragm to *A. laevis* venom and acetylcholine. (A) addition of 0.033-mg/ml acetylcholine to the bath; (B) wash; (C) addition of 0.5-μg/ml *A. laevis* venom to the bath; (D) addition of 0.033-mg/ml acetylcholine; (E–F) wash; (G) addition of 0.0033-mg/ml acetylcholine.

postsynaptic site via competition with acetylcholine at the receptor sites. This was tested by two methods. First, antagonism of the neuromuscular block by the use of an anticholinesterase (prostigmine) which partially relieved the block as shown in Fig. 6. And second by use of the denervated sensitized diaphragm in which the muscle contraction elicited by acetylcholine could be blocked by prior addition of the venom. In both cases the postsynaptic block spontaneously reversed following washing of the venom from the preparation.

The venoms studied showed marked effects on the neuromuscular activity and seem to act in a manner similar to that reported for the venom of the sea snake, *Laticauda semifasciata* (Cheymol *et al.*, 1971). The potent inhibition of neuromuscular activity by these sea snake venoms appears to be spontaneously reversible in contrast to other snake venoms such as α-bungarotoxin and cobra toxin which irreversibly block the neuromuscular activity (Changeux *et al.*, 1970, Lester, 1972).

ACKNOWLEDGMENTS

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A Fluorescent Study of the Neurotoxic Effect of Cobrotoxin on the Cholinergic Reaction of Acetylcholine with Synaptic Membranes

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INTRODUCTION

The properties of many snake venoms have been described by a number of researchers. The two-dimensional structure of cobrotoxin extracted from the Taiwan cobra (*Naja naja atra*) has been established (Yang *et al.*, 1969a,b), which permits studies of structure-activity relationships. Preceding studies on the chemical modifications of cobrotoxin have suggested that the intact Trp-29, Tyr-25, Lys-47, and Glu-21 are essential for its full activity (Chang and Hayashi, 1969; Chang *et al.*, 1971a,b,c). Since the complete structure of the toxin has been determined, modification of the functional groups of amino acids in the toxin can give us further information about the neurological blocking mechanism of the toxin as related to chemical nerve transmission.

Reasonably successful attempts to extract the cholinergic receptors from the

electroplax of the electric eel or electric torpedo have been reported (Eldefrawi and Eldefrawi, 1972; Klett *et al.*, 1973; Martinez-Carrion and Raftrey, 1973; Meunier *et al.*, 1972). The toxin extracted from snake venoms are supposed to have a blocking effect on the peripheral cholinergic action of ACh (acetylcholine). Meunier *et al.* (1972) reported the results of labeling the cholinergic receptors present in the excitable membranes of *Electrophorus electricus* with the toxin purified from *Naja nigricollis*.

We investigated the toxic activity of native cobrotoxin and its chemically modified forms as related to the cholinergic receptors in the synaptic membranes derived from the rat brain cortices. The identification of the cholinergic receptor of the guinea pig cerebral cortex was reported by Bossman (1972). However, there has not been sufficient information available in this field.

Our investigation was performed by the method of fluorometry using a hydrophobic biomembrane probe, 1-anilinonaphthalene-8-sulfonate sodium salt (ANS). Several investigations have been reported on the fluorescent examination of the cholinergic receptors (Kasai *et al.*, 1969; Kikuno and Sekiya, 1974; Sekiya and Kikuno, 1972; Sekiya *et al.*, 1973a,b; Weber and Borris, 1971). The application of fluorometry to the cholinergic reaction of ACh with the synaptic membranes extracted from rat brains proved to be a sensitive method of observation (Kikuno and Sekiya, 1974; Sekiya and Kikuno, 1972; Sekiya *et al.*, 1973a,b).

In this report, it is proposed that Lys-47, Arg-30, and Arg-33 in the cobrotoxin peptide loop play essential roles in the toxic function as related to the cholinergic reaction of ACh with the synaptic membranes derived from the central nervous system.

MATERIALS AND METHODS

Synaptic Membranes. SM (synaptic membranes) were prepared according to the method of Azcurra *et al.* (1967) with a slight modification. Brain cortices were extracted from Wistar strain rats (male, 150–200 g). The nonnuclear fraction was rehomogenized after osmotic shock with deionized water. The mitochondrial fraction was treated with 0.1% triton X-100 for 30 min prior to discontinuous sucrose density-gradient centrifugation (0.8 M–0.9 M–1.0 M–1.2 M). 0.9 M and 1.0 M sucrose layer fractions were collected as the synaptic membrane fractions and suspended in 0.1 M Tris-HCl buffer, pH 7.4.

1-Anilinonaphthalene-8-sulfonate. ANS sodium salt for albumin analysis was purchased from Tokyo Kasei. The purity of ANS was determined by one spot on TLC at concentration of 10^{-4} M and 10^{-5} M. Since Mg^{2+} ions inhibit the cholinergic reaction of ACh (Sekiya and Kikuno, 1974), we did not use Mg salt of ANS, which is more commonly used. ANS has an insignificantly small fluorescence peak at 515 nm in 0.1 M Tris-HCl buffer, pH 7.4 with excitation at 365 nm.

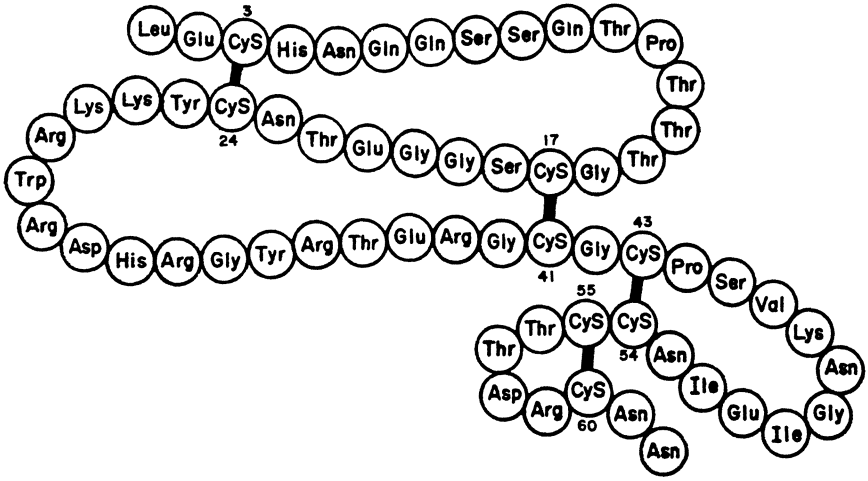

Fig. 1. The structure of Cbtx (Yang *et al.*, 1969a,b).

Acetylcholine. Acetylcholine chloride (Wako Pure Chemicals, Osaka) was used as a solution in 0.1 M Tris-HCl buffer, pH 7.4. The titration of ACh on SM was performed routinely with a 1-μl microsyringe (Jintan Termo, Tokyo).

Fluorometry. Fluorometry with ANS as a hydrophobic probe was performed with a Hitachi 203 fluorospectrometer (a Spinco Bowmen type) without calibration. As a standard fluorescent solution, quinine sulfate in 0.1 N H_2SO_4 was prepared. Quartz cells with 1-cm lightpath were used. All of the experiments were carried out at room temperature (25°C).

Cobrotoxin. Cobrotoxin (Cbtx) was purified by one of the authors, C. C. Yang. The procedures for modification of Cbtx have been reported in other papers (Chang *et al.*, 1969; 1971a,b,c). We used Cbtx modified at Lys-27, Lys-27 and 47, Arg-28, Arg-28 and 33, or Arg-28, 30 and 33. The toxins were dissolved in 0.1 M Tris-HCl buffer, pH 7.4 (Fig. 1).

Protein Measurement. Protein measurement was performed according to the method of Lowry *et al.* (1951).

RESULTS

Titration of ACh on SM. As shown in Fig. 2, ANS fluorescence of SM was quenched on titration with ACh in a concentration range from 6.4×10^{-6} M to 5.6×10^{-3} M. No change of the fluorescence peak at 475 nm, with excitation at 365 nm, was observed during the titration. 3 mM Ca^{2+} accelerated the reaction of ACh as expressed in terms of fluorescence quenching. 3mM Ca^{2+} increased

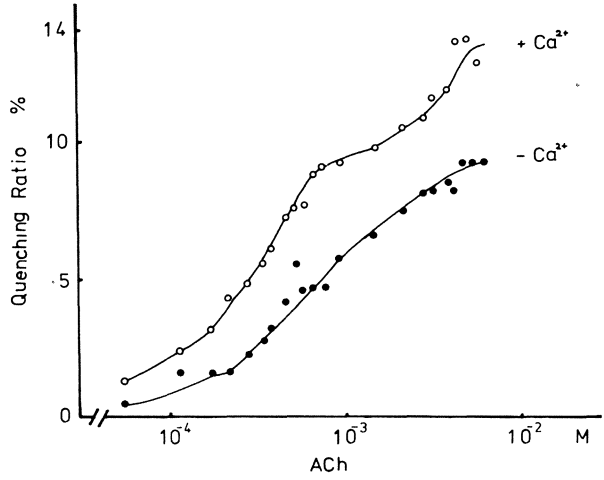

Fig. 2. The titration curves of SM with ACh using ANS as a probe. 3 ml of an SM suspension (0.10 mg/ml) in 0.1 M Tris-HCl buffer, pH 7.4, was titrated with 0.001 ml volumes of an ACh solution delivered with a 1-μl microsyringe to a total volume of 0.02 ml. The ANS concentration was 4.0×10^{-5} M. (black circles) No addition of Ca^{2+}; (white circles) with Ca^{2+} (3 mM). Fluorescence excitation at 365 nm and observation at 480 nm. The quenching ratio was calculated as: $(f_0 - f)/f_0 \times 100\%$, where f_0 is the fluorescence before addition of ACh and f is the fluorescence after addition of ACh. All the subsequent titration was carried out as described above if not indicated otherwise.

ANS-SM complex fluorescence by a factor of 1.5. The influence of Ca^{2+} on the fluorescence has been reported (Sekiya and Kikuno, 1974). In the absence of Ca^{2+} ions, the reaction of ACh with SM decreased and in several cases, no quenching occurred. In the presence of Ca^{2+}, the quenching curve revealed apparent double phases of the reaction. The kinetics of each phase were distinct. The apparent dissociation constant K_{app} was of a magnitude of 10^{-4} M for the first phase and 10^{-3} M for the second. The results suggest that the reaction ACh with SM involves multiple reactions dependent on multiple cholinergic reaction sites.

Cbtx Effect on ACh Reaction with SM. Cbtx at a concentration of 5.0×10^{-5} M inhibited the reaction of ACh with SM. The quenching ratio decreased to 56% of that in the absence of Cbtx, as shown in Fig. 3. Cbtx was preincubated with 3 ml of an SM suspension for 15 min before the suspension was titrated with ACh. ANS fluorescence was quenched approximately 6% by Cbtx itself. The reaction curves of ACh revealed the single phase character in the presence of the toxin. This result suggested that conformational rearrangement of the cholinergic receptors in SM might have occurred, which could decrease the ACh-SM reactivity as observed by the fluorescence quenching. The inhibitory capacity of Cbtx was proportional to the concentration of the toxin in a range from 5.0×10^{-6} M to 4.0×10^{-5} M as shown in Fig. 4.

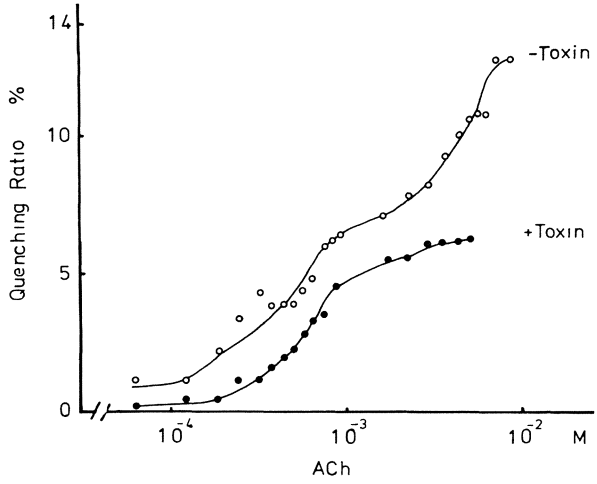

Fig. 3. The effect of native Cbtx on the reaction of ACh with SM. (white circles) In the absence of the toxin; (black circles) in the presence of the toxin (5.4×10^{-5} M). Both experiments were carried out with addition of 3mM Ca^{2+}. The quenching ratio was calculated as indicated in Fig. 2., SM was 0.10 mg/ml.

Effects of Modified Cbtx on the Reaction of ACh with SM. Cbtx modified at Lys-27 retained its inhibitory capacity, while the toxin modified at Lys-27 and 47 lost the toxicity, as shown in Fig. 5. The toxic nature of modified Cbtx (Lys-27) was similar to that of the native Cbtx inducing the single phase titration curve shown in Fig. 3, but the double-phase character of the ACh reaction was preserved in the presence of Cbtx modified at Lys-27 and 47. This result clearly

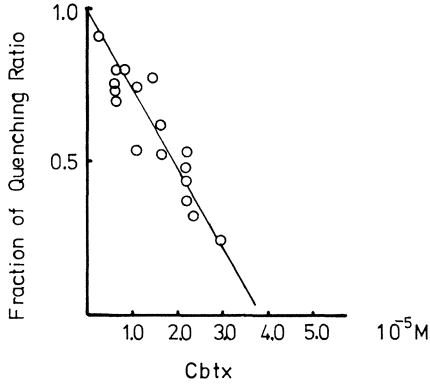

Fig. 4. The Cbtx effect on the reaction of ACh with SM in response to the concentrations of the toxin. The fraction of the quenching ratio is the ratio of quenching without the toxin to that with the toxin. In these titration experiments eserine sulfate (2.5×10^{-4} M) was added to the SM suspension as an inhibitor of AChE (Sekiya *et al.*, 1973a).

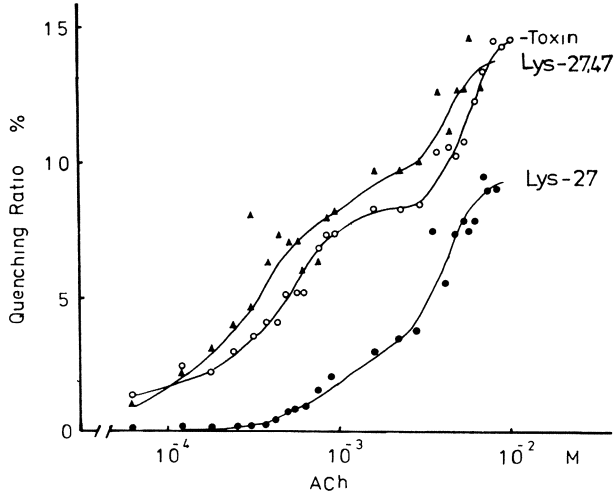

Fig. 5. The effects of the modified Cbtx. (white circles) In the absence of Cbtx; (black circles) in the presence of the toxin modified at Lys-27 (4.0×10^{-5} M); (black triangles) in the presence of the toxin modified at Lys-27 and 47 (4.0×10^{-5} M). The toxins were preincubated with 3 ml of a SM suspension (0.10 mg/ml) for 15 min before the ACh titration. All of the experiments were carried out with addition of 3 mM Ca^{2+}. The quenching ratio was calculated as indicated in Fig. 2.

indicated that Cbtx modified at Lys-27 and 47 lost the capacity to react with the cholinergic receptors in SM. It was demonstrated that Lys-47 was one of the functional basic amino acid residues related to the toxic nature of Cbtx.

Cbtx modified at Arg-28 or Arg-28, 33 preserved the toxic nature. Arg-28 modified Cbtx showed toxicity even at a concentration of 5.0×10^{-6} M. Cbtx modified at Arg-28 and 33 at a concentration of 4.0×10^{-5} was toxic, and preserved the double-phase character of the ACh reaction, as shown in Fig. 6. Cbtx modified at Arg-28, 30, and 33 was nontoxic at a concentration of 4.5×10^{-5} M. These results indicated that Arg-30 and Arg-33 were the functional basic amino acid residues.

Lethal Activities and Inhibitory Capacity of Modified Cbtx. As shown in Table 1, the inhibitory capacities of modified Cbtx correlated with their lethal activities. Cbtx modified at Arg-28, 30, and 33 was not toxic even at a concentration of 4.7×10^{-5} M; however, Cbtx modified at Arg-28 preserved the inhibitory capacity even at such a low concentration as 5.0×10^{-6} M. The toxic nature of Cbtx modified at Arg-28 disappeared when the concentration exceeded 2.0×10^{-5} M. We cannot explain the phenomenon as yet. Cbtx modified at Arg-28 and 33 preserved the toxicity, but its quenching curves preserved the

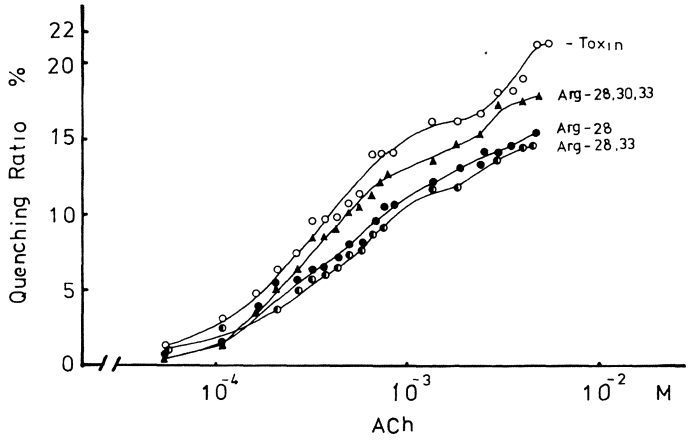

Fig. 6. The effects of the modified Cbtx. (white circles) in the absence of the toxin; (black circles) in the presence of the toxin modified at Arg-28 (0.5×10^{-5} M); (half-black circles) the toxin modified at Arg-28 and 33 (4.0×10^{-5} M); (black triangles) the toxin modified at Arg-28, 30, and 33 (4.7×10^{-5} M). The toxins were preincubated with 3 ml of an SM suspension (0.10 mg/ml) for 15 min before ACh titration. All of the experiments were carried out with addition of 3 mM Ca^{2+}. The quenching ratio was calculated as indicated in Fig. 2.

double-phase character shown in Fig. 6. This suggests that Cbtx modified at Arg-28 and 33 has a weaker effect on the conformation of the cholinergic receptors than does Cbtx modified at Arg-28 or at Lys-27. The lethal activities of Cbtx appear to be closely related to its biochemical reaction with the cholinergic receptors, in which the ACh recepting sites are distributed in an unknown structural conformation.

Table 1. The Lethal Activity and Inhibitory Effect of Cbtx

Site of modification	Concentration, M $\times 10^{-5}$	Lethal activity,[a] %	Inhibition rate,[b] %
No modification	5.4	100	53
Lys-27	4.0	100	36
Lys-27, 47	4.0	0	10
Arg-28	0.5	100	30
Arg-28, 30	4.0	22.6	31
Arg-28, 30, 33	4.7	3.1	12

[a] The lethal activity was expressed in percentage of LD of Cbtx to mice, 1.1 μg/mouse.
[b] The inhibition rate (%) was calculated as the ratio of the quenching ratio obtained in the absence of toxin to that in the presence of the respective toxin.

DISCUSSION

The functions of the basic and aromatic amino acid residues in cobrotoxin have been demonstrated by Chang *et al.* (1971a,b,c) and Yang *et al.* (1969a,b) who performed lethality and immunological determinations.

In this report, it was shown that the basic amino acid group has a functional role in blocking the reaction of the cholinergic receptor with ACh. The toxic effect of cobrotoxin could be attributed to its diminishing the recepting capacity of ACh at the synaptic junctions.

It was suggested that in the cobrotoxin peptide loop there may be two major essential components responsible for the blocking reactions; one component includes Arg-30 and Arg-33 and the other Lys-47 (see Fig. 1). Cobrotoxin did not inhibit AChE of SM derived from rat brain at a concentration of 5.0×10^{-5} M (unpublished data); therefore, cobrotoxin might react directly with the cholinergic sites in SM.

In another case of cholinergic blocking, *d*-tubocurarine chloride showed a blocking effect similar to that of cobrotoxin; *d*-tubocurarine chloride blocked the cholinergic reaction of ACh with SM at a magnitude of concentration of 10^{-5} M (Fig. 7). In the figure, the double-phase character was not clearly shown in the quenching curve without *d*-TC; such disadvantage occurred from the

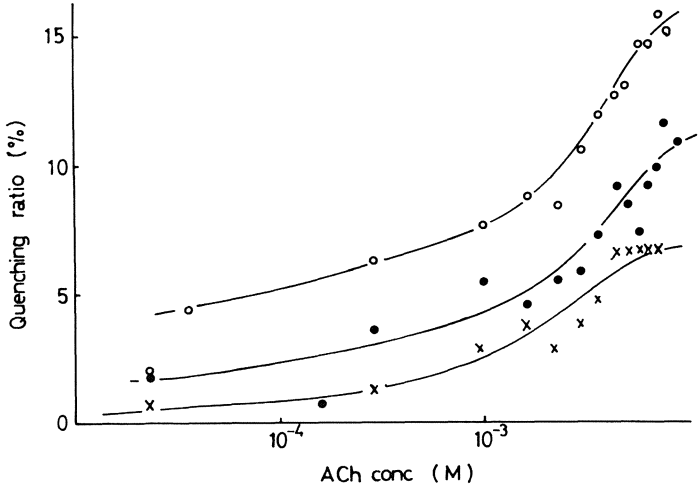

Fig. 7. The effect of *d*-tubocurarine on the reaction of ACh with SM. (white circles) No addition of *d*-TC; (black circles) with *d*-TC (5.4×10^{-6} M); (×) with *d*-TC (2.7×10^{-5} M). All the experiments were carried out with addition of eserine sulfate (2.5×10^{-4} M) as an inhibitor of AChE. 3 ml of an SM suspension (0.06 mg/ml) in 0.1 M Tris-HCl buffer, pH 7.4, was titrated with 0.01-ml volumes of an ACh solution delivered with a 50-μl microsyringe to a total volume of 0.15 ml in the presence of 3 mM Ca^{2+}.

delivery of a larger volume for titration (0.01 ml) than that employed in other experiments (0.001 ml for each titration as indicated in Fig. 2). *d*-Tubocurarine has two positively charged N^+ sites, which are supposed to react with the anionic sites of the cholinergic receptors. We cannot conclude that cobrotoxin has the same molecular configuration as that of *d*-tubocurarine, but the results obtained may support our conclusion that blocking of the cholinergic reactions is due to the multiple reactions of multiple cholinergic receptor sites with the toxin.

We have tried unsuccessfully to extract SM from rat brains in a pure state rich in cholinergic receptors. The ACh and Cbtx concentrations used in our experiments were somewhat higher than those usually used in *in vivo* or *in situ* physiological experiments or in the experiments on the binding of snake toxins to the extracted receptors. The concentration to be used, we however presume, should depend on the purity of the SM preparation. Our experiments, however, are as comprehensive as *in vitro* experiments.

SUMMARY

The toxic nature of cobrotoxin was investigated with a fluorometry with 1-anilinonaphthalene-8-sulfonate (ANS) as a hydrophobic probe. The inhibitory effect of cobrotoxin was demonstrated in titration of ACh on the synaptic membranes derived from rat brain cortices. The toxic nature of modified cobrotoxin was also investigated. Cobrotoxin modified at Lys-27, Lys-27, 47, Arg-28, Arg-28, 33, or Arg-28, 30, 33 were used. The toxin modified at Lys-27, 47 or Arg-28, 30, 33 lost its toxicity. The results showed that Lys-47, Arg-30 and Arg-33 are the functional basic amino acids, and that there are two essential components in the cobrotoxin peptide loop. The inhibitory effects of the native and modified toxins correlated fairly well with their lethal activities.

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Blocking Action of Snake Venom Neurotoxins at Receptor Sites to Putative Central Nervous System Transmitters

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INTRODUCTION

In recent years an increasing number of snake venoms or their constituent subfractions have been successfully developed as probes or other pharmacological tools in areas of clinical and basic research far beyond those of treatment for snake bites. Some of the identified factors which have contributed in this manner include enzymatically operating fractions such as the amino acid esterase termed "Arvin," found in venom of *Agkistrodon rhodostoma*, and used to reduce levels of plasma fibrinogen (Sharp *et al.*, 1968), and certain nonenzymatic proteins which interfere with the normal functioning of vertebrate myoneural junctions. The high degree of specificity and strong binding affinity for acetylcholine receptors of the alpha fractions of several elapid snake venoms have been used to label, extract, and partially purify the membrane protein which carries the cholinergic receptor site in certain tissues (Meunier *et al.*, 1972).

While the alpha fractions are powerful probes which have greatly accelerated research and understanding of the cholinergic processes, they have as yet had little impact on central nervous system phenomena since the only well-defined cholinergic synapse in the vertebrate CNS is that of the motoneuronal axon collaterals to the Renshaw cell. Several other central transmitter systems are suspected, however, for which comparable high-affinity binding agents are not yet available. These include the catecholamines such as dopamine and octopamine,

other biogenic amines such as serotonin, and several small molecules including histamine and various mono- and dicarboxylic amino acids. The widespread systemic effects and profound alterations of sympathetic and parasympathetic responses which often accompany envenomation, especially by crotalid and viperid snakes, suggest that there may be some components of these venoms which interfere with normal CNS processing and that they may do so through a protein-protein interaction at one of the sites of noncholinergic transmission. In order to test this hypothesis, and in hopes of adding to the store of useful pharmacological tools for studying neural phenomena, a general survey was undertaken of the actions of certain venoms on various putative transmitter receptor sites in central nervous system tissue.

The vast number, small size, and bewildering complexity of neurons within the vertebrate CNS present great technical difficulties for the researcher intent upon single-unit analysis and elucidating the pharmacology of these systems. The problems of cell identification, intracellular recording, and drug application are compounded by the trauma owed to substantial skull or spinal cord dissection, and maintenance of the necessary life support systems such as circulation and respiration during the course of experimentation. However, such difficulties are minimal with preparations using invertebrate animals while the fundamental characteristics of membrane excitability, synaptic transmission, and both sensory and motoneuronal functions remain the same (Bullock and Horridge, 1965). One of the most successful of invertebrate preparations to be exploited in recent years has been that of the central ganglia of nonprosobranch molluscs such as *Aplysia* or *Helix*. In these animals the large size and peripheral location of indivdual cell bodies within the central nervous system allows easy penetration by intracellular recording electrodes and direct access by applied drugs and other compounds to specific units of neural tissue which can be recognized and identified as functional components from one animal to the next in the living preparation (Parmentier and Case, 1972; Parmentier, 1973). In addition to the ready accessibility of these neurons it has long been recognized that their membranes contain specific, pharmacologically distinct, receptor sites to most of the compounds which have been suggested as transmitters in vertebrate CNS tissue. We hoped that by studying the neuropharmacology of a relatively simple ganglion in detail we might learn some principles of venom interactions which are sufficiently general to be applicable to other neural aggregates, including those of vertebrates which are currently not as accessible to such direct investigations. This paper presents the results of a series of experiments on the possible blocking action of several snake venoms at receptor sites on *Aplysia* central neurons.

MATERIALS AND METHODS

Abdominal, cerebral, pleural and pedal ganglia from *Aplysia californica* (Pacific Bio-Marine, Venice, California) or *Aplysia dactylomela* (Marine Speci-

mens Unlimited, Marathon, Florida) were removed from the animal and pinned to the Sylgard (Dow Corning) bottom of a lucite dish which was fitted with perfusion apparatus and filled with high-Mg^{2+} (150 mM) sea water to block spontaneous transmitter release from the nerve terminals. The connective tissue surrounding the ganglia was slit with a razor blade to expose the individual cell bodies, and single neurons were impaled with a double-barreled glass microelectrode (R = 5–10 MΩ) drawn from Theta tubing (National Institutes of Health Glass Shop) and filled by injection with 2 M KAc. One barrel monitored membrane potential using a Bak high-impedance amplifier with output to a Tektronix 3A3 preamplifier and 565 oscilloscope, then to a Brush recorder. The second barrel was used to pass current to preset the membrane potential and to monitor changes in membrane resistance by injecting square pulses of constant current into the cell.

Putative transmitters were dissolved in distilled water at 1 M concentrations (pH 3–4) and placed in barrels of a five-barreled iontophoretic electrode which had been bumped at the tip to give a resistance of 2–5 MΩ for each barrel. The experimental compounds were acetylcholine, dopamine, octopamine, histamine, and serotonin. The control panel for iontophoretic current was designed to pass a constant charge set between 0–1000 nc and used solid state internal logic to automatically vary the duration of each pulse in order to assure that the same total charge was passed from each barrel (J. Willis, in preparation). This capability made possible a meaningful comparison of the responses at one cell to several transmitters. The iontophoretic electrode was positioned near the cell body from which recordings were being made, and each compound was tested in turn for its effect on that particular neuron.

Crude snake venoms were dissolved in high-Mg^{2+} sea water containing 2 mg/ml of bovine serum albumen and added directly to the recording bath by syringe once control responses to the transmitters had been established. Preparations were normally exposed for 30 min, followed by at least an equal amount of wash time under continuous perfusion. The following venoms were acquired from noncommercial sources: *Crotalus durissus terrificus* was obtained from the U.S. Army Venom Research Laboratory at Ft. Knox, Kentucky; *Pelamis platurus* and an unknown species of *Micrurus* were gifts of Dr. Hershel Flowers, San José, Costa Rica; *A. rhodostoma* venom was collected with the help of Dr. Clifford Roberts, U.S. Army Medical Research Group, Kuala Lumpur, Malaysia. Venom from *Laticauda semifasciata* was purchased from Biologicals Unlimited, Baltimore, Maryland, and all other venoms were purchased from Miami Serpentarium Laboratories, Miami, Florida.

Physical separation of the venom components was accomplished by ion-exchange chromatography on a carboxymethyl Sephadex C-25 column, 25 X 340 mm, equilibrated with 0.01 M phosphate buffer at pH 6.0. Elution was continued with the 0.01 M buffer until after passage of the initial peak, which emerged at the void volume of the column. Then having shown that no additional com-

ponents of the whole venom were eluted at this pH or up to a phosphate concentration of 0.06 M, elution was continued with a logarithmic gradient from 0.06 M phosphate, pH 6.40, to 0.06 M phosphate, pH 8.00, using a 100-ml mixing chamber (Broomfield and Currie, 1969). The samples were spectrophotometrically monitored and active peaks were pooled and lyophilized. Separate fractions were then dissolved in a minimum of distilled water and placed again on a Sephadex column which had been equilibrated with the high-Mg^{2+} sea water. When eluted from the column the venom components were free of buffer salts and their physiological effects accurately determined on the ganglion cells.

RESULTS

The results of ninety-three experiments using venom from ten snakes on three cholinergic and two dopaminergic responses on *Aplysia* central neurons are summarized in Table 1. Data from a third dopaminergic response and from responses to serotonin, histamine, and octopamine are incomplete due to the relative infrequency of these sites and will be discussed separately later in this section.

Elapidae. 400 μg/ml of venom from *Bungarus multicinctus*, which was taken as a standard for cholinergic block during these experiments, was able

Table 1. The Effects of Ten Snake Venoms on Five Putative Transmitter Receptor Sites Found on *Aplysia* Central Neurons

	Acetylcholine			Dopamine	
	Na	K	Cl	Na	K
Elapidae					
Bungarus multicinctus	B[a]	B	B	–	–
Micrurus sp.	B	–[c]	–	–	–
Naja naja	B	–	B	–	–
Hydrophidae					
Laticauda semifasciata	↓[b]	–	↓	–	–
Pelamis platurus	↓	–	B	–	–
Crotalidae					
Crotalus durissus terrificus	B	–	↓	–	B
Agkistrodon rhodostoma	–	–	–	–	–
Bothrops atrox	–	–	–	–	–
Viperidae					
Echis carinatus	↓	–	B	–	–
Vipera russelli	↓	–	–	–	B

[a] B, total block.
[b] ↓, reduction.
[c] –, no effect.

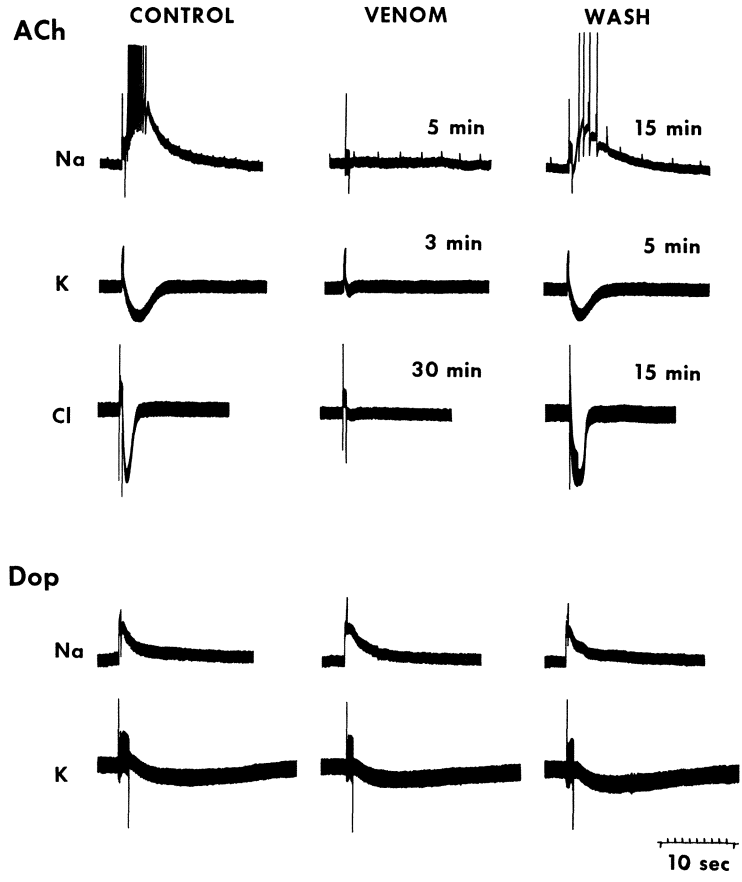

Fig. 1. The effect of 400 μg/ml *Bungarus multicinctus* venom on five putative transmitter responses on *Aplysia* central neurons. Each experiment was conducted on a separate neuron. All responses were in the millivolt range, with spike peaks off scale. Indicated times refer to periods spent in venom or wash perfusion. Stimulus artifacts indicate onset of transmitter application. Note the reversible block of all three cholinergic receptor responses.

to rapidly and reversibly eliminate responses at all three ionic channels controlled by acetylcholine, but had no effect on either sodium or potassium fluxes induced by dopamine activation (Fig. 1). The block was most rapid on the ACh-K^+ response, being complete as well as reversible in less than five minutes, while the ACh-Na^+ block and recovery took somewhat longer. While the *Micrurus* and the *Naja naja* venoms, at the same concentration, both blocked the ACh-Na^+ response, neither affected the ACh-K^+ response and only the *N. naja* venom was effective at the hyperpolarizing chloride channel. None of the elapid snake venoms interfered with dopamine responses.

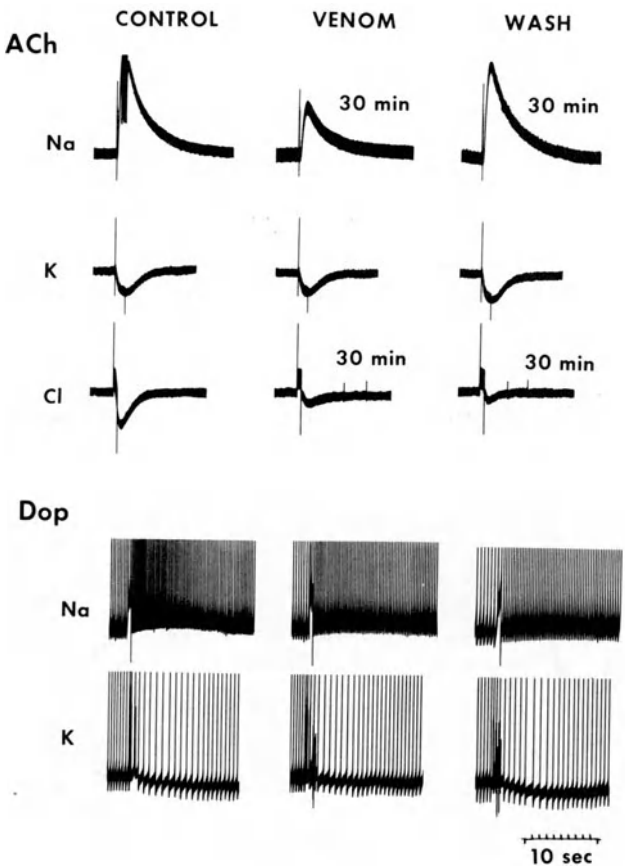

Fig. 2. The effect of 400 μg/ml *Laticauda semifasciata* venom on five putative transmitter responses on *Aplysia* central neurons. Note the incomplete cholinergic blocks to sodium and chloride channels, and the unaffected ACh–K^+ response.

Hydrophidae. Among the sea snakes both *Laticauda* (Fig. 2) and *Pelamis* reduced the response to ACh-activated sodium flux, but neither could totally block this response in a time course (30 min) or concentration (400 μg/ml) comparable to the bungarotoxin. As with the elapids the ACh-K^+ response remained resistant to the effects of the venom but the chloride responses were effectively reduced by *Laticauda* and blocked by *Pelamis*. In both of these instances the chloride responses could not be recovered by washing although sodium sensitivity was returned.

Crotalidae. One of the three crotalid snake venoms was effective against two classes of acetylcholine responses, and also produced the first indication of a dopamine receptor block. *Crotalus durissus terrificus*, a Brazilian rattlesnake

with a known neurotoxic syndrome associated with its bite (Rosenfeld, 1971), reversibly blocked the ACh-Na^+ response and significantly reduced the ACh-Cl^- response (Fig. 3). This figure also shows the dopamine-potassium channel reverisbly blocked by *C. d. terrificus* venom. However, conductance test pulses (not shown) indicated that the membrane resistance of several of the cells studied with this venom were drastically reduced within comparable time courses to those producing the dopamine block, probably by some additional lytic factor in the venom which operates directly on the membrane phospholipid structure. Fractionation of the venom yielded several peaks of which the nonlytic fractions no longer had a blocking effect on the dopamine-potassium

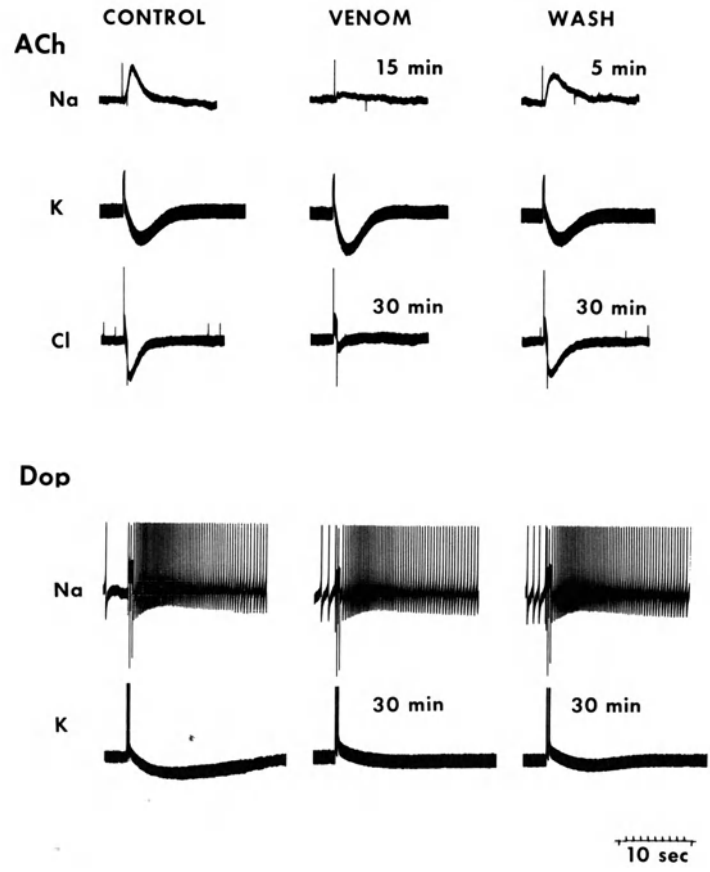

Fig. 3. The effects of 400 μg/ml *Crotalus durissus terrificus* venom on five putative transmitter responses on *Aplysia* central neurons. Note the nonpersistent block of the sodium and chloride responses induced by acetylcholine, and the block of the dopamine-potassium response.

receptor complex. Venoms from *Bothrops atrox* and *A. rhodostoma* had no effect on either the acetylcholine-controlled or dopamine-controlled responses.

Viperidae. Venom from *Echis carinatus* which, like *C. d. terrificus*, has been reported to interfere with peripheral respiration in vertebrates (Chapman, 1968), reduced the ACh-Na^+ response and blocked the ACh-Cl^- response but, as was the case with all venoms tested except *B. multicinctus*, did not affect the potassium response to acetylcholine (Fig. 4). The chloride block was quite rapid and not reversible despite extended washings. *E. carinatus* venom did not affect dopamine responses.

Figure 5 presents the data collected from experiments with *Vipera russelli* venom. While there was a slight reduction of both the sodium and chloride

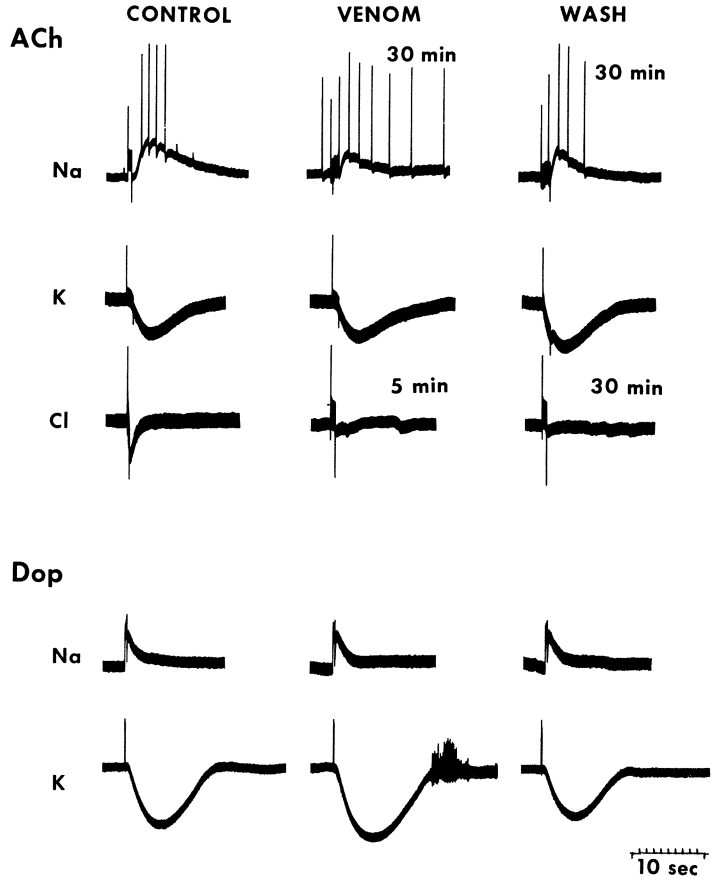

Fig. 4. The effect of 400 μg/ml *Echis carinatus* venom on five putative transmitter responses on *Aplysia* central neurons. Note the persistent block of the ACh-Cl^- response.

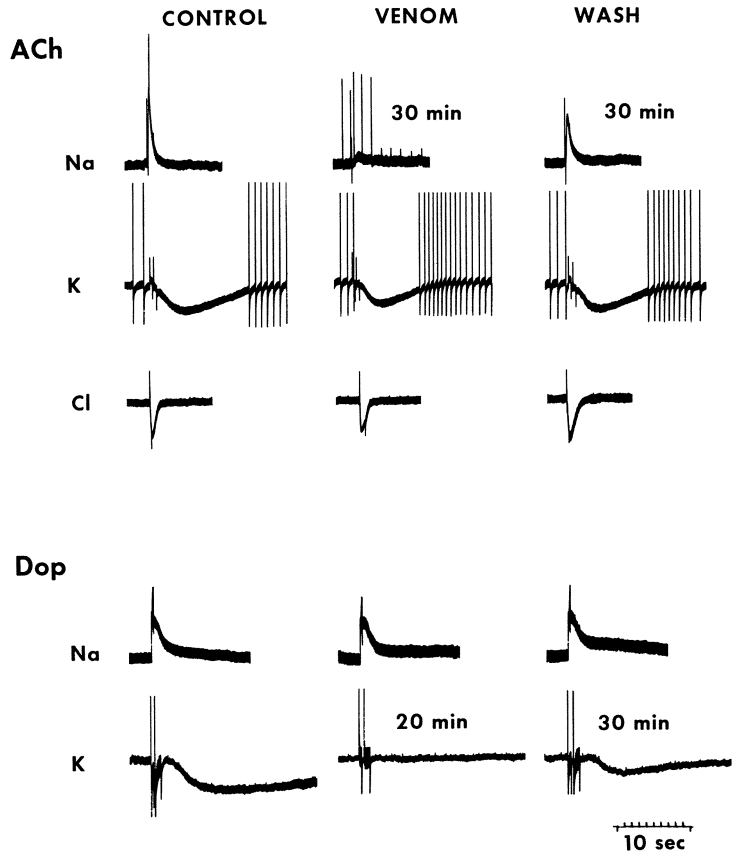

Fig. 5. The effect of 400 μg/ml *Vipera russelli* venom on five putative transmitter responses on *Aplysia* central neurons. Note the total block and partial recovery of the dopamine induced potassium response.

responses to acetylcholine the most interesting effect of this venom was its elimination of the potassium flux following dopamine iontophoresis. Data gathered from neurons in seven different animals gave consistent results and the block appeared sufficiently definite to warrant fractionation of the venom. The resulting elution diagram, shown in Fig. 6, reveals several complex peaks which have yet to be fully resolved. However, preliminary results suggest that fractions IV or V may be responsible for part or all of the dopamine blocking effect.

Additional Results. During the course of these experiments only fourteen neurons were located which carried receptor sites to any ionophore controlled by receptors to octopamine, serotonin, or histamine, and only five dopamine-chloride responses were recorded. Of these only single experiments support the

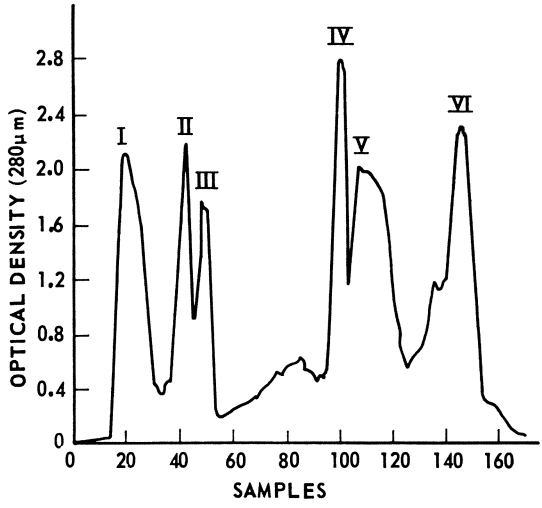

Fig. 6. Elution diagram of *Vipera russelli* venom.

observations that (1) *C. d. terrificus* venom may block the histamine-potassium response; (2) *B. multicinctus* venom may reduce the histamine-sodium and the dopamine-chloride responses; and (3) *V. russelli* venom may selectively eliminate potassium responses to serotonin and octopamine as well as to dopamine.

DISCUSSION

The prevailing hypothesis explaining cholinergic receptor mechanisms in molluscan central neurons recognizes three separate and pharmacologically distinct receptor-ionophore complexes which may be distinguished by the particular current-carrying ion activated by acetylcholine at each site (Swann and Carpenter, 1975). The resulting shift in membrane potential is thus determined by Donnan distribution requirements regarding the mobile ions and will ordinarily be expressed as a rapid depolarization following sodium fluxes, a rapid hyperpolarization following chloride fluxes, and a more prolonged hyperpolarization after activation of potassium ions (Kehoe, 1972). Usually only one form of cholinergic receptor-ionophore complex will be found on any given neuron but occasionally biphasic responses are observed, both spontaneously and following iontophoresis of the acetylcholine. Recently this concept has been extended to include other putative transmitter systems (Ascher, 1972; Carpenter and Gaubatz, 1974, 1975), including all five compounds surveyed during this study.

The results in Table 1 confirm several previous observations concerning

cholinergic reception, both in molluscs and in vertebrates, and also pose several new lines of inquiry. The clinical syndromes of peripheral neuromuscluar block for *C. d. terrificus* and *E. carinatus*, which would involve disruption of known cholinergic transmission systems, were supported by these single-unit membrane studies and argue for a postsynaptic effect for the cholinergic blocking fraction in this venom. However, further work is necessary to sort out definite receptor-site effects as opposed to more general effects of membranolytic components.

The observation that *E. carinatus* venom irreversibly blocks one of the acetylcholine responses suggests that its binding affinity might be sufficiently high for use in extraction of the receptor protein in the molluscan CNS, although its neurotoxic syndrome in vertebrates does not appear to be clinically significant. In comparison, the lack of complete cholinergic block at the sodium channel by venom from the sea snakes was surprising in light of the high toxicity levels reported for this family of snakes against vertebrate systems. It is likely that these discrepancies reflect subtle differences in the forms of cholinergic receptors that have developed in various types of neural tissue and that the venom properties could be extremely valuable in sorting out these differences.

The results in Table 1 show that, with the exception of *B. multicinctus*, none of the venoms affected the potassium efflux response. This supports previous experiments which demonstrated that this particular complex has a unique pharmacological spectrum and that the receptor as it exists in molluscs is not directly comparable to known cholinergic receptors in vertebrate tissue. The ACh-Na^+ and ACh-Cl^- complexes, on the other hand, compare rather closely to nicotinic receptors found on sketetal muscle and autonomic ganglia, respectively, and this subdivision is supported by the present experiments. All venoms which interfered with the sodium or the chloride response, with only the exception of *Micrurus*, blocked or reduced both responses. However, the postjunctional membrane of vertebrate skeletal muscle, where Darwinian selection of these venoms presumably took place, is selectively permeable only to sodium and potassium, not to chloride. This suggests that either the active sites of the various venom proteins are unable to clearly differentiate between the two complexes or that the chloride-receptor complex which functions in the less organized molluscan tissue may have been retained and modified as higher neural systems evolved. The failure of *Micrurus* venom to block chloride fluxes in this regard may suggest a fundamental variation in the structure or functioning of coral snake neurotoxin.

The demonstration of a possible blocking fraction in the venom of *V. russelli*, if confirmed, will provide adrenergic-receptor physiologists with several new possibilities for experimentation, including selective blocking of dopaminergic pathways in the caudate nucleus and disruption of the proposed cholinergic-dopaminergic link functioning within the supraesophageal ganglion. Experiments

have already begun in our laboratory to survey the blocking effect of this venom on tissue cultured spinal neuron responses to adrenergic compounds. The observation that the potassium channel was the only dopamine channel affected by *V. russelli* or *C. d. terrificus* is insufficient, by itself, to provide information on the structure or functioning of these receptors. However, additional venom experiments in conjunction with known blocking compounds for these complexes (curare for Dop-Na^+; ergometrine for Dop-K^+) may lead to a more definitive hypothesis concerning these complexes.

The value of the *V. russelli* fraction in the extraction of a dopamine receptor protein will depend on the degree of its binding affinity for the tissue receptor to be investigated. α-Bungarotoxin, for example, has a relatively high dissociation constant from cholinergic sites in the molluscan CNS and can be washed off with little difficulty (Shain *et al.*, 1974). However, it is essentially irreversibly bound to vertebrate postjunctional tissue. While the results of these experiments indicate that the Dop-K^+ block by *V. russelli* venom is reversible by washing this may not be the case with other dopaminergic receptors. In addition, further surveys of viperid snake venoms may turn up fractions with a higher binding affinity than has been indicated here, and a more comprehensive survey of venom concentrations might also uncover levels of effect that would be better suited for pharmacological dissection of the neural receptor systems.

The snake venoms surveyed in these experiments constitute an extremely small percentage of the potentially valuable, naturally occurring toxins which are now available to neurophysiologists. In the future close collaboration between clinical and research scientists should result in an ever-increasing number of refined, highly selective compounds for use in a wide variety of medical and experimental situations. The molluscan CNS preparation, as an extremely subtle discriminator of receptor-site pharmacology, could prove to be of considerable value as a screening device for these future pharmacological tools as they are developed.

SUMMARY

A general survey of crude venom from snakes of four different families revealed that several venoms which are known to operate at peripheral cholinergic junctions in vertebrates also interfere with cholinergic receptor sites in molluscan CNS tissue, and that venom from one snake, *V. russelli*, may contain a fraction which selectively interferes with dopamine receptor sites. Several additional experiments concerning the structure and mechanisms of both cholinergic and dopaminergic transmission are proposed and certain snake venoms are suggested for further investigation as possibly valuable pharmacological probes in future experiments of receptor pharmacology.

ACKNOWLEDGMENTS

We wish to thank Mr. Gary Gaubatz for his fine technical assistance during the course of these experiments, and Dr. James Willis for the use of his recently designed iontophoretic unit. Dr. Clarence Broomfield provided consistently high-quality advice and assistance concerning fractionation of the venoms, and Wendy Parmentier helped to prepare the manuscript.

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Species Differences in Reversibility of Neuromuscular Blockade by Elapid and Sea Snake Neurotoxins

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INTRODUCTION

In the previous paper, it has been demonstrated that the neuromuscular (N-M) block produced by long-chain neurotoxins (α-bungarotoxin and toxin A) is much more irreversible than that produced by short-chain neurotoxins (cobrotoxin and erabutoxin b) (Lee *et al.*, 1972a). Since the former neurotoxins differ from the latter in having more residues of valine and alanine, and fewer residues of glutamic acid in their molecules, it has been suggested that the richness in hydrophobic amino acids of their molecules may account for the irreversibility of the N-M blockade.

On the other hand, it was also shown that the skeletal muscle of the frog (*Rana tigrina*) was extremely resistant to erabutoxin b and that the N-M block produced by cobrotoxin as well as by toxin A in the muscle of this species was reversible. These findings are contradictory to those of Tamiya and Arai (1966) who have found that erabutoxins a and b produce an irreversible N-M block in the muscle of *Rana nigromaculata*, indicating that there might be some species differences, even within the same genus, in susceptibility to and reversibility of the N-M block by these neurotoxins.

In this connection, it is of interest to note that the skeletal muscle of the cat is highly resistant to the N-M blocking action of cobra venom but not so to that of *Bungarus multicinctus* venom (Lee and Tseng, 1969). So far, no information

is available on the reversibility of the N-M block produced by different neurotoxins in the muscle of this species.

The present investigation was undertaken in order to obtain more information on species differences in reversibility of N-M blocking produced by these neurotoxins.

MATERIALS AND METHODS

α-Bungarotoxin. The venom of *B. multicinctus* was first fractionated into eight major fractions on a column of CM-Sephadex C-50 as previously described (Lee *et al.*, 1972b). α-Bungarotoxin was obtained from fraction II by rechromatography on a CM-cellulose column. Its LD_{50} in mice was 0.15 μg/g if given intraperitoneally and 0.21 μg/g if given subcutaneously.

Cobrotoxin. The venom of *Naja naja atra* was first fractionated into twelve fractions on a column of CM-Sephadex C-50 as previously described (Lee *et al.*, 1968). The principal neurotoxin (cobrotoxin) was obtained from fraction VIII by rechromatography on a CM-cellulose column. Its LD_{50} in mice was 0.07 μg/g if given intraperitoneally and 0.09 μg/g if given subcutaneously.

Toxin A. Toxin A, isolated from the venom of Indian cobra (*Naja naja*) by means of CM-cellulose column chromatography (Nakai *et al.*, 1970; Nakai *et al.*, 1971), was kindly supplied by Dr. K. Hayashi, Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan. Its LD_{50} in mice was 0.15 μg/g (Nakai *et al.*, 1970).

Erabutoxin b. Erabutoxin b isolated from the venom of *Laticauda semifasciata* was a gift from Dr. N. Tamiya, Department of Chemistry, Tohoku University, Sendai, Japan. Its LD_{50} in mice was 0.15 μg/g (Tamiya and Arai, 1966).

Phrenic Nerve-Diaphragm Preparation. Phrenic nerve-diaphragm preparations were isolated from adult rats (Long-Evans) and kittens (200-400 g) according to the method described by Bülbring (1946). The preparation was suspended in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 11.9 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, and 5.6 mM glucose) which was constantly aerated with 95% O_2 + 5% CO_2 at 37°C. Indirect and direct supramaximal stimuli were alternately applied every 10 sec with rectangular pulses of 0.1 and 0.5 msec, respectively. The contractions were recorded isometrically with a Grass FT-03 force displacement transducer and a Grass model 5 polygraph.

Sciatic Nerve-Sartorius Muscle Preparation of the Frog. Three species of frogs, *R. tigrina var. pantherina*, *Rana narina*, and *Rana plancyi*, indigenous to Taiwan were used. The excised sciatic nerve-sartorius muscle preparation was mounted in 30 ml of aerated frog Ringer's solution (110 mM NaCl, 4.1 mM KCl, 1.6 mM $CaCl_2$, 4.2 mM $NaHCO_3$, and 4.0 mM glucose) at room temperature (23-28°C). Indirect and direct supramaximal stimuli were applied alternately

every 10 sec with rectangular pulses of 0.1 and 0.5 msec, respectively, and the contractions were recorded isometrically as described for the phrenic nerve-diaphragm preparation.

Biventer Cervicis Nerve-Muscle Preparation of the Chick. Isolated biventer cervicis nerve-muscle preparation (Ginsborg and Warriner, 1960) was suspended in 20 ml of Krebs' solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, and 11.2 mM glucose) which was bubbled with 95% O_2 + 5% CO_2 at 37°C. The preparation was indirectly stimulated with supramaximal rectangular pulses of 0.5 msec at a frequency of 0.1 Hz and the isometric contractions were recorded as described for the diaphragm preparation.

RESULTS

Phrenic Nerve-Diaphragm Preparation of the Kitten. As shown in Fig. 1, the phrenic nerve-diaphragm preparation of the kitten was much less sensitive to erabutoxin b and cobrotoxin than to α-bungarotoxin and toxin A. The order of their potency in blocking N-M transmission in the kitten diaphragm was found to be α-bungarotoxin > toxin A > cobrotoxin >> erabutoxin b. After N-M blockade, the preparation was washed every 10 min with Tyrode's solution. As shown in Figs. 2a and b, the N-M block produced by either erabutoxin b or cobrotoxin could be easily reversed by washing, whereas that by toxin A was reversed only slowly by repeated washings (Fig. 2c). By contrast, the N-M block by α-bungarotoxin was irreversible; it could not be reversed even by repeated washings for four hours (Fig. 2d).

Sciatic Nerve-Sartorius Muscle Preparation of the Frog. In the previous paper, it was found that the sartorius muscle of *R. tigrina* was extremely resistant

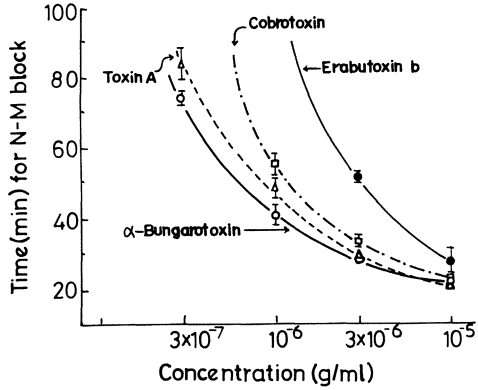

Fig. 1. Relationship between concentration (g/ml) of neurotoxins and time (min) for a complete N-M block of the kitten phrenic nerve-diaphragm preparation. The vertical bar denotes the standard error of each mean value of 4-6 experiments.

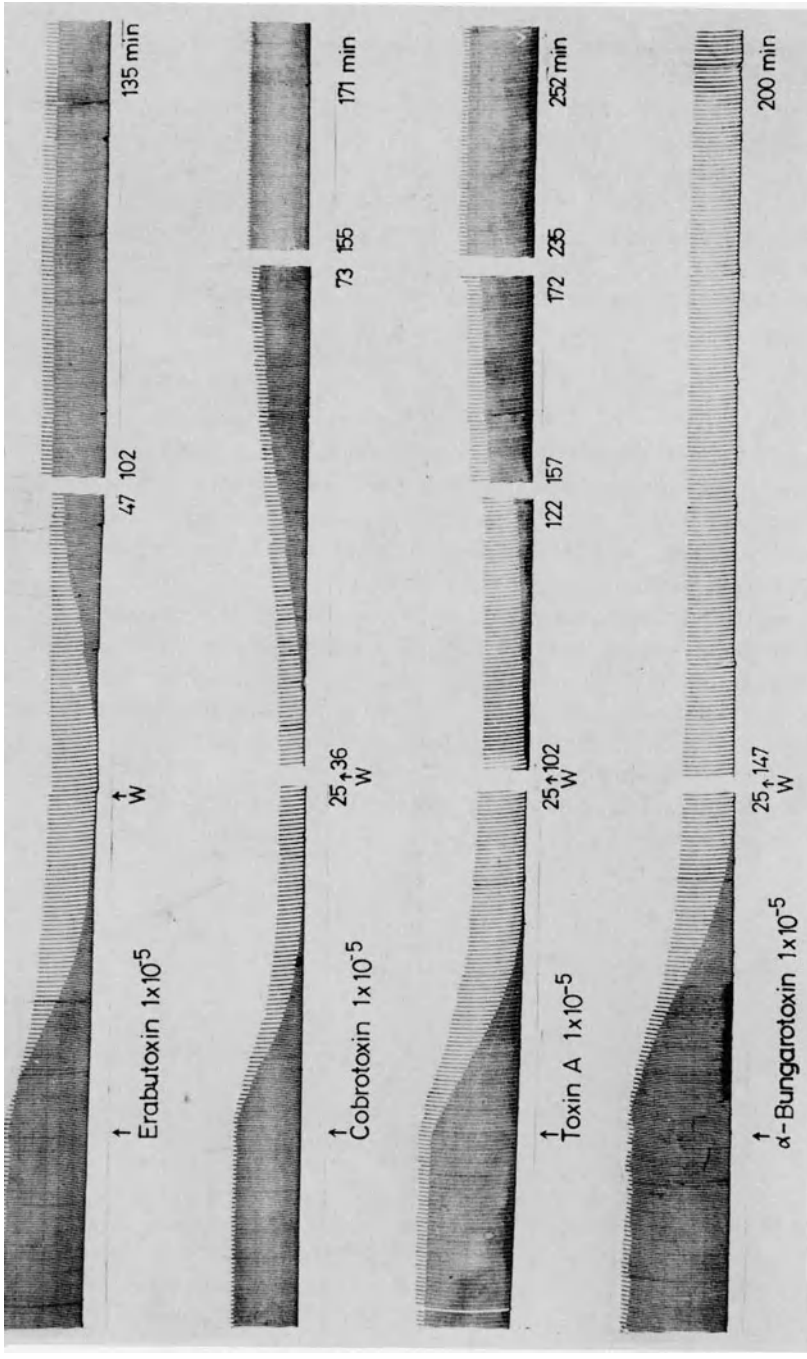

Fig. 2. Comparison of reversibility of N-M blocking by different neurotoxins in the kitten phrenic nerve-diaphragm preparations. The preparation was washed (starting at the arrow with W) every 10 min with Tyrode's solution after N-M block.

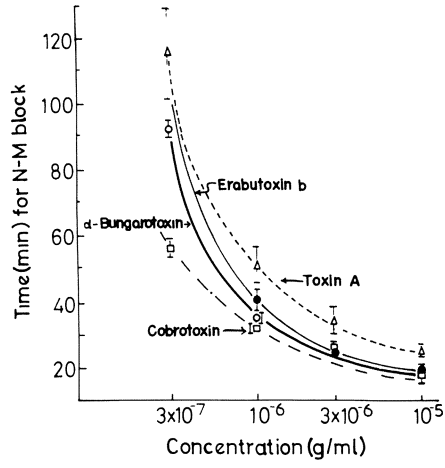

Fig. 3. Relationship between concentration (g/ml) of neurotoxins and time (min) for complete N-M blocking of the sartorius muscle preparation of *Rana narina*.

to erabutoxin b and also relatively insensitive to cobrotoxin (Lee *et al.*, 1972a). The order of potency in producing N-M blocking was quite different from that found in the rat diaphragm and the chick biventer cervicis muscle preparations, being α-bungarotoxin $\geqq$ toxin A > cobrotoxin >> erabutoxin. Moreover, the N-M block produced by cobrotoxin as well as that by toxin A in the muscle of this species of the frog were reversible, wheras in the rat diaphragm and the chick biventer cervicis muscle the N-M block by toxin A was irreversible.

In the present study, the N-M blocking action of these four neurotoxins was compared in the sartorius muscle preparations of *R. narina* and *R. plancyi*. Unlike the muscle of *R. tigrina*, the sartorius muscles of these two species were found to be quite susceptible to both erabutoxin b and cobrotoxin. The order of potency in producing N-M blocking in the sartorius muscle of *R. narina* was cobrotoxin > α-bungarotoxin $\geqq$ erabutoxin b > Toxin A (Fig. 3); whereas that of

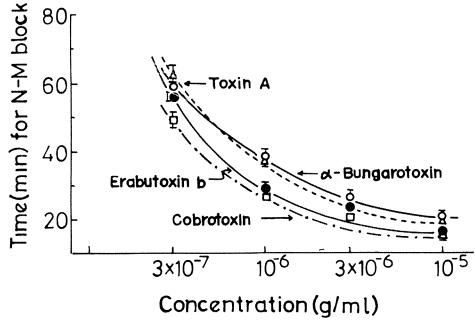

Fig. 4. Relationship between concentration (g/ml) of neurotoxins and time (min) for complete N-M blocking of the sartorius muscle preparation of *Rana plancyi*.

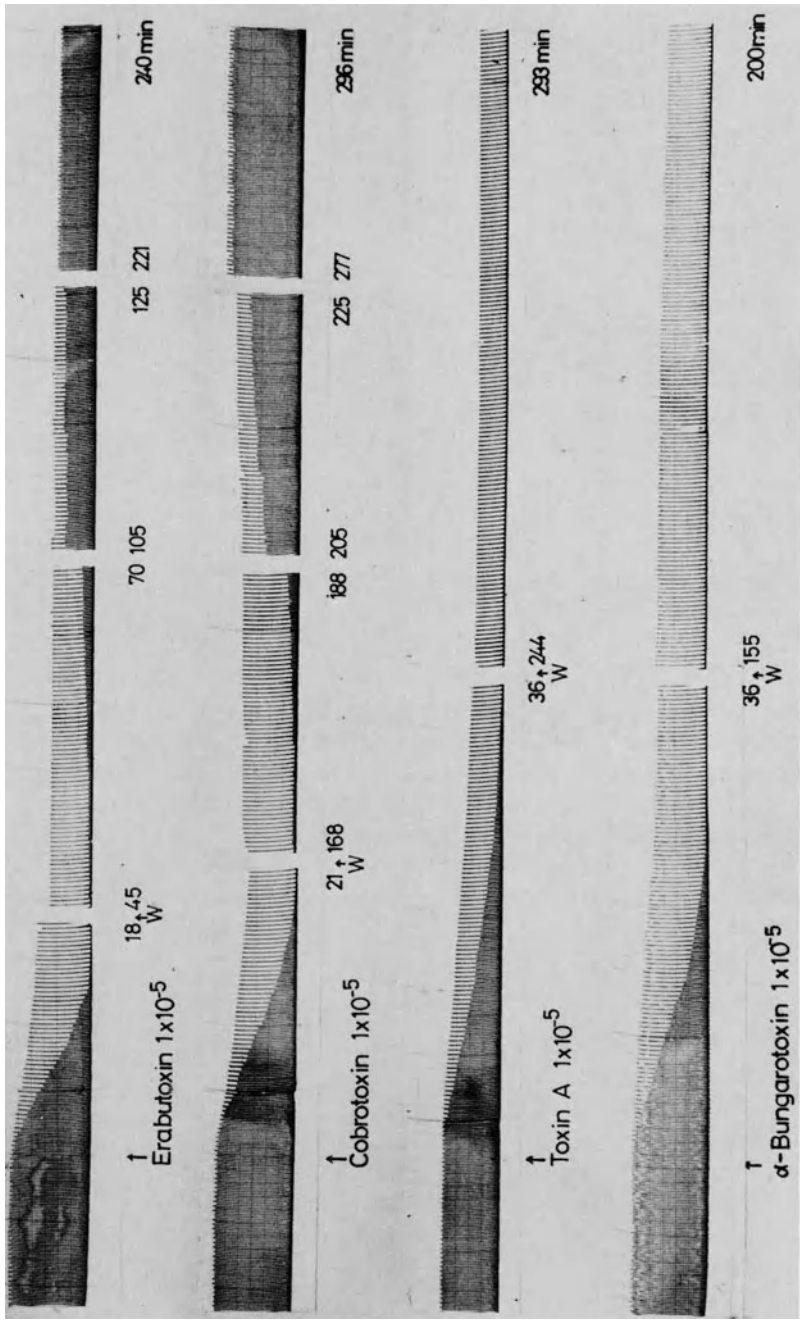

Fig. 5. Comparison of reversibility of N-M blocking by different neurotoxins in the sartorius muscle preparation of *Rana narina*. The preparation was washed (starting at the arrow with W) every 10 min with frog Ringer's solution.

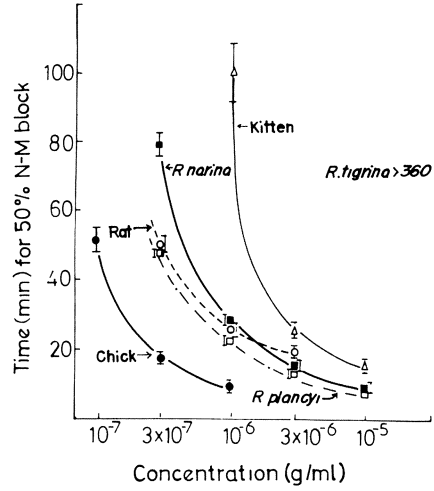

Fig. 6. Relationship between concentration (g/ml) of erabutoxin b and time (min) for 50% N-M blocking of nerve-muscle preparations from different animal species.

R. plancyi was cobrotoxin $\geqq$ erabutoxin b > Toxin A = α-bungarotoxin (Fig. 4). As shown in Fig. 5, the N-M block produced by either erabutoxin b or cobrotoxin in the muscle of *R. narina* was slowly reversible, whereas that by either α-bungarotoxin or toxin A was irreversible even after prolonged washing. Unlike the muscle of *Rana narina*, however, all four of these neurotoxins produced an irreversible N-M block in the muscle of *R. plancyi*.

Species Differences in Susceptibility to N-M Blocking Action. In order to compare the susceptibility of nerve-muscle preparations from different species to the N-M blocking action of these neurotoxins, the time needed for a 50% N-M block was plotted against concentrations for each neurotoxin.

As shown in Figs. 6-9, the orders of susceptibility of nerve-muscle preparations from different species to these four neurotoxins are as follows:

1. Erabutoxin b (Fig. 6): chick > *R. plancyi* $\geqq$ rat > *R. narina* > kitten >> *R. tigrina*.

2. Cobrotoxin (Fig. 7): chick > *R. plancyi* > rat $\geqq$ *R. narina* > kitten > *R. tigrina*.

3. Toxin A (Fig. 8): chick > *R. plancyi* > rat $\geqq$ kitten > *R. narina* > *R. tigrina*.

4. α-Bungarotoxin (Fig. 9): chick > *R. plancyi* > rat $\geqq$ kitten $\geqq$ *R. narina* > *R. tigrina*. Thus, without exception, the chick biventer cervicis muscle was the most susceptible while the sartorius muscle of *R. tigrina* was the most resistant to each neurotoxin. The kitten diaphragm preparation was also quite resistant to erabutoxin b and cobrotoxin but not particularly resistant to toxin A and α-bungarotoxin. Its susceptibility to the latter two neurotoxins was rather close to that of the rat diaphragm preparation. It is also interesting to note that susceptibilities of three species of frogs to these neurotoxins are quite different from

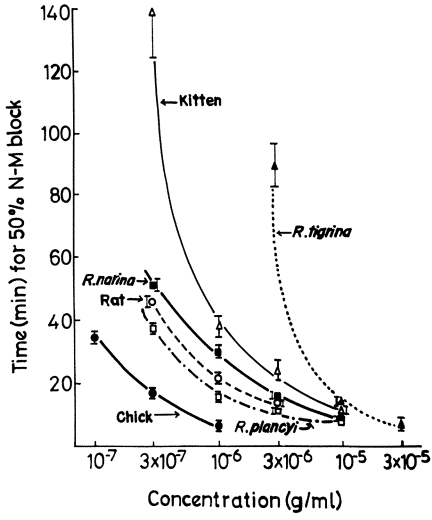

Fig. 7. Relationship between concentration (g/ml) of cobrotoxin and time (min) for 50% N-M blocking of nerve-muscle preparations from different animal species.

one to another. The muscle of *R. plancyi* was the most susceptible, followed by that of *R. narina*, whereas that of *R. tigrina* was the most resistant, especially to erabutoxin b.

Species Differences in Reversibility of N-M Block. In order to compare reversibility of the N-M blocking produced by these neurotoxins in nerve-muscle preparations from different species, the time needed for 50% recovery from the complete N-M block was measured after repeated washings every 10 min.

As shown in Table 1, the N-M block produced by either cobrotoxin or erabutoxin b was reversible in the nerve-muscle preparations of the following species in this order: *R. tigrina* > kitten > rat > *R. narina*. The N-M block pro-

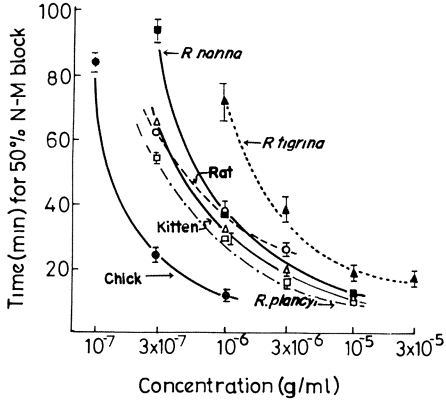

Fig. 8. Relationship between concentration (g/ml) of toxin A and time (min) for 50% N-M blocking of nerve-muscle preparations from different animal species.

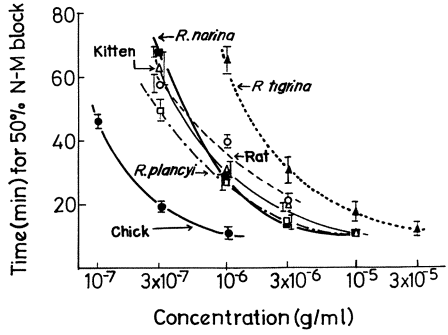

Fig. 9. Relationship between concentration (g/ml) of α-bungarotoxin and time (min) for 50% N-M blocking of nerve-muscle preparations from different animal species.

duced by erabutoxin b in these preparations was more easily restored than that by cobrotoxin. On the other hand, no recovery was found in the chick biventer cervicis and the sartorius muscle of *R. plancyi* even after repeated washings for several hours. The N-M block by toxin A was slowly reversible only in the sartorius muscle of *R. tigrina* and the kitten diaphragm; no recovery was found in the rat diaphragm, the sartorius muscle of both *R. narina* and *R. plancyi*, and the chick biventer cervicis muscle. The N-M block by α-bungarotoxin was always irreversible; no recovery was found in the nerve-muscle preparation of every species thus far tested.

DISCUSSION

As clearly shown in the present study, reversibility of N-M blocking produced by snake neurotoxins depends not only on the kind of toxins used but also on the species of animals whose muscle is to be tested.

The elapid and sea snake neurotoxins with curarelike action (so-called postsynaptic toxins) can be classified into two groups according to their amino acid compositions (Lee, 1972; Strydom, 1973). Neurotoxins belonging to the first group (short-chain neurotoxins) are composed of 61-62 residues of 15-16 amino acids in a single peptide chain crosslinked by four disulfide bridges; whereas those belonging to the second group (long-chain neurotoxins) consist of 71-74 residues of 17-18 amino acids with five disulfide bridges. Erabutoxin b and cobrotoxin belong to the first group, while toxin A and α-bungarotoxin belong to the second group. The previous finding that the N-M block produced by α-bungarotoxin and toxin A is much more irreversible than that by cobrotoxin and erabutoxin b (Lee *et al.*, 1972a) is further substantiated by the present study. Thus, while the N-M block produced by cobrotoxin and erabutoxin b is reversible in the muscles of the kitten, rat, *R. tigrina*, and *R. narina*, that produced by toxin A in the muscle of the rat, chick, *R. narina* and *R. plancyi*, as well as that by α-bungarotoxin in every muscle preparation so far tested, is irreversible. Be-

Table 1. Time (min) for 50% Recovery from Complete Neuromuscular Block[a]

Species	Concentration, g/ml	Erabutoxin b	Cobrotoxin	Toxin A	α-Bungarotoxin
Kitten diaphragm	3×10^{-6}	23.0 ± 5.0(3)	77.0 ± 16.9(4)	143.0 ± 11.9(4)	No recovery
	10^{-5}	37.4 ± 6.7(5)	79.7 ± 21.8(3)	178.0 ± 3.2(4)	
Rana tigrina sartorius	10^{-5}	No complete block	14.3 ± 2.2(4)	164.0 ± 10.1(4)	No recovery
	3×10^{-5}	No complete block	21.0 ± 3.5(3)	250.3 ± 53.8(4)	
Rat diaphragm	10^{-6}	136.0 ± 9.8(4)	207.7 ± 10.5(4)	No recovery	No recovery
Rana narina sartorius	10^{-6}	142.7 ± 5.8(3)	225.5 ± 5.2(4)	No recovery	No recovery
Chick biventer cervicis	10^{-6}	No recovery	No recovery	No recovery	No recovery
Rana plancyi sartorius	10^{-6}	No recovery	No recovery	No recovery	No recovery

[a]Data are presented as mean ± S.E.M. Figures in parentheses denote number of experiments.

sides, the N-M block by α-toxin from *Naja nigricollis*, consisting of 61 amino acid residues, has been reported to be reversible (Tazieff-Depierre and Pierre, 1966), while that by toxin T_3 from *Naja naja siamensis*, consisting of 71 residues, has been shown to be irreversible (Lester, 1970). From these findings it can be concluded that the N-M block produced by the long-chain neurotoxins is much more irreversible than that by short-chain neurotoxins. The present study also demonstrates that α-bungarotoxin is most irreversible, followed by toxin A, and that erabutoxin b is more reversible than cobrotoxin.

With regard to susceptibility to and reversibility of N-M blocking by snake neurotoxins, the skeletal muscles (or more precisely the motor endplates) of different species can be classified into the following three categories.

1. Muscles with low susceptibility to snake neurotoxins, especially to erabutoxin b and cobrotoxin, as exemplified by the sartorius muscle of *R. tigrina* and the kitten diaphragm. The N-M block in this group is mostly reversible, except in the case of α-bungarotoxin.

2. Muscles with intermediate susceptibility to snake neurotoxins, as exemplified by the sartorius mucle of *R. narina* and the rat diaphragm. The N-M block in this group is slowly reversible if produced by the short-chain neurotoxins, but it is irreversible if produced by the long-chain neurotoxins.

3. Muscles with high susceptibility to snake neurotoxins, as exemplified by the sartorius muscle of *R. plancyi* and the chick biventer cervicis muscle. Every neurotoxin so far tested produces irreversible N-M block in this group.

From these findings, it may be concluded that the less susceptible the muscle is to the neurotoxin, the more reversible will be the N-M block and *vice versa*. It is rather surprising to find that the susceptibility of the frog muscle to snake neurotoxins differs so much from one species to another. So far no explanation can be given for such species differences.

SUMMARY

Reversibility of the N-M block produced by four postsynaptic snake neurotoxins has been compared in nerve-muscle preparations of different species.

Erabutoxin b and cobrotoxin produce reversible N-M blocking in the muscles of the kitten, rat, *R. tigrina*, and *R. narina* but irreversible blocking in those of the chick and *R. plancyi*.

Toxin A produces irreversible N-M block in the muscles of most species except the kitten and *R. tigrina*.

α-Bungarotoxin produces irreversible N-M block in every muscle preparation so far tested.

It is concluded that the N-M block produced by the long-chain neurotoxins (α-bungarotoxin and toxin A) is much more irreversible than that by short-chain neurotoxins (cobrotoxin and erabutoxin b), and that the less susceptible is

the muscle to the neurotoxins, the more reversible will be the N-M block and *vice versa*.

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Comparison of Actions of Cobra Cardiotoxin and Scorpion Toxin on the Chick Biventer Cervicis Muscle

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INTRODUCTION

Most toxins isolated from snake and scorpion venoms are basic polypeptides, composed of 57–66 amino acid residues in a single chain crosslinked by four disulfide bonds (Lee, 1972; Miranda *et al.*, 1970). However, their pharmacological actions are rather different. The postsynaptically acting snake neurotoxins possess a curarelike action without any direct effect on the muscle or nerve endings (Lee, 1972), while scorpion venoms induce muscle twitchings and neuromuscular blockade, owing to their effect on the muscle or through the motor nerve (Zlotkin and Shulov, 1969). Unlike snake neurotoxins, cobra cardiotoxin (CTX) induces contracture of skeletal muscle, neuromuscular blockade, and irreversible membrane depolarization (Lee *et al.*, 1968). Recently, Miranda *et al.* (1970) have succeeded in isolating eleven toxins from scorpion venoms. The most toxic fraction, namely, toxin II from the venom of *Androctonus australis Hector,* can induce muscle contracture and spontaneous contractions. The purpose of this investigation is to compare the muscle contracture induced by CTX and toxin II and to elucidate their possible mode of action by studying their interactions with cations and certain drugs.

MATERIALS AND METHODS

Cardiotoxin. Cardiotoxin (CTX) was isolated from the venom of *Naja naja atra* according to the method described by Lee *et al.* (1968). Fraction XII (CTX) was rechromatographed twice on a CM-cellulose column in order to eliminate the trace of phospholipase A.

Toxin II. Toxin II, which was isolated from the venom of *A. a. Hector* according to the method described by Miranda *et al.* (1970), was kindly supplied by Professor F. Miranda, Laboratoire Biochimie Médecine, Faculté de Médecine, Marseille, France.

β-Bungarotoxin. β-Bungarotoxin (β-BuTX) was isolated from the venom of *Bungarus multicinctus* according to the method described by Lee *et al.* (1972).

Skeletal Muscle Preparation. Chicks at the age of 4–8 days were used in all experiments. The chick biventer cervicis nerve-muscle preparation was isolated according to the method of Ginsborg and Warriner (1960). The muscle was suspended in 10 ml of Krebs-Ringer bicarbonate solution (118.1 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.1 mM glucose) which was constantly aerated with 95% O_2 + 5% CO_2 at 37°C. The muscle indirectly stimulated through the tendon with supramaximal rectangular pulses of 0.5 msec at a rate of 6 per min. The contraction was isometrically recorded with a force-displacement transducer and a Nihon Kohden recorder, model RM-25. Paired muscles were used: one as the control in normal Krebs solution and the other in the modified Krebs solution or normal Krebs solution containing various drugs (1–1.8 mM procaine HCl, 0.3–3 μM tetrodotoxin, 10^{-5} g/ml of *d*-tubocurarine or β-BuTX). The muscle was preincubated with the drug or the modified Krebs solution for 20 min followed by the addition of 1.5 μM CTX or 0.04 μM toxin II. The time to reach peak tension and peak tension were recorded and calculated as a percentage of the control. The significance of the difference between the control and treated muscles was tested by Student's *t* test. The number of experiments per group was at least three as indicated in the results.

Preparation of Modified Krebs Solution. The modified Krebs-Ringer bicarbonate solution was prepared as follows: Ca^{2+}-free Krebs solution was prepared by omitting $CaCl_2$; high-Mg^{2+} Krebs solution by increasing the amount of $MgCl_2$ from 1.2 mM to 10 mM; high-Ca^{2+} Krebs solution by increasing $CaCl_2$ from 2.5 mM to 12 mM, omitting KH_2PO_4 and reducing the amount of $NaHCO_3$ to one half in order to prevent the formation of precipitation; and low-Na^+ Krebs solution by replacing NaCl with an isosmotic sucrose.

Uptake of $^{45}Ca^{2+}$ in the Chick Biventer Cervicis Muscle. The muscle preparation was incubated in Krebs solution containing $^{45}Ca^{2+}$ (2μCi/ml) in the presence or absence of 1.5 μM CTX or 0.04 μM toxin II at 25°C for 30 min. The muscle was then rinsed with normal Krebs solution three times, each for 5 min.

Radioactivity of $^{45}Ca^{2+}$ in the muscle was measured with a Packard liquid scintillation counter model 3320, after the muscle was blotted with filter paper, weighed, and digested with protosol (New England Nuclear Inc., U.S.A.).

Measurement of Membrane Potentials. Resting membrane potentials were measured by the conventional microelectrode recording method using the isolated chick biventer cervicis muscle. The organ bath contained 20-ml Krebs solution at 30–32°C and was aerated with 95% O_2 + 5% CO_2. The microelectrodes filled with 3 M KCl had a resistance of 5–20 MΩ. Recordings were made only from the superficial muscle fibers.

RESULTS

Actions of CTX and Toxin II on the Chick Biventer Cervicis Muscle. Both CTX and toxin II were potent enough to induce contracture in the chick biventer cervicis muscle. Figures 1 and 2 show the representative tracings of CTX and toxin II contracture. CTX was less potent than toxin II and the threshold

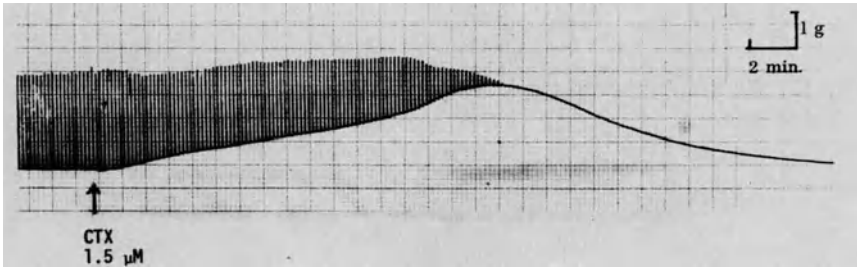

Fig. 1. Record of isometric twitch and contracture tension of the chick biventer cervicis muscle to supramaximal indirect stimulation through the tendon in the presence of 1.5 μM CTX.

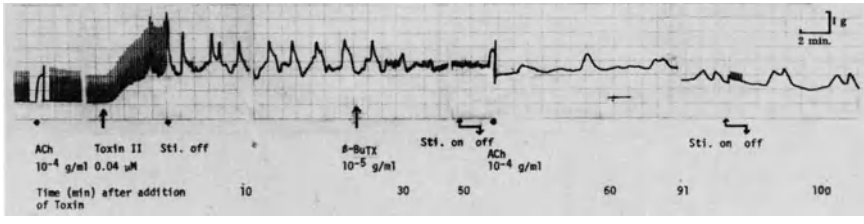

Fig. 2. Toxin II induced contracture and spontaneous twitchings in the chick biventer cervicis muscles. After the peak tension of toxin II contracture was obtained, the indirect stimulation was turned off. Spontaneous twitchings were recorded isometrically, which were inhibited by β-BuTX. After the response to ACh was tested, both toxin II and β-BuTX were washed out and spontaneous twitchings re-appeared.

concentrations required to induce a prominent contracture were 0.45 μM and 0.014 μM respectively. Toxin II, but not CTX initially increased the amplitude of twitch response to indirect stimulation. In addition to the muscle contracture, both toxins blocked the neuromuscular transmission. Toxin II also induced spontaneous contractions in this muscle, but CTX did not. The dose-response curves of CTX and toxin II contracture are shown in Fig. 3. The peak tension increased progressively over the concentration ranges 0.45-1.5 μM for CTX and 0.0014-0.14 μM for toxin II, whereas the time to reach peak tension was proportionally shortened.

Effects of Ca^{2+} on the Actions of CTX and Toxin II. CTX contracture was markedly accelerated and toxin II contracture moderately by a Ca^{2+}-free medium (Tables 1 and 2). The time to reach peak tension was shortened. However, spontaneous contractions induced by toxin II were completely abolished by the Ca^{2+}-free medium but reappeared as soon as Ca^{2+} (2.5 mM) was added to the Ca^{2+}-free medium (Fig. 4). On the other hand, the high-Ca^{2+} (12 mM) medium abolished the action of CTX completely but only partially inhibited toxin II contracture (Tables 1 and 2). The neuromuscular blocking action of toxin II was completely inhibited by a high-Ca^{2+} medium but reappeared as soon as the high-Ca^{2+} medium was replaced with normal Krebs solution (Fig. 5).

Effects of High Mg^{2+} and Low Na^{+} Media on the Actions of CTX and Toxin II. A high-Mg^{2+} (10 mM) medium accelerated CTX contracture but inhibited toxin II contracture (Tables 1 and 2). The neuromuscular blockade induced

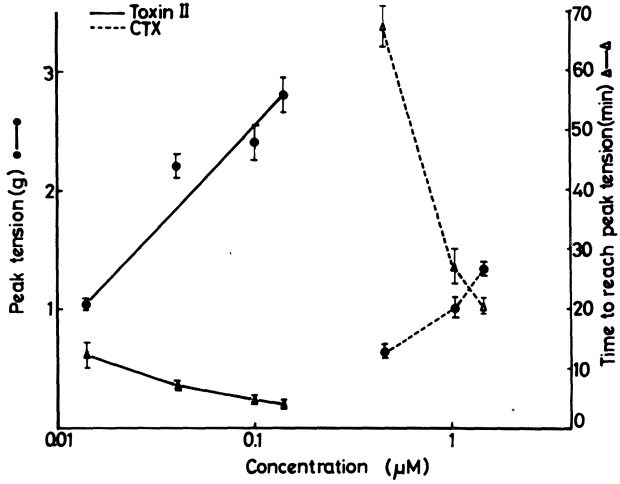

Fig. 3. Dose-response curves of CTX and toxin II contracture in chick biventer cervicis muscles. Peak tension (circles) and the time to attain peak tension (triangles) over the concentration range 0.014-0.14 μM of toxin II (circles, solid line) and 0.45-1.5 μM CTX (triangles, dashed line) are shown.

Table 1. Effects of Various Cations and Drugs on Cardiotoxin Contracture in Chick Biventer Cervicis Muscles[a]

Pretreatment	Concentration	No. of exp.	TPT, min	PT, %
Control	–	21	20.2 ± 1.3	100.0 ± 4.3
Ca^{2+} free	0	3	3.5 ± 0.7[b]	80.7 ± 12.9
High Ca^{2+}	12 mM	5	>120	0
High Mg^{2+}	10 mM	9	10.7 ± 1.0[b]	101.7 ± 7.2
Low Na^{+}	60 mM	6	20.5 ± 1.3	84.6 ± 3.7
Procaine	1.8 mM	8	25.6 ± 3.2	96.7 ± 6.0
TTX	3 μM	3	15.5 ± 3.8	91.3 ± 5.5
d-TC	10^{-5} g/ml	3	18.8 ± 2.6	95.2 ± 4.2
β-BuTX	10^{-5} g/ml	7	16.7 ± 3.9	121.0 ± 6.7

[a]The chick biventer cervicis muscle is suspended in normal Krebs solution and stimulated through the tendon with supramaximal rectangular pulses of 0.5 msec at a rate of 6 per min. Various cations or drugs are added 20 min prior to the addition of 1.5 μM CTX. The time to reach peak tension (TPT) and peak tension (PT) are recorded. Peak tension is calculated as the percentage of the control. Data are presented as mean ± S.E.
[b]$p < 0.05$.

by the high-Mg^{2+} medium was slightly antagonized by toxin II (Fig. 6). Spontaneous contractions induced by toxin II were unaffected by the high-Mg^{2+} medium. On the other hand, the low-Na^{+} (60 mM) medium had no effect on CTX contracture, while it partially inhibited toxin II contracture (Tables 1 and 2). Spontaneous contractions were apparently unaffected by the low-Na^{+} medium.

Table 2. Effects of Various Cations and Drugs on Toxin II Contracture in Chick Biventer Cervicis Muscles[a]

Pretreatment	Concentration	No. of exp.	TPT, min	PT, %
Control	–		7.1	100
Ca^{2+} free	0	7	3.9 ± 2.2[b]	124.0 ± 12.0
High Ca^{2+}	12 mM	5	19.0 ± 2.0[b]	90.0 ± 16.0
High Mg^{2+}	10 mM	6	16.4 ± 2.1[b]	89.0 ± 33.0
Low Na^{+}	60 mM	5	16.5 ± 0.8[b]	108.0 ± 13.0
Procaine	1.0 mM	7	40.1 ± 3.6[b]	85.0 ± 13.0[b]
TTX	0.3 μM	7	complete inhibition	complete inhibition
d-TC	10^{-5} g/ml	4	15.1 ± 2.0[b]	80.0 ± 8.0[b]
β-BuTX	10^{-5} g/ml	5	7.8 ± 1.0	101.0 ± 17.0

[a]The chick biventer cervicis muscle is suspended in normal Krebs solution and stimulated through the tendon with supramaximal rectangular pulses of 0.5 msec at a rate of 6 per min. Various cations or drugs are added 20 min prior to the addition of 1.5 μM CTX. The time to reach peak tension (TPT) and peak tension (PT) are recorded. Peak tension is calculated as the percentage of the control. Data are presented as mean ± S.E.
[b]$p < 0.05$.

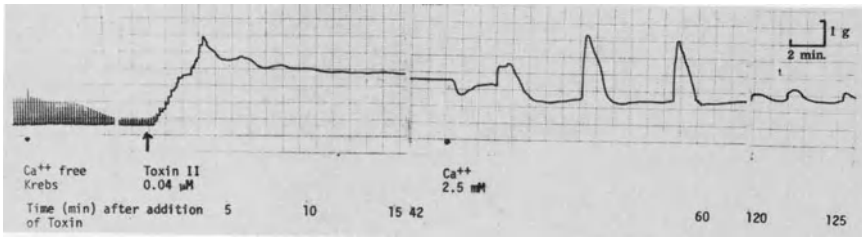

Fig. 4. Effect of a Ca^{2+}-free medium on the actions of toxin II on the chick biventer cervicis muscles. Toxin II contracture was slightly accelerated but spontaneous twitchings were abolished by the Ca^{2+}-free medium. Spontaneous twitchings reappeared after the addition of 2.5 mM Ca^{2+}

Effect of Procaine and Tetrodotoxin (TTX) on the Actions of CTX and Toxin II. As shown in Table 1, both procaine and TTX had no significant effect on CTX contracture. By contrast, both procaine and TTX inhibited the action of toxin II (Table 2), whether they were added before toxin II or at the peak tension of toxin II contracture. After washing, toxin II contracture reappeared.

Effects of *d*-Tubocurarine (*d*-TC) and β-Bungarotoxin on the Actions of CTX and Toxin II. As shown in Table 1, both *d*-TC and β-BuTX were also without significant effect on CTX contracture. However, *d*-TC slightly inhibited toxin II contracture and delayed the appearance of spontaneous contractions. Although β-BuTX did not inhibit toxin II contracture, it partially inhibited spontaneous contractions induced by toxin II (Fig. 2).

Effects of CTX and Toxin II on the Uptake of $^{45}Ca^{2+}$ in the Chick Biventer Cervicis Muscles. As shown in Table 3, the uptake of $^{45}Ca^{2+}$ in the chick

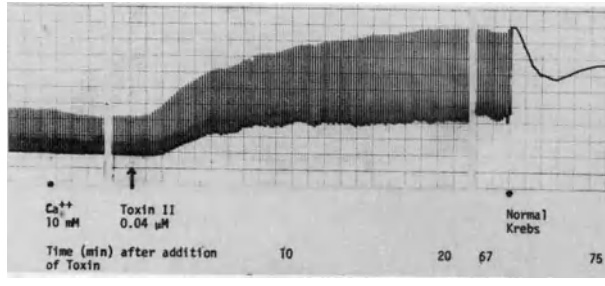

Fig. 5. Effect of a high-Ca^{2+} medium on the actions of toxin II on the chick biventer cervicis muscles. The twitch responses to indirect stimulation were markedly potentiated by toxin II in a high-Ca^{2+} medium. Toxin II contracture was slightly inhibited but the neuromuscular blocking action of toxin II was abolished by a high-Ca^{2+} medium. When the high-Ca^{2+} medium was replaced by normal Krebs solution neuromuscular blockade reappeared.

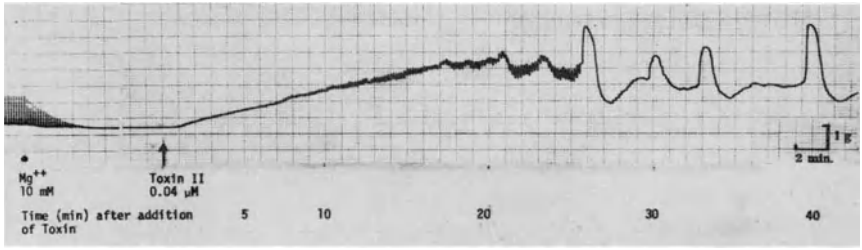

Fig. 6. Effect of a high-Mg^{2+} medium on the actions of toxin II on the chick biventer cervicis muscles. Twitch responses to indirect stimulation was abolished by a high Mg^{2+} medium. After the addition of 0.04 μM toxin II, twitch responses were partially recovered. Toxin II contracture was inhibited but spontaneous twitchings were not inhibited by a high-Mg^{2+} medium.

biventer cervicis muscle was markedly increased by CTX but only slightly by toxin II.

Effects of CTX and Toxin II on the Membrane Potential of the Chick Biventer Cervicis Muscles. Both CTX and toxin II decreased the membrane potential profoundly. As shown in Fig. 7, CTX decreased the membrane potential progressively from -66.5 ± 2.5 to -11.4 ± 1.2 mV in 2 hr, while toxin II decreased the membrane potential from -63.5 ± 1.9 to -35.0 ± 1.6 mV in 5 min and then no further decrease was observed in 2 hr.

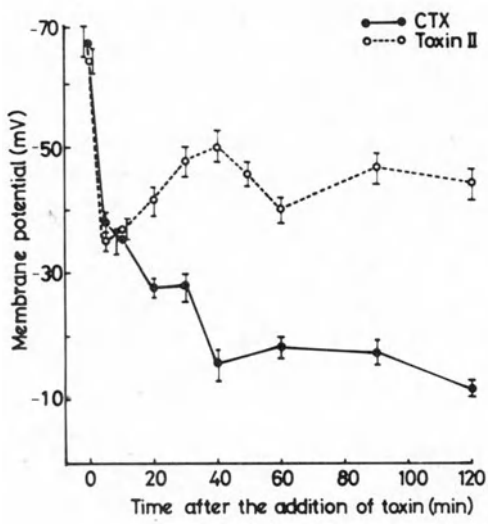

Fig. 7. Time course of effects of CTX and toxin II on the resting membrane potential of surface fibers of the chick biventer cervicis muscles. Each point is the mean of 15–33 individual fibers obtained in three experiments. Vertical lines indicate S.E.

Table 3. Effects of CTX and Toxin II on the Uptake of $^{45}Ca^{2+}$ in Chick Biventer Cervicis Muscles[a]

	No. of exp.	Muscle, cpm/mg	%
Control	9	281 ± 38	100 ± 15
CTX	9	1005 ± 67	372 ± 24[b]
Toxin II	7	424 ± 34	151 ± 12[b]

[a]The muscle preparation is incubated in Krebs solution containing $^{45}Ca^{2+}$ (2 μCi/ml) in the presence or absence of 1.5 μM CTX (or 0.04 μM toxin II) at 25°C for 30 min. The muscle is then rinsed with normal Krebs solution 3 times, each for 5 min. The radioactivity of $^{45}Ca^{2+}$ in the muscle is measured by a Packard liquid scintillation counter, after the muscle is blotted by filter paper, weighed and digested. Data are presented as mean ± S.E.
[b]$p < 0.05$.

DISCUSSION

Although toxin II and CTX were isolated from different kinds of animal venoms, namely scorpion and cobra venom, respectively, some similarities were found in their chemical structure. Both toxins are single polypeptides crosslinked by four disulfide bonds and have molecular weight around 7000. Moreover, their pharmacological actions are similar in many respects. They are potent in inducing contracture of the skeletal muscle, membrane depolarization, and blockade of neuromuscular transmission (Lee *et al.*, 1968; Zlotkin and Shulov, 1969). Therefore, it would be interesting to see if they induce muscle contracture by a common mechanism of action.

Comparison of the contracture induced in the chick biventer cervicis muscle revealed that toxin II is about 50 times more potent than CTX. The amplitude of twitch responses to the indirect stimulation was initially potentiated by toxin II but not by CTX. In addition, toxin II also produced spontaneous contractions in this muscle preparation, but CTX did not. The membrane potential of the chick biventer cervicis muscle was decreased by both toxins. CTX contracture was potentiated by either a Ca^{2+}-free or high-Mg^{2+} medium, inhibited completely by a high-Ca^{2+} medium, but unaffected by a low-Na medium, procaine, TTX, *d*-TC or β-BuTX. In contrast, toxin II contracture was moderately potentiated by a Ca^{2+}-free medium, inhibited by high Ca^{2+}, high-Mg^{2+}, and low-Na^{+} media, procaine, TTX and *d*-TC, but unaffected by β-BuTX. All of these findings suggest that the mode of actions of CTX and toxin II are distinct from each other.

It has been demonstrated that Ca^{2+} is important for muscle contraction as well as for the release of neurotransmitter (Sandow, 1965; Katz and Miledi, 1968), while Mg^{2+} is antagonistic to Ca^{2+} (Hubbard *et al.*, 1968). Mg^{2+} at a

concentration of 5 mM or higher exerts inhibitory actions on the acetylcholine response to the denervated rat diaphragm (Freeman and Turner, 1969) and on the transmitter release from nerve endings (Del Castille and Katz, 1954; Hubbard *et al.*, 1968). In this experiment, a high-Mg^{2+} medium, which almost completely inhibited twitch responses to indirect stimulation and partially inhibited toxin II contracture suggested a partial role of the acetylcholine release from the motor nerve endings in inducing contracture. The partial inhibitory action of *d*-TC on toxin II contracture also suggests this possibility. On the other hand, toxin II contracture could be induced in the Ca^{2+}-free medium so that extracellular Ca^{2+} was not required for the contracture. Procaine, which has been shown to inhibit the release of Ca^{2+} from the sarcoplasmic reticulum (Feinstein, 1963; Johnson and Inesi, 1969), antagonized toxin II contracture. Therefore, it is inferred that toxin II induces the muscle contracture by acting on the nerve endings as well as by a direct action on the muscle, possibly by releasing the membrane-bound Ca^{2+}.

Tetrodotoxin, which is a specific inhibitor of Na^+ influx (Narahashi, 1972), abolished toxin II contracture. Lowering the extracellular Na^+ concentration also inhibited toxin II contracture. These results are in agreement with those obtained with tityustoxin, another purified scorpion toxin, and the venom of *Leiurus quinquestriatus;* TTX could inhibit acetylcholine release from the rat brain induced by tityustoxin (Gomez *et al.*, 1973); and lowering of the extracellular Na^+ concentration antagonized the depolarizing action of the venom of *L. quinquestriatus* on the single myelinated frog nerve (Adam *et al.*, 1966). It is conceivable that all of these toxins exert their effects by increasing membrane Na^+ permeability.

A high-Ca^{2+} medium inhibited toxin II contracture and abolished the neuromuscular blocking action of toxin II. When a high-Ca^{2+} medium was replaced with normal Krebs solution, the neuromuscular blocking action reappeared immediately. These results confirm the findings that the high-Ca^{2+} medium antagonized the venom of *L. quinquestriatus* in depolarizing the nerve fibers (Adam *et al.*, 1966) and also in inducing contracture in the isolated skeletal muscle (Adam and Weiss, 1959).

Spontaneous contractions induced by toxin II was Ca^{2+} dependent. This effect was abolished by a Ca^{2+}-free medium and re-appeared by the addition of 2.5 mM Ca^{2+}. Since *d*-TC and β-BuTX could only partially inhibit this effect, it is possible that spontaneous contractions are partly initiated from presynaptic nerve endings and partly from a direct action on the muscle. This effect could also be attributed to the increase of membrane Na^+ permeability, since it was inhibited by TTX completely.

Although a high-Ca^{2+} medium, procaine, and TTX exhibited the profound inhibition on toxin II contracture and spontaneous contractions, these inhibitory effects could be removed by washing. This finding indicates that these inhibitors do not prevent the binding of toxin II to the tissues.

CTX contracture was explicitly potentiated by Ca^{2+}-free and high-Mg^{2+} media, while completely inhibited by the high-Ca^{2+} medium. It is evident that extracellular Ca^{2+} is not required for CTX contracture. A high-Mg^{2+} medium potentiated CTX contracture, probably by displacing Ca^{2+} from the membrane binding site (Hagiwara and Takahashi, 1967), which decreased Ca^{2+} content of the muscle membrane as observed in the Ca^{2+}-free medium. Neither TTX nor lowering the extracellular Na^+ concentration inhibited CTX contracture. Thus, it is suggested that CTX induces contracture by releasing membrane-bound Ca^{2+} rather than increasing the Na^+ permeability of the cell membrane.

In summary, although CTX and toxin II possess some similarities in their chemical structure and pharmacological actions, they do not share a common mechanism of action. By contrast, toxin II appears to have a mechanism of action similar to that of batrachotoxin, which is a steroidal toxin isolated from the skin of a colombian frog, *Phyllobates aurotaenia* (Albuquerque *et al.*, 1971), in spite of their difference in the chemical structure.

SUMMARY

Both cobra cardiotoxin (CTX) and scorpion toxin II are potent in inducing contracture, membrane depolarization, and blockade of neuromuscular transmission of the chick biventer cervicis muscle. In addition, toxin II induces spontaneous contractions in this muscle preparation. CTX increases $^{45}Ca^{2+}$ uptake markedly but toxin II only slightly increases $^{45}Ca^{2+}$ uptake.

CTX contracture is potentiated by either Ca^{2+}-free or high-Mg^{2+} (10 mM) media, inhibited completely by a high-Ca^{2+} (12 mM) medium, but unaffected by a low-Na^+ medium, procaine, tetrodotoxin, *d*-tubocurarine, or β-bungarotoxin. By contrast, toxin II contracture is moderately potentiated by a Ca^{2+}-free medium, inhibited by high Ca^{2+}, high-Mg^{2+} and low-Na^+ media, procaine, tetrodotoxin, and *d*-tubocurarine, but unaffected by β-bungarotoxin. All of these findings suggest that CTX induces contracture by releasing membrane-bound Ca^{2+}, while toxin II does so by increasing the Na^+ permeability of the cell membrane.

Spontaneous contractions induced by toxin II are abolished by the Ca^{2+}-free medium. This effect is also inhibited partially by *d*-tubocurarine or β-bungarotoxin and completely by tetrodotoxin or procaine. These results suggest that toxin II induces spontaneous contractions by increasing the Na^+ permeability of the nerve endings as well as the muscle.

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Enhancement of the Cobra Venom Direct Lytic Factor by Prostaglandins and Related Synergistic Phenomena on Pulmonary Microvascular Events

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INTRODUCTION

The synergism of phospholipase A with the direct lytic factor (DLF), the most basic small molecular-weight protein isolated from Elapid venoms, has initially been studied in the hemolysis of washed erythrocytes (Condrea *et al.*, 1964a). It is now accepted that the presence of both DLF and phospholipase A is responsible for the hemolytic effect of cobra venoms. Furthermore it has been shown that DLF is the only component of *Naja naja* venom which has the ability to produce the type of microvascular lesion which can readily be observed after topical application of the full venom on the pulmonary pleural surface of dogs (Bonta *et al.*, 1969; 1972b). This type of microvascular damage was initially described as a local hemorrhage (Bonta *et al.*, 1970; 1973) though recent and as yet uncompleted microscopical studies point in the direction of vascular congestion accompanied by permeability changes (G. M. Böhm, personal communication). In view of the continuing uncertainty as to the exact ultrastructural character of the pulmonary microcirculatory lesion by cobra venom, it will be mentioned throughout the present paper as microvascular lesion or damage.

Phospholipase A does not share this property of DLF, to induce the vascular event mentioned above, but the enzyme can reinforce the vascular damaging effect of the lytic protein (Bonta *et al.*, 1972b; 1973).

The strongly cationic character of DLF is of recognized significance in causing hemolysis. Other basic proteins, including melittin, protamine, and histone are also known to mimick the hemolytic property of DLF (for references see: Condrea *et al.*, 1972a; Kaiser *et al.*, 1972). Bee venom and melittin were also reported as producers of microvascular lesions similar to those caused by DLF (Bhargava *et al.*, 1970; Bonta *et al.*, 1972a). In this paper we study certain histones for DLF-like activity on pulmonary vessels.

Phospholipase A can cleave phospholipids to yield fatty acids other than lysolecithin. These fatty acids can serve as precursors for prostaglandins, when the necessary synthetizing enzyme(s) are present in the tissue. Lung tissue is rich in prostaglandin-synthetase. Perfusion of lungs with phospholipase A promptly leads to the appearance of prostaglandins in the perfusate, and in fact phospholipase A has been suggested as the triggering enzyme in prostaglandin release (Kunze and Vogt, 1971). We, therefore, investigated if prostaglandins, besides phospholipase A proper, can also enhance the microvascular event induced by DLF in pulmonary tissue. Some results were briefly mentioned earlier (Bonta *et al.*, 1973), but other investigators have not as yet considered prostaglandins as potential or partial mediators of phospholipase A in its synergistic action with DLF.

MATERIALS AND METHODS

Mongrel dogs of either sex were used, after pentobarbitone anaesthesia, to make open-thorax preparations and thus expose the lungs. Filter paper disks of 5-mm diameter were soaked in solutions of the materials to be studied for vascular effects and applied for 3-10 min (specified in the tables) to the pulmonary pleural surface. The onset of action was measured and the intensity of the lesion was rated on a nonparametric scale ranging from 0.5-3. Earlier experience has shown that the two events are correlated and thus for reasons of brevity we present only results of the intensity of the lesions induced. When the interaction of two materials was studied the substance which preceded the second one in administration was applied by using filter paper disks of 15-mm diameter. The large surface of the canine lung permitted the simultaneous induction of several lesions and thus enabled us to make paired comparisons between different treatments. Details of the above method have been previously described (Bonta *et al.*, 1970).

N. naja venom, lot NSOTL, was commercially obtained (Miami Serpentarium Laboratories) and according to the seller was of Thailand cobra origin. We used

this venom to prepare phospholipase A and DLF, using a slight modification of the ion-exchange chromatography procedure of Brisbois *et al.* (1968). The purity of the materials obtained in this manner was checked by disk electrophoresis. Another DLF preparation, coded as siamensis 11 + 12 was a pool of two subfractions of the most basic fraction obtained from chromatography of Thailand cobra venom on the cation exchanger Bio-Rex 70 (Karlsson *et al.*, 1971; D. Eaker, personal communication). The DLF of *Hemachatus hemachatus* (African Ringhals cobra) venom was in fact the hemolytic basic protein denoted earlier as fraction 12B (Fryklund and Eaker, 1973). Prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2) were obtained from Upjohn Co. (Kalamazoo). Histones of calf thymus origin were obtained from Sigma. One of the histones was lysine rich and the other arginine rich. The third histone was lyophilized, but apart from this no details were known about it. Crystalline lysolecithin *ex ovo* was of a commercial source (Koch Light). The above materials were dissolved in Sörensen phosphate buffer (pH 7.38). Arachidonic acid (Sigma) was dissolved in sodium bicarbonate. None of the solvents produced any observable effect and they did not interfere with the effect of any of the above substances. The solutions were prepared freshly before each use.

RESULTS AND DISCUSSION

The microvascular damaging effect of DLF from *N. naja* venom and the enhancement of this effect by phospholipase A from the same source has been shown (Bonta *et al.*, 1972b), as the results shown in Table 1 confirm. For comparison, the effects of the lytic proteins siamensis 11 + 12 and fraction 12B are also demonstrated. In macroscopic appearance no qualitative difference was found between the lesion produced by any one of the lytic factors. As the preparation siamensis 11 + 12 was in fact a pool of two materials, it is not inconceivable that only one of them is the vascular damaging substance. This may be a reason for the slightly smaller effect as compared to that of highly purified DLF. Experiments with the two separate components of siamensis 11 + 12 will be needed to clarify the situation. Also, fraction 12B, when administered alone, displayed a relatively small effect. Furthermore, Table 1 shows that following the administration of phospholipase A a marked potentiation is evident, irrespective of the source of the lytic factors.

A physicochemical property of DLF is its basic character as measured by isoelectric point. Owing to its positive charge DLF binds to negatively charged biomembranes (e.g., the surface of erythrocytes or platelets and subcellular structures as lysosomes) and alters the arrangement of surface structures, thus rendering substrates susceptible to phospholipase A (Condrea *et al.*, 1964b; Kaiser *et al.*, 1972). However, protamine (also a basic protein), though imitating

Table 1. Comparison of Different DLF Preparations for Microvascular Lesion Inducing Effect in Dog Pulmonary Tissue[a]

DLF preparation	Intensity of vascular lesion		
	DLF alone	Phospholipase A pretreatment	Potentiation average
Purified DLF, *Naja naja*	10.5	24	
	5.5	10.5	
	3	7.5	120 %
Siamensis 11 + 12 pooled fraction	1	9	
	6	16.5	
	6	22.5	380 %
Fraction 12B, *Hemachatus haemachatus*	6	10.5	
	1.5	10	280 %

[a]DLF, 4 mg/ml, was applied for 3 min. Phospholipase A, 4 mg/ml, was applied for 10 min immediately followed by DLF. On two dogs all three DLF components were tested, on a third dog only DLF of *N. naja* and siamensis 11 + 12 were compared. Owing to the small number of observations individual results rather than mean values and SEM are given. For rating scale of intensity and other technical details see the Materials and Methods section.

the hemolytic effect of DLF, failed to duplicate its synergistic action with phospholipase A (Condrea *et al.*, 1964a). Bee venom, melittin, protamine, and histone also mimicked the enzyme activating effect of DLF on the erythrocyte membrane (Condrea *et al.*, 1972b). Some synthetic basic copolymers were proven to be more potent hemolytic agents than DLF, and promoted phospholipid splitting by phospholipase A (Klibansky *et al.*, 1968). Pulmonary vessel lesions, qualitatively similar to those produced by DLF, were found with bee venom and melittin (Bhargava *et al.*, 1970; Bonta *et al.*, 1972a). We thus conducted pilot studies designed to discover if other basic proteins might display this effect and if phospholipase A could enhance it. The results of a few experiments with histones are shown in Table 2. The large dose (two and half times that usually required with DLF) and the long contact with the tissue (three times that of DLF) was necessary, as otherwise no effect whatsoever was observed. At though even under these excessive conditions the effects are far from impressive. In the case of the histone of unknown amino acid composition and the arginine-rich histone, the potentiation by phospholipase A also lacked statistical significance. As all DLF proteins, so far isolated, are known for their high content of lysine (Lee, 1972), we might have expected that the lysine-rich histone would display a marked effect. We may speculate on the reasons for the absence of it: (1) the specific orientation of the lipophilic groups, the abundance of which is characteristic of DLF proteins (Fryklund and Eaker, 1973) and is necessary for penetration into the lipid part of the biomembrane, might not have been directed to the outside

of the molecule; (2) the structural requirements for attack of the vessel wall membrane(s) may differ from those necessary to alter membranes of other cells (e.g., erythrocytes). In turn this implies differences in physicochemical arrangement of membrane surface structures. The present results, together with those obtained earlier with bee venom and melittin, leave little doubt that pulmonary microvessels have negatively charged membranes thus being liable for attack by such strongly cationic molecules as DLF or melittin. The latter two apparently possess additional structural requirements, which the histones, or at least those of calf thymus origin, do not share. Since other types of histones exist, it is still possible that others do have, in addition to the positive charge, the necessary amino acid arrangement to imitate the effect of DLF on vessels.

The microvascular lesion induced by DLF on *N. naja* venom is qualitatively

Table 2. Effect of Histones Alone and in Combination with Phospholipase A on Dog Pulmonary Microvessels[a]

Substance	Intensity of vascular lesion	
	Without phospholipase A	Phospholipase A pretreatment
Histone of unknown amino acid composition	0	2
	10	14
	0	10
	0	0
	0	13
	0	0
Histone lysine rich	0	10.5
	0	0
	0	3
	0	6
Histone arginine rich	8	5
	3	5
	4	5
DLF, *Naja naja*	6.3 ± 1.7	15 ± 2.2

[a]Histones, 10 mg/ml, were applied for 10 min. Phospholipase A (*N. naja*), 4 mg/ml, was applied for 3 min. immediately followed by histone. DLF, 4 mg/ml, was applied for 3 min. Conditions of phospholipase A pretreatment were similar to those with the histones. The DLF values (6 observations, mean and SEM) are presented for the sake of comparison. They were not all necessarily obtained on the same dogs as with the histone values, but on each dog where histone was tested one DLF experiment was concomitantly run. For the histone experiments four dogs in total were used. The histone values are individual results.

indistinguishable from that produced by the full venom. Quantitatively however, there is a marked difference in that the isolated DLF can only produce approximately half the effect of the full venom (Table 3). Considering that the DLF content of the full venom is certainly not more than 55% on a dry weight basis (Lee, 1972) and that in our experiments the two materials were applied in equal doses, the difference in activity is even more striking. Further, we observed that when the tissue was exposed to phospholipase A (itself not producing the vascular damage) prior to the administration of DLF, the total effect of the full venom could readily be retrieved. Occasionally the combined application of phospholipase A and DLF induced a lesion which was more pronounced than if the full venom had been administered. Whether or not this was due to the absence of a phospholipase A inhibitor, known to be isolated from cobra venom (Braganca *et al.*, 1970), or simply resulted from the fact that the total amount of material was higher when the tissue was exposed to the two separated venom components rather than the full venom, cannot be determined from the present data.

For a better understanding of the above potentiation of DLF by phospholipase A, we considered that the lipid-splitting enzyme may act in two different, though simultaneously occurring ways. Aside from lysolecithin formation, phospholipase A liberates fatty acids from the lecithin molecule, part of which, being unsaturated, may serve as precursors for prostaglandin formation. Lysolecithin may alter membrane permeability characteristics as shown from its potent hemolytic effect. The prostaglandins, in particular PGE_1, are known to enhance vascular permeability (Kaley and Weiner, 1971). We tested the possibility

Table 3. Potentiation of *Naja naja* DLF on Dog Pulmonary Microvessels[a]

Potentiator	*Naja naja* full venom, 2–4 mg/ml	DLF, 2–4 mg/ml	Potentiation average
None	16 ± 0.5(27)	8.5 ± 0.75(25)	
Phospholipase A, 2–4 mg/ml		17.5 ± 1.45(12)	110%
None	16.8 ± 1.8(6)	6.8 ± 0.85(5)	
Lysolecithin 2–4 mg/ml		11.0 ± 0.85(5)	65%
None	18 ± 1.5(13)	8 ± 0.75(12)	
Prostaglandin E_1 0.01 mg/ml		13.5 ± 1.0(12)	70%

[a]The results (intensity of lesion) are mean values ± SEM. The numbers in brackets are the number observations. The potentiators were applied for 10 min, immediately followed by DLF for 3 min. In each case the potentiator without the DLF was concomitantly run, but since none of the potentiators, when given alone, produced any effect in any one of the experiments, these zero values are not taken up in the table. The dose of the full venom was either 2 or 4 mg/ml, depending on the sensitivity of the dog, but was kept constant within each dog. The dose of DLF, phospholipase A, and lysolecithin was always identical with the dose of the full venom.

of either of the two above mechanisms occurring by trying to mimic the event by replacing the lipid-splitting enzyme with lysolecithin on the one hand and prostaglandins on the other.

As shown in Table 3 both of the agents, though each was devoid of a visually observable effect by itself, did enhance the activity of DLF. In each case however, the effect was clearly lower than if the tissue had been exposed to phospholipase A. As for lysolecithin, we knew from earlier experience (Bonta *et al.*, 1969) that it does not produce the type of vascular lesion caused by venom by itself. In this context the synergism between phospholipase A and DLF, and the lack of the effect of lysolecithin when given alone is all the more remarkable, as lysolecithin is a powerful hemolytic agent even in the absence of DLF. The fact that lysolecithin under the present experimental conditions was only enhancing but not by itself displaying the effect, suggests that the mechanisms underlying the lysolecithin activity on cell lysis on the one hand and pulmonary microvascular lesion on the other are different.

The effect of DLF under conditions of pretreatment of the tissue with PGE_1 was intermediate between that of observed after DLF alone and of that occurring after the joint action with phospholipase A. The latter, when cleaving phospholipids, may also release precursors of prostaglandins other than PGE_1. It was of interest then to study the potentiation of DLF with another prostaglandin. A comparison between the effect of PGE_1 and PGE_2, structurally differing from each other in one double bond of the side chain, is shown in Table 4. With each of the two prostaglandins the potentiation was of similar magnitude, but on a weight basis PGE_2 was definitely less active. The dose needed to obtain the enhancement of the DLF effect was 50 times higher with PGE_2 than with PGE_1.

Table 4. Comparison of Potentiation by Two Prostaglandins on DLF Microvascular Lesioning Effect in Dog Pulmonary Tissue[a]

Prostaglandin	*Naja naja* full venom, 2 mg/ml	DLF, 2 mg/ml	Potentiation average
None	18 ± 1.5(13)	8 ± 0.75(12)	
PGE_1, 0.01 mg/ml		13.5 ± 1.0(12)	70%
None	19 ± 1.0(23)	8.5 ± 0.5(23)	
PGE_2, 0.1 mg/ml		9.5 ± 1.2(5)	10%
PGE_2, 0.5 mg/ml		14.0 ± 2.0(7)	65%

[a]The results (intensity of lesion) are mean values ± SEM. Numbers in brackets are the number of observations. Prostaglandins were applied for 10 min, immediately followed by DLF for 3 min. In each case, prostaglandin doses were followed by DLF concomitant doses, but since in no case did the prostaglandins alone produce any effect, these zero values are not taken up in the table. In some occasions PGE_1 and PGE_2 experiments were concomitantly run.

Even with a dose of 0.5 mg/ml of PGE_2 only reinforcement of the DLF activity, but no effect of its own was observed. Not shown in Table 4 are the results of pilot experiments with $PGF_{2\alpha}$, which in doses of up to 0.1 mg/ml also had no effect when given alone, and was unable to potentiate DLF. A higher dose of $PGF_{2\alpha}$ was not tested, as unfortunately we had no access to a more concentrated solution of this compound. Regarding induction of increased vascular permeability, $PGF_{2\alpha}$ has been reported as ineffective (Kaley and Weiner, 1971).

To further test the possible involvement of prostaglandin formation in the potentiating effect of phospholipase A, we studied the effect of an intermediate product in the chain of prostaglandin synthesis. One suitable candidate would have been bishomo-γ-linolenic acid, a precursor of PGE_1. As we had no access to this substance, we substituted arachidonic acid, which having one more double bond yields PGE_2. The results as presented in Table 5 demonstrate that, in contrast with the observations with either phospholipase A or the prostaglandins, there was a slight but clearly dose-dependent effect present when arachidonic acid was administered alone. In a dose being devoid of effect on its own, arachidonic acid failed to potentiate DLF, while in a concentration to yield effect by its own virtue the joint action with DLF was additive rather than synergistic. Thus it appears that arachidonic acid is not a suitable tool to investigate the role of prostaglandin release in the interaction of phospholipase A with DLF. While there is little doubt that arachidonic acid may serve as a prostaglandin precursor, there are observations to indicate that it also yields metabolites other than PGE_2 (Kunze, 1970).

In a recent experiment we observed that indomethacin, a recognized inhibitor of PG synthesis, did partially inhibit the enhancement of DLF by phospholipase A. As it was only a single observation, it will need confirmation before it can be used as an argument.

Table 5. Effect of Arachidonic Acid Alone and in Combination with DLF on Dog Pulmonary Microvessels[a]

Arachidonic acid	Arachidonic acid alone	DLF alone, 4 mg/ml	Combined administration
None	–	9 ± 1.0(21)	–
0.1 mg/ml	0(6)	–	10.5 ± 1.0(6)
1.0 mg/ml	0.75(8)	–	11.0 ± 0.8(8)
5.0 mg/ml	5.5 ± 1.0(8)	–	17.5 ± 1.5(8)

[a] Results (intensity of lesion) are mean values ± SEM. Numbers in brackets are the number of observations. With combined administration arachidonic acid was applied for 10 min, immediately followed by DLF for 3 min.

While the synergistic action of phospholipase A and DLF has, by others, been mainly studied in isolated cell systems or the membranes thereof, our experiments show that it applies to more complexly organized systems such as pulmonary vessels as well. Further, the above experiments indicate that phospholipase A is not singular in reinforcing the effect of DLF, at least not when vessels in pulmonary tissue are concerned. Lysolecithin and prostaglandins of the E type each share this property of phospholipase A. Lysolecithin, in our experiments, behaves differently when cell lysis is involved, since in the latter condition it is highly effective when given alone, while on pulmonary vessels it is not. In fact cell lysis is unlikely to be involved in the vascular lesions studied. The prostaglandins, E_1 and E_2, also qualitatively imitated the enhancing effect of phospholipase A. Since, however, neither lysolecithin nor the prostaglandins quantitatively yielded the extent of potentiation displayed by phospholipase A, the latter appears to exert a more complex effect than any of the other substances on their own. In turn this might mean that simultaneous release of lysolecithin and prostaglandins is involved when phospholipase A acts as potentiator of DLF on lung vessels. Still another possibility is that lysolecithin and the prostaglandins, though imitating the potentiating effect of phospholipase A, are in fact not involved when the latter is administered. The presented results are not decisive for the exclusion of either possibility and work is in progress to clarify the situation. Finally, it should be pointed out that the interaction of prostaglandins with DLF has not yet been studied in experiments other than those presently described.

SUMMARY

The direct lytic factor (DLF) is the only component of *N. naja* venom which reproduced the microvascular lesion caused by the full venom after topical application on pulmonary tissue. Quantitatively, DLF is less potent than the full venom in inducing pulmonary microvessel lesions. The phospholipase A component of the venom does not produce microvascular lesion, but does enhance the effect of DLF so that the latter matches or eventually surpasses the effect of the full venom.

The DLF component of *H. haemachatus* venom is qualitatively similar to that of *N. naja* venom in inducing vascular lesions. Phospholipase A of *N. naja* not only enhanced this effect of DLF of its own species but also of the other species studied.

Histones of calf thymus origin were hardly able to imitate the vascular effect of DLF. Phospholipase A slightly enhanced the low activity of a histone rich in lysine, but not that of the histone rich in arginine. The results suggest that

besides cationic charge and high content of lysine, some other characteristic is required to imitate the DLF effect on pulmonary microvessels.

Lysolecithin does not produce pulmonary vascular lesion, but enhances the effect of DLF on this event. The enhancement, however, is smaller than that induced by phospholipase A.

Neither prostaglandin E_1 nor prostaglandin E_2 induces lesion of pulmonary vessels, but both potentiate the effect of DLF. PGE_1 is more potent in enhancing DLF than is PGE_2. The potentiation by prostaglandins is partial as compared to that by phospholipase A. Arachidonic acid itself produces microvascular lesions and its joint action with DLF is additive rather than synergistic.

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Bacterial and Snake Venom Phospholipases: Enzymatic Probes in the Study of Structure and Function in Bioelectrically Excitable Tissues

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INTRODUCTION

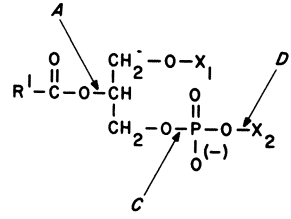
Many of a cell's most vital processes are associated with its membrane systems: plasma, mitochondrial, nuclear, and so on. The characteristic function of bioelectrically excitable tissue such as nerve and muscle, i.e., the conduction of an electrical signal, is accomplished by the rapid and reversible alteration of the electrical potential across their plasma membranes. In addition to this specialized property, electrically excitable membranes are also the site of maintenance of selective cellular permeability, active transport processes, and numerous other functions which are characteristic of all cells. If we are to understand how a nerve, muscle, or synapse can carry out these multiple activities, we must first attain a better understanding of the organization and functioning of the major constituents of their plasma membranes. Those of us working with membranal phospholipids like to believe the quote of Thudichum (1884), that pioneer in phospholipid chemistry who said that "Phosphatides are the centre, life and chemical soul of all bioplasm . . .". It has, however, been difficult to prove specific functions for phospholipids in the nervous system, even though phospholipids do have an active metabolism and rapid turn over both in brain and peripheral nerves (Ansell, 1973; Hawthorne, 1973). If phospholipids were just

the inert cement of the membranes, one would wonder what this metabolism accomplishes. In quantitative terms phospholipids are the major lipid constituent of bioelectrically excitable tissues.

This chapter reviews some of my attempts to analyze phospholipid function and organization in the membranes of bioelectrically excitable tissues. The rationale of my experimentation has been to produce quantitatively known modifications in phospholipids and to relate these changes to any structural modifications or functional alterations which are simultaneously observed. Snake venoms and bacterial toxins have been extremely useful tools in these studies since they are the richest known sources of phospholipases A and C, respectively; the two enzymes which I have used to hydrolyze membranal phospholipids. Prior to discussing how these studies have helped clarify our understanding of organization and function in peripheral axons and synapses and the central nervous system, we must briefly review phospholipid chemistry and the suggested functions, which have been proposed by others, for phospholipids. Much of the experimental data on which this review is based have been published and appropriate references are given in the text.

PHOSPHOLIPID CHEMISTRY

The formulas of a few representative phospholipids and the points of hydrolytic action by phospholipases are shown in Fig. 1. Note that they are phosphoglycerides with a fatty acid ester at the β or 2 position of the glycerol backbone. In addition the "phosphatidyl-" compounds also have a fatty acid ester at the α or 1 position. Phosphatidylcholine (PC), also referred to as lecithin, has choline substituted on the phosphorus and is essentially neutral, existing at physiological pH in the zwitterion form. Phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA) have a net negative charge and are acidic phospholipids; the phosphate grouping can be considered completely ionized at physiological pH since it has a pK_a of between 1 and 3. There are also di- and triphosphoinositides which have a phosphate grouping at the 4 or the 4 and 5 positions, respectively, of the inositol molecule. In addition to these phosphatidyl compounds, all of which contain a fatty acid ester at the 1 or α position there are also phosphatidal compounds or plasmalogens which have an α,β unsaturated ether at the α position. Therefore, we find choline, ethanolamine, serine, and inositol plasmalogens. Two other major phospholipids which are not shown in Fig. 1 are cardiolipin or diphosphatidyl glycerol (which has two phosphatidic acid molecules joined by the three carbons of glycerol) and sphingomyelin (which resembles choline plasmalogen, except that at the 2 position of the glycerol backbone there is an NHCOR instead of OCOR, and that at the 1 position there is a particular C_{15} chain). This description is, how-



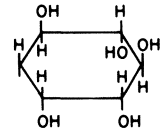
	X_1	X_2
Phosphatidylcholine		$CH_2CH_2N^+(CH_3)_3$
Phosphatidylethanolamine		$CH_2CH_2N^+H_3$
Phosphatidylserine	$\begin{matrix}O\\\|\\C-R\end{matrix}$	$CH_2CH(N^+H_3)COO^{(-)}$
Phosphatidic acid		—
Phosphatidylinositol		
Phosphatidalcholine	CH=CH-R	$CH_2CH_2N^+(CH_3)_3$

Fig. 1. Structure of several glycerophosphatides. A, C, and D indicate the points of hydrolysis of phospholipase A_2, C, and D, respectively. R is the hydrocarbon chain.

ever, very incomplete since what gives them their variety and partial liquid character in the membrane are their fatty acids. It has, for example, been suggested that the instability of myelin in certain clinical disorders is caused by a decrease in long-chain fatty acids (O'Brien, 1965). It is known that the fatty acids are organ specific, so that if you compare the fatty acid pattern in the lungs of 10 different species you will find that they are very similar, but markedly different from that of brain in these 10 species, although the brain species pattern are also similar to each other (Van Deenen, 1965). It thus appears that fatty acids have specific functions to fulfill.

Since I will be referring to information derived from the degradation of phospholipids, I will briefly review what is known of the catabolism of phospholipids, that is, the action of phospholipases.

Phospholipase A splits off the fatty acid ester at the β or 2 position leaving behind a lysophosphatide; a compound with only one fatty acid. These lysophosphatides have detergent properties and affect many tissues including nerve tissue (Zeller, 1951; Tobias, 1955; Morrison and Zamecnik, 1950; McArdle *et al.*, 1960; Rosenberg and Condrea, 1968). Therefore, the effects of phospholipase A may be either directly due to disruption of phospholipids or indirectly due to production of lysophosphatides. Phospholipase A activity is present in most mammalian tissues but its richest sources are snake venoms. Phospholipase C splits off the phosphorylated base, also called the polar head group, leaving a diglyceride. Both the phosphorylated base and diglyceride are inert on biological systems. While small activities of phospholipase C are found in mammalian tissues, the richest sources are bacterial toxins, especially *Clostridium perfringens* and *Bacillus cereus*.

Their structure, being a combination of hydrophobic fatty acids and hydrophilic phosphate groups, makes them admirably suited to form part of a membrane separating one aqueous phase from another. As we will see later all theories of membrane structure do attach a major significance to the phospholipid component. The limited scope of this chapter does not allow a detailed description of the phospholipid composition of various membranes, but you will notice in Table 1 that there are quite marked differences in composition from tissue to tissue, the reasons for which we do not as yet know. Some of the observations we can make concerning the distribution of phospholipids are as follows: most of the ethanolamine phospholipid is in the plasmalogen form (in contrast to all

Table 1. Content and Distribution of Major Phospholipids in Various Tissues[a]

	Lobster leg nerve	Monkey sciatic	Skeletal muscle	Pig kidney
Lipids, % dry wt.	8	40	11	17
Phospholipids, % dry wt.	6	19	5	14
% Distribution of major phospholipids				
PC	40	15	44	36
PS	9	16	4	7
PE	8	4	15	17
PE Plasmalogen	23	30	7	14
PC Plasmalogen	0	0	12	3
PS Plasmalogen	0	2	–	3
PI	2	4	4	3
Cardiolipin and PA	3	1	6	4
SM	15	28	6	13

[a] Modified from various tables and references in Ansell *et al.*, 1973.

other phospholipids); di- and triphosphoinositides are mainly localized in the brain; sphingomyelin is in higher concentration in myelin, whereas lecithin is primarily found in gray matter; the phospholipid composition of nonmyelinated peripheral nerves resemble that of gray matter, whereas myelinated peripheral nerve tissue has a composition similar to that of white matter.

SUGGESTED FUNCTIONS OF NEURONAL PHOSPHOLIPIDS

The Hokins have for many years studied the possible involvement of phospholipids in active transport processes, and one of their early suggestions was that phosphorylation involved diglyceride which was converted to phosphatidic acid (Hokin and Hokin, 1959). In contrast it is now established that the phosphate group is attached to the carboxyl of a glutamic acid residue in the protein. Nevertheless, many studies indicate that phospholipid is necessary for the activity of the sodium transport ATPase (for a review of this data, see Hawthorne, 1973). In addition, the fundamental observations of the Hokins would have to be explained by any theory of phospholipid function. For example, in Fig. 2 you will note an example of their finding that ACh increases the turnover of phosphatidic acid and phosphatidylinositol in such tissues as salt glands, submaxillary glands, pancreas, and brain.

Suggestions have also been made that phospholipids have a role in the genesis of bioelectricity, that is in allowing Na and K ions to move down their concentration gradients during initiation of an action potential in contrast to active transport, where Na is pumped out of the cell against its concentration gradient. The interesting observations of Larrabee and his colleagues (Larrabee *et al.*, 1963; Larrabee and Leicht, 1965; Larrabee, 1968) remain the best evidence in support of the proposal that synaptic functioning is associated with changes in phospholipids. He found that electrical stimulation of preganglionic nerves to the sympathetic superior cervical ganglia increased incorporation of ^{32}P orthophosphate and ^{14}C into phosphatidylinositol, and to a lesser extent lecithin, but not PA. ACh had a similar effect on slices of ganglia. The effects appear to be postsynaptic since incorporation into axonal phospholipids was not affected by electrical stimulation, nor did degeneration of presynaptic nerve terminals alter labeling in ganglia, while curare abolished the PI effect. The exact significance of this effect, which is not likely to be related to Na transport, remains unknown. Hokin suggests that it is involved with protein transport within the neuron or with an effect of depolarization on intracellular membranes. On the basis of autoradiographic studies he claims that the effects Larrabee observed occur primarily in the cytoplasm of the neuron rather than in the synaptic membrane (Hokin, 1965).

Goldman (1964) has also proposed that phosphate groups are essential for

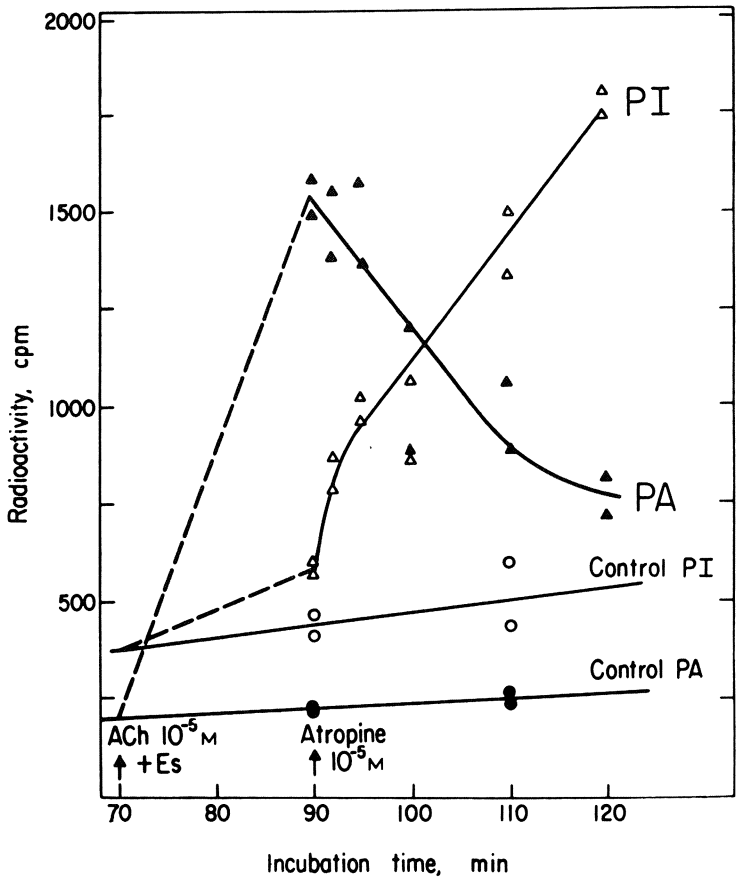


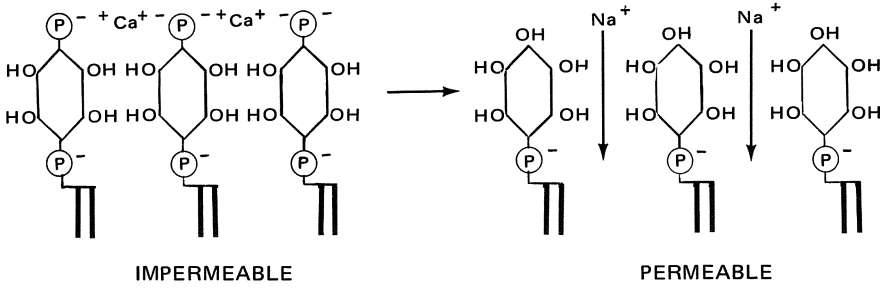
Fig. 2. Radioactivity in phosphatidic acid (PA) and phosphatidylinositol (PI) after addition of acetylcholine and atropine to salt gland slices. Circles show radioactivity of PA in the absence of acetylcholine and atropine, with triangles showing results in their presence (Hokin and Hokin, 1964).

the generation of bioelectricity. He proposed that they act as exchange sites through which ions pass. Their affinity for different cations would depend on their particular molecular configuration, which in turn would depend on the electrical field strength across the membrane. He suggests three major configurations: one binding Ca, one Na, and one K. In some ways this is similar to the ideas of Tasaki (1968), who specifies a fixed negative charge in the membrane, the membrane itself behaving as a cation exchanger. In the resting state Ca^{2+} would be bound and then displaced during activity. Since the phosphorus grouping of phospholipids constitutes one of the major sources of negative charges in the membrane, one might suppose phospholipids to be one of the major ex-

change sites. When most people speak about the function of phospholipids they do not consider the glycerol portion, they are really talking about the polar head groups, that is the phosphorylated bases of the phospholipids.

There is also the proposal that acidic phospholipids are drug receptor sites, for example, for local anesthetics. Feinstein (1964) has done much to develop this idea, which is in some ways similar to the proposals of Goldman. Evidence was brought forth to show that local anesthetics and calcium can compete for binding to the charged polar head groups of phospholipids. This may be of special importance since calcium ions play a role in maintaining the excitability of nervous tissue. Of course in all types of binding studies one has the problem of specificity.

Hawthorne and Kai (1970) specifically propose that it is the dephosphorylation of polyphosphoinositides which is responsible for the release of calcium and the depolarization of neuronal membranes, while rephosphorylation allows reuptake of calcium and repolarization of membrane (Fig. 3). At rest the axolemma would contain calcium bound to polyphosphoinositides and the axolemma would be impermeable to sodium ions. Depolarization, perhaps just the change



1. REST	CALCIUM TPI: MEMBRANE IMPERMEABLE	–
2. DEPOLARIZATION	TPI → DPI → PI	Monoesterase active; kinase inactive (Ca^{++})
3. CALCIUM LOST SODIUM ENTERS	MEMBRANE PERMEABLE	–
4. RECOVERY: CALCIUM REGAINED	PI → DPI → TPI	Kinases active (low Ca^{++}); monoesterase inactive (Na^{+}); sodium pump active

Fig. 3. Suggested steps in neuronal excitation and recovery. TPI, triphosphoinositide; DPI, diphosphoinositide; PI, phosphatidylinositol (Hawthorne and Kai, 1970).

in charge of the membrane, would be sufficient to allow the phosphomonoesterase of the axoplasm to hydrolyze the membrane polyphosphoinositides. The phosphomonoesterases would be active under the conditions of low Na, whereas the kinases would be inactive because of the high calcium concentration. As a result of the hydrolysis of polyphosphoinositides, calcium is lost, sodium enters, and the membrane becomes permeable. During the recovery process TPI would be resynthesized in the membrane because the kinases become active in low calcium, whereas the monoesterases are inactive in high sodium. While we have no direct evidence for this theory, we do know that the ions have the effects described.

Durrell *et al.* (1969) have proposed a theory to explain the relationships between the effects of ACh and phospholipid metabolism. It is similar to the theory of Hawthorne and Kai although it does not specify polyphosphoinositides as the key phospholipids. It is, however, another attempt to explain the interesting observations of the Hokins and Larrabees. The proposed scheme is as follows:

1. Diglyceride-P-X $\xrightarrow{\text{ACh}}$ Diglyceride + P-X
2. Diglyceride + AT^{32}P $\longrightarrow$ Diglyceride-^{32}P + ADP
3. Diglyceride-^{32}P + CTP $\longrightarrow$ Diglyceride-^{32}P-P-C + PP
4. Diglyceride-^{32}P-P-C + In $\longrightarrow$ Diglyceride-^{32}P-In + CMP

Diglyceride-P-X is any phospholipid except phosphatidic acid, while diglyceride-^{32}P-P-C represents beta ^{32}P-CDP-diglyceride. In represents 1-myoinositol and diglyceride-^{32}P-In represents ^{32}P-phosphatidylinositol. ATP and ADP are adenosine tri- and diphosphate, respectively; CMP, CDP, and CTP represent cytidine mono-, di-, and triphosphates, respectively; and ACh is acetylcholine. It is assumed that the diglyceride moiety is stable and remains attached to the membrane throughout the sequence of reactions. Reactions 3 and 4 are the generally recognized pathways for the synthesis of PI. Reaction 2 is one of the three pathways for the synthesis of PA in brain. Reaction 1 is a phospholipase-C type reaction. Durrell proposes that reaction 1 is the rate-limiting step which is stimulated by ACh. Reaction 2 is rapid compared to reactions 3 and 4. Low concentrations of ACh would primarily increase PI incorporation of ^{32}P without much change in ^{32}P-labeled PA, because reaction 1 is still rate limiting. At higher concentrations of ACh reaction 1 may be stimulated so much that reactions 3 and 4 become rate limiting and ^{32}P-labeled PA would accumulate. This agrees with results on salt glands and brain homogenates. Any PL, except PA, could be the substrate for reaction with ACh (1), although in line with the ideas of Hawthorne and Kai, he suggests that polyphosphoinositides may be the preferred substrate. Durrell obtained some evidence that ACh stimulates the breakdown of inositol phosphatides about 50%, an effect which was blocked by atropine.

The theory of Durrell does not specify a mechanism by which ACh increases hydrolysis of phospholipids, although in his review article (Durrell *et al.*, 1969) he does discuss the theory of Watkins (1965) which could be integrated into the proposal. Watkins was also intrigued with the possible functions of phospholipids and noted the similarity of structure and charge distribution in acetylcholine, GABA, and glutamic acid to that of the polar head groups of lecithin, PE, and PS, respectively (Fig. 4). He proposed that at restricted portions of the membrane these transmitters replace the polar head portion of the corresponding lipid by competing with the phospholipid for binding to the protein. The displacement of the lecithin and release of a divalent metal ion such as Ca^{2+} could cause a change in protein configuration. As you see there is (1) electrostatic binding of the quaternary NH_4 group of lecithin to an anionic protein side chain; as well as (2) a divalent metal ion bridge between the lipid PO_4 group and a second anionic group of the protein; and finally (3) coordinate bonding between the double-bonded O_2 atom of the PO_4 group and a peptide group of the protein chain. Acetylcholine competes with phosphatidylcholine for sites 1 and 3. It is also possible that ACh plus lecithin form a complex liberating free protein. Similar ideas were proposed for GABA and glutamate with their respective phospholipids.

Rather than hypothesizing about what the ACh receptors are, there have been many recent attempts, and progress made, towards the isolation of the pure ACh receptor. In regard to our discussion, the work of DeRobertis (1971) is of special interest since he claims to have isolated from several different sources (brain, electric tissue, etc.) a phospholipid-protein complex which has receptor properties. His material, however, appears fundamentally different from that isolated by other workers in the field (Changeux, Miledi, O'Brien, Raftery, and others). He used $CHCl_3$-CH_3OH to extract a ^{14}C ACh or curare binding component which was separated using a discontinuous gradient (Sephadex LH-20) column. However, Levinson and Keynes (1972) have claimed that the results on the Sephadex column were artifactual and due to solvent fronts. They show a similar pattern with pure ^{14}C ACh and pure phospholipids (no protein), and with tissues not thought to have any ACh receptor.

It appears then that specific roles for phospholipids of the nature discussed till now have not been proven. A stronger case can probably be made for a general role of phospholipids in the maintenance of structural and permeability properties of membranes. The studies of Green and Fleischer with mitochondria indicate that there often is no specific role for phospholipid, but that it provides a proper medium and therefore has multiple functions (Green and Fleischer, 1963; Fleischer and Fleischer, 1967; Lester and Fleischer, 1961). They found that acetone-water solutions, which extracted most of the mitochondrial phospholipid, and phospholipases caused a loss in the ability to transfer electrons. This activity could be restored by adding back to the membrane relatively large

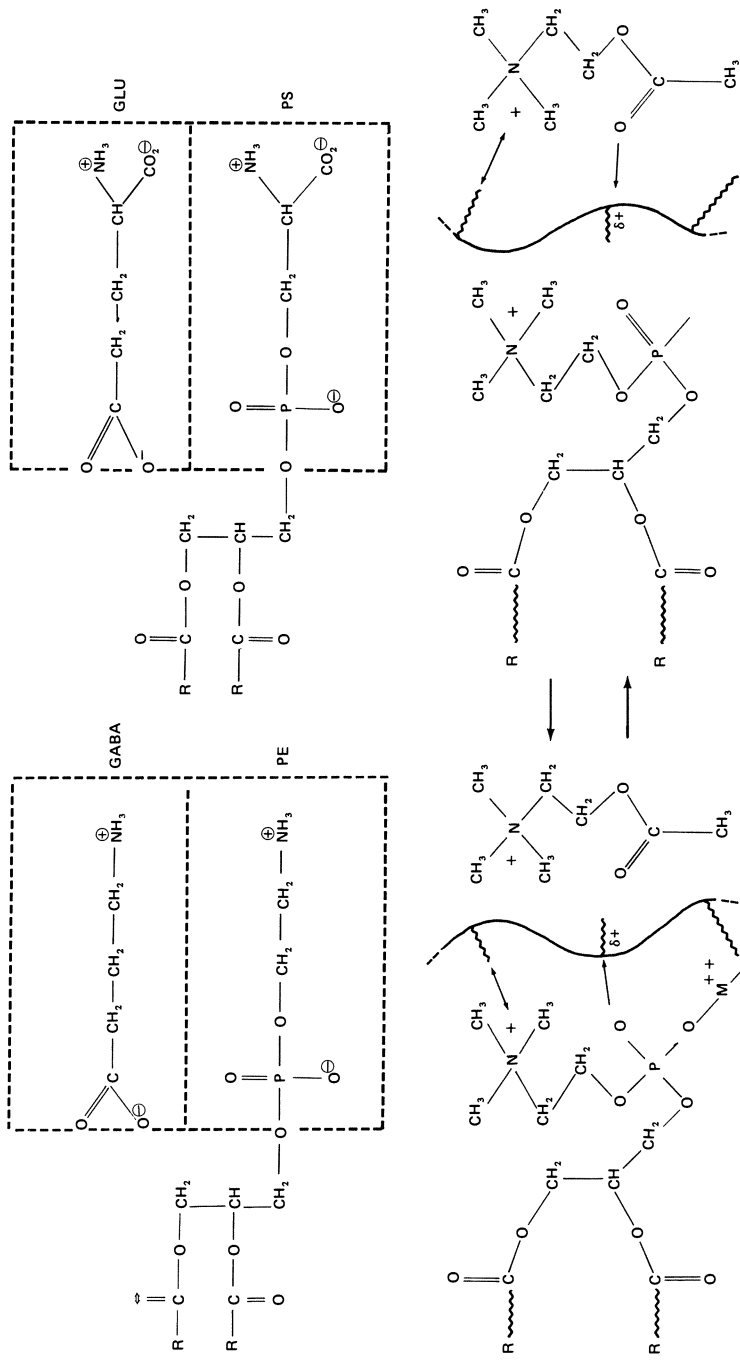


Fig. 4. Similarity in structure and charge distribution of gamma-aminobutyric acid (GABA) and the terminal residue of phosphatidylethanolamine (PE) and of glutamic acid (GA) and the terminal residue of phosphatidylserine (PS) (upper part of figure). Dissociation of a hypothetical lecithin–protein complex by acetylcholine (lower part of figure) (Watkins, 1965).

amounts of any phospholipid. The phospholipid appeared to protect the reactants leading to the synthesis of ATP from the hydrolytic effects of water.

It has often been suggested that the essential contribution of lipid in membranes is to allow passage of fat soluble materials and prevent water soluble materials from penetrating. In several studies it has been found that disruption of membranal phospholipids will increase permeability. Tobias (1960) had studied the effects on lobster nerves of trypsin, phospholipase C, and venom solutions used as a source of phospholipase A. He concluded that the chemical name of membrane resistance and capacitance is more likely to be phospholipid than protein. Brown (1940) found that a snake venom increased the permeability of frog atria to water and Hadidian (1956) found that using venom there was an increased penetration of procaine into nerve cells. Various studies (Whittaker, 1961; McArdle *et al.*, 1960; Braganca and Quastel, 1953; Petrushka *et al.*, 1959) have indicated that phospholipase A can release various enzymes from brain slices in their active forms. These studies indicated that phospholipids may be essential in the maintenance of membrane permeability.

EFFECTS OF PHOSPHOLIPASES ON THE SQUID GIANT AXON

I became especially interested in membrane permeability about 13 years ago when I wanted to alter the permeability of axonal membranes. Nachmansohn (1959, 1969) had attributed the changes in membrane conductance to Na and K which occur during electrical activity as being due to the action of acetylcholine on a receptor protein in the membrane. One of the objections to this theory was the failure of acetylcholine, curare, and other quaternary nitrogen derivatives to affect conduction in nerve axons in contrast to their powerful action on junctions. It had been shown, for example, in the squid giant axon, that the lipid insoluble ACh cannot penetrate through the membrane. We, therefore, thought that if ACh is indeed essential for axonal conduction, it should be possible to demonstrate the effects of ACh if we could reduce the permeability barriers surrounding the axolemma.

In our studies we elected to use the squid giant axon (Fig. 5) which is a large single nonmyelinated fiber. It is relatively easy to insert a microelectrode into the axon and measure resting and action potentials. This preparation also has the unique advantage that its axoplasm can be extruded in sufficient amounts to allow penetration studies to be carried out.

We first tested, on the squid axon, lipid soluble tertiary nitrogen compounds such as atropine and physostigmine (Rosenberg and Ehrenpreis, 1961; Rosenberg and Podleski, 1962, 1963) which interact with the ACh system at junctions, since if the ACh system is present in the axon then the permeability barriers should not interfere in the action of these compounds. The minimal active concentration of these compounds, that is the minimal concentration required to

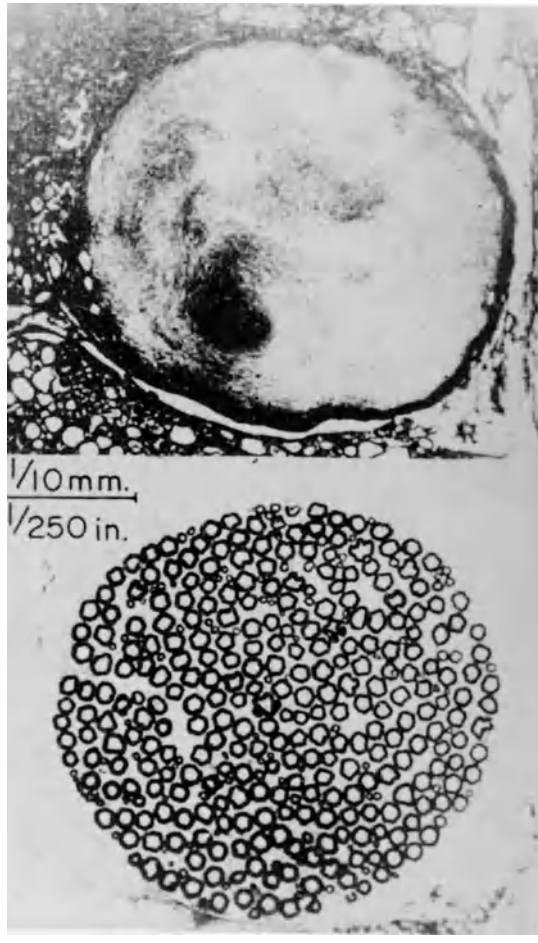


Fig. 5. Cross section of the squid giant axon (*top*) and the rabbit sciatic nerve (*bottom*); same magnification (Young, 1952).

block conduction, was not more than 10-fold greater on the squid axon than on the electroplax synapse. In marked contrast all lipid insoluble compounds tested, such as ACh, curare, and decamethonium, were completely inert on the squid axon. In attempting to reduce the permeability barriers surrounding the squid axon, various enzymes, detergents, and other compounds in the highest concentrations having no effect on conduction were applied to the axon for 30 min, after which the axon was exposed to normal sea water for 15 min and then 10^{-3} M curare was applied for 30 min (Rosenberg and Ehrenpreis, 1961; Rosenberg and Podleski, 1962, 1963; Rosenberg, 1965). Curare was thus used as a

test compound to indicate sensitization of the axon. Curare had no effect on conduction following exposure of the axon to digitonin, hyaluronidase, trypsin, lipase, lysozyme, desoxycholate, Span, Tween, and dimethyl sulfoxide. However, following exposure to certain snake venoms, curare reversibly blocked conduction. An axon rendered sensitive to the action of curare remained so for the entire survival time of the preparation (approximately 6 hr). There were great variations in potency and effectiveness of the venoms. Of about 25 venoms tested, the cottonmouth moccasin venom was one of the most potent whereas the venom of the Eastern diamondback rattlesnake was inactive.

An example of the effect of curare following venom pretreatment (Rosenberg and Podleski, 1963), as observed with intracellular electrodes, is shown in Fig. 6. The venom itself did not block conduction, the preparation was still conductive when placed in normal sea water, while curare rapidly blocked conduction with little or no effect on the resting potential. This agrees with the findings at junctions, where it blocks without depolarization. Similar effects were obtained with ACh, except that in high concentrations ACh depolarized the membrane.

Decamethonium, benzoylcholine, and other cholinergic compounds were also rendered active by venom pretreatment (Rosenberg and Ehrenpreis, 1961;

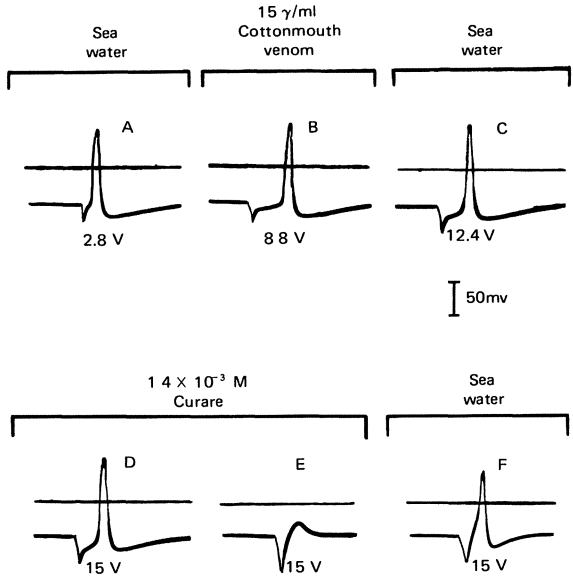


Fig. 6. Effect of curare on the resting and action potential of the squid giant axon following exposure to cottonmouth moccasin venom. A, control; B, after exposure to 15 μg/ml venom for 15 min; C, 15 min after return to sea water (stimulus voltage remained constant for this period); D, E, 4, and 8 min after exposure to 1.4 mM curare; F, 22 min after return to sea water (Rosenberg and Podleski, 1963).

Rosenberg and Podleski, 1962, 1963). In contrast, choline and many non-cholinergic compounds were equally inactive both before and after venom pretreatment. The inactivity of choline is of special interest since it indicates that the effects of ACh are rather specific. Choline is also inactive at synapses.

We interpreted our venom effects as being due to a reduction of the permeability barriers surrounding the conducting membrane, thereby allowing curare and the other compounds to penetrate. To check whether this was true we exposed control or venom-treated squid axons to ^{14}C-labeled compounds for one hour after which the axoplasm was extruded and ^{14}C assayed (Fig. 7; Rosenberg and Hoskin, 1963). You will notice that the lipid soluble tertiary

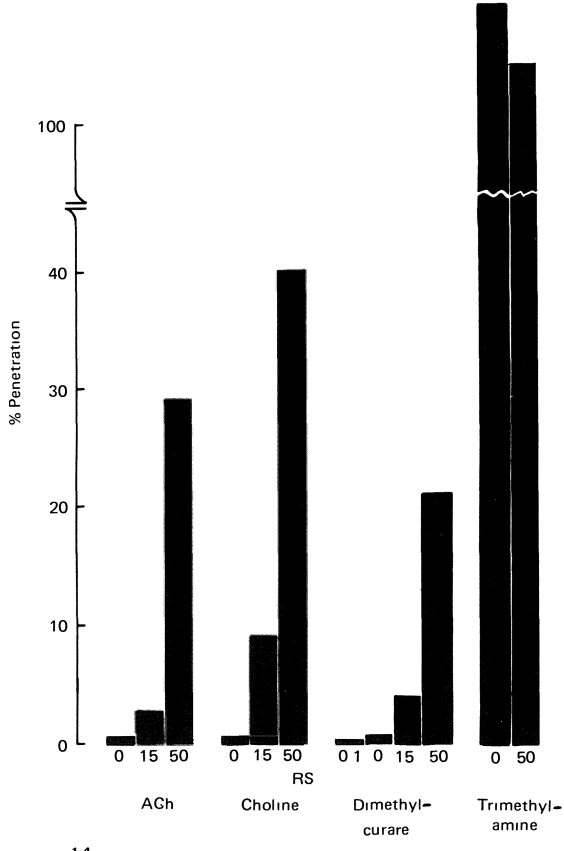


Fig. 7. Penetration of ^{14}C-labeled ACh, choline, dimethylcurare, and trimethylamine into the axoplasm of the squid giant axon with and without exposure to cottonmouth moccasin venom. The percentage indicates the radioactivity of the axoplasm compared with that in the outside fluid. The figures below the columns indicate the μg of venom/ml. (Rosenberg and Hoskin, 1963).

nitrogen derivative, trimethylamine, readily penetrated in contrast to the other compounds, whose low levels of apparent penetration may be due to contamination during extrusion. The penetration markedly increased following pretreatment with 15 and 50 μg/ml cottonmouth venom. 15 μg/ml was the concentration used as the pretreatment of the axons, while 50 μg/ml itself blocked conduction. Even 1000 μg/ml Eastern diamondback rattlesnake venom had no effect on penetration nor did it sensitize the axons to curare. Cottonmouth venom seemed to nonspecifically increase the permeability to any poorly penetrating compound (Hoskin and Rosenberg, 1965), including sucrose, glutamate, GABA, DOPA, Dopamine, and serotonin. These compounds, however, had no effect on conduction even after venom pretreatment.

We were of course interested in seeing what the axons looked like after venom treatment (Martin and Rosenberg, 1968). The venom produced marked vesiculation and fragmentation of the Schwann cell, whereas the axolemma itself did not appear to be markedly affected (Fig. 8). The Schwann cell may then be the site of the permeability barrier which is disrupted by venom.

The increased permeability and sensitization to acetylcholine produced by venom is not limited to the squid giant axon but has also been observed in lobster nerve, eel electroplax, and frog muscle (Rosenberg and Dettbarn, 1964, 1967). We have thus been able to demonstrate, after reduction of permeability barriers, a component of the axonal membrane which responds to ACh.

We were especially interested in studying the component of the venom responsible for increasing permeability. Various venom components including hyaluronidase, protease, phosphodiesterase, 1-amino acid oxidase (a direct lytic factor from venom), and a neurotoxic fraction from venom were all inactive in our test system (Rosenberg and Ehrenpreis, 1961; Rosenberg and Podleski, 1962; Rosenberg and Ng, 1963; Condrea and Rosenberg, 1968). The first indication we had that phospholipase A might be the active component was when we boiled venom solutions at acid and alkaline pH (Rosenberg and Podleski, 1962). We confirmed (Rosenberg and Ng, 1963) earlier reports that phospholipase A is the only known enzymatic component of venom resistant to boiling at an acid pH, although it is destroyed by boiling at alkaline pH (Hughes, 1935; Braganca and Quastel, 1953; Magee and Thompson, 1960). After cooling and readjusting pH, the acid-heated venom solution was almost as active as the control venom solution on electrical activity of axons and in rendering axons sensitive to curare, whereas the alkaline-heated solutions were inactive.

A partially purified preparation of PhA was isolated from ringhals (*Hemachatus hemachatus*) venom by electrophoresis (Condrea and Rosenberg, 1968; Condrea *et al.*, 1967). This isolated fraction increased permeability, sensitized axons to curare and ACh, and in higher concentrations blocked conduction of axons. 10 μg/ml rendered the squid axon sensitive to ACh and curare and 50 μg/ml blocked conduction. PhA produced vesiculations of the Schwann cell

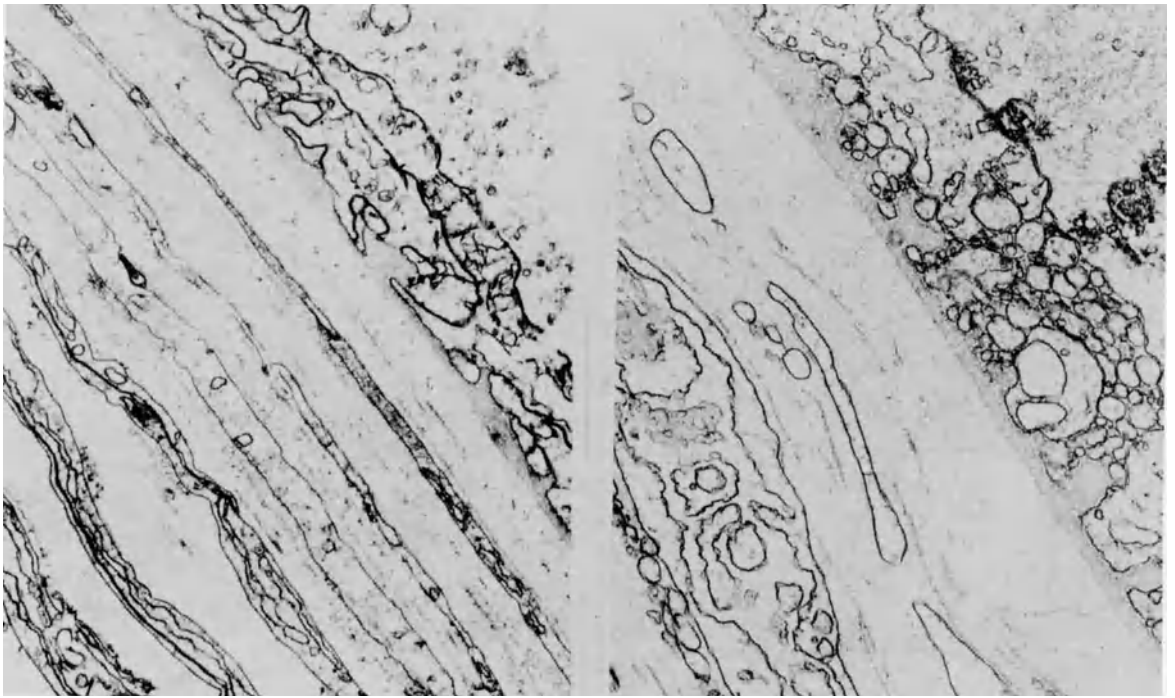


Fig. 8. Electron micrographs of squid giant axons. *Left*, control; *right*, after treatment with 100 μg/ml of *Naja naja* venom. $KMnO_4$–Pb stains used. Axoplasm is shown in the upper right-hand corners, while connective tissue extends from lower left-hand corners to upper left and lower right corners (diagonally). Basement membrane, Schwann cell, and plasma membrane can be seen between connective tissue and axoplasm. Note vesiculation of Schwann cell in venom-treated axon (Robertson, and Rosenberg, unpublished observations).

similar to those reported for whole venoms (Martin and Rosenberg, 1968), and almost completely destroyed the permeability barrier to the penetration of radioactive ACh (Condrea and Rosenberg, 1968).

At that point in our studies we came to a tentative incorrect conclusion, that phospholipids are essential for conduction and permeability properties of the squid axon. To obtain further information on these points we analyzed the phospholipids of the squid giant axon (Rosenberg and Condrea, 1968; Condrea and Rosenberg, 1968). We developed a two-dimensional thin-layer chromatographic technique for the separation of phospholipids after a Folch chloroform-methanol extraction. The individual phospholipids were scraped from the plates and the percent of phospholipid hydrolysis was calculated from the phosphorus measurements on each of the separated phospholipid and lysophospholipid spots. We found about 25%-50% splitting of the major phospholipids (Rosenberg and Condrea, 1968; Condrea and Rosenberg, 1968). A conclusion that this may represent the critical level of phospholipid splitting beyond which functional effects are observed, was not possible to make however because of the following results.

In most of our studies we used giant axons containing adhering small nerve fibers for convenience. The observation was later made that cottonmouth moccasin venom is inert on giant axons dissected free of adhering small nerve fibers, neither increasing permeability nor rendering axons sensitive to curare and ACh (Martin and Rosenberg, 1968; Rosenberg and Hoskin, 1965; Rosenberg and Podleski, 1962). A partially purified sample of PhA was also inactive on finely dissected axons (Condrea and Rosenberg, 1968). A likely explanation for these findings is that in the closely dissected axon there is less substrate on which PhA can act and therefore less lysophosphatide is formed. To check this possibility, ringhals venom was incubated with giant axons containing adhering small nerve fibers, and a lipid extract of this incubate was diluted in sea water and applied to finely dissected axons. This lysophosphatide mixture blocked conduction, rendered axons sensitive to ACh, and increased the penetration of ACh (Rosenberg and Condrea, 1968). A synthetic sample of lysolecithin had similar effects. We concluded that the enzymatic formation of lysophosphatides within the membrane is responsible for the increased permeability of the squid giant axon produced by venoms. In all studies using PhA it is necessary to dissociate the effects of PL splitting per se from the secondary effects of evolved toxic products.

Various venoms and isolated PhA resulted in approximately identical percentages of splitting of PL in giant axons with or without adhering small nerve fibers (Table 2), although blocking conduction only in those preparations containing small nerve fibers (Condrea and Rosenberg, 1968). In the finely dissected preparation, however, only about 1/50 as much lysophosphatide was produced at the same percentage of splitting as in the crudely dissected preparation. This

Table 2. Effect of Phospholipases A and C on Phospholipids in Intact Squid Giant Axons[a]

	mg/ml	Preparation	% Hydrolysis			
			PC	PE	PS	SM
PhA	0.05	GA+[b]	48	37	10	0
	0.05	GA[c]	26	39	10	0
	0.05	Ax[d]	36	50	–	–
PhC	2.0	GA+	55	29	13	52
	20.0	GA+	84	50	3	84
	20.0	GA	56	27	7	100
	20.0	Ax	54	21	–	–

[a] Modified from Rosenberg and Condrea, 1968.
[b] GA+ represents giant axon containing adhering small nerve fibers.
[c] GA represents finely dissected giant axons free of adhering small nerve fibers.
[d] Ax represents axoplasm extruded from intact giant axons.

explained why in the finely dissected preparation PhA did not block conduction, increase permeability, vesiculate the Schwann cell, or render the axon sensitive to curare or ACh even when the PhA was used in high concentrations. These observations surprised us, because it meant that the axons were apparently functioning normally even though 25%–50% of their phospholipids were hydrolyzed.

As a more critical test for phospholipid function in the squid axon we used PhC, since the hydrolytic products of its action on phospholipids are not toxic. Also PhC splits the phospholipids at perhaps a more critical point in the molecule, if the polar head groups are really the essential part of the phospholipid. We found that concentrations of PhC as high as 20 mg/ml had no effect on the action potential of the squid axon, did not increase penetration of ACh, nor render the axons sensitive to curare or ACh. This was observed despite the fact, as shown on Table 2, that PhC split a greater percentage of the phospholipids than PhA did (Rosenberg and Condrea, 1968). Note that the splitting by PhC was similar in crudely or finely dissected axons, and that the splitting extended into the axoplasm. These results then indicated that axonal conduction and membrane permeability could be maintained in the presence of extensive phospholipid splitting, up to 80% lecithin and 100% sphingomyelin. Our results could have been criticized, however, on the basis that, although the phospholipids were split, the split products could have remained in the membrane in close proximity to each other and still be able to function in the normal fashion. To text this possibility we measured diglyceride and lipid and aqueous phosphorus values as measures of phospholipids and phosphorylated bases of the phospholipids.

We did this in the axon, envelope, axoplasm, and incubation solutions during and after exposure to PhC (Rosenberg, 1970). Some of the results for whole axons incubated for 30 min in PhC or sea water and then another 30 min in sea water are shown in Fig. 9. 50% of the total phospholipids were hydrolyzed by PhC with 66% of the evolved phosphorylated bases appearing in incubate I and the remaining 34% appearing in incubate II. None of the evolved phosphorylated bases remain in the axon. In contrast, 87% of the evolved diglyceride remains within the axon while 5% appears in incubate I and 8% in incubate II. We were able to conclude, taking into account all of our results, that the axon appeared to function normally even when over 60% of the polar head groups of the phospholipids were depleted from the membrane. Almost all of the diglyceride produced remains attached to the membrane.

These results then appeared to cast some doubts on theories attributing vital roles in axonal conduction or membrane permeability to intact phospholipids or their polar head groups. Our results may also indicate something about the structural organization of membranes. Various models of membrane structure are shown in Fig. 10. According to the Danielli-Davson paucimolecular model and Robertson's unit membrane hypothesis the polar head groups of the phospholipids would primarily be expected to interact with the external protein layers by hydrophilic, that is electrostatic forces (Danielli and Davson, 1935; Robertson, 1960, 1966). In contrast, more recent theories including the protein-crystal model of Green and the fluid mosaic model of Singer propose that the protein component of the membrane extends into the interior of the membrane in close

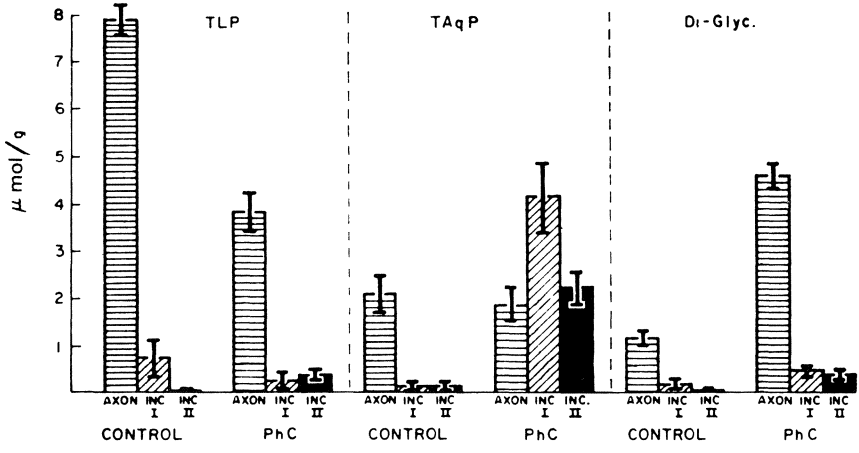


Fig. 9. Total lipid phosphorus (TLP), total aqueous phosphorus (TAqP), and diglyceride (Di-Glyc) in control and phospholipase C (PhC) treated axons. Results for axons are presented as μmol/g, while for incubates it is μmol/g of axon to which the incubates were exposed. The bars indicate ± 1 standard error (Rosenberg, 1970).

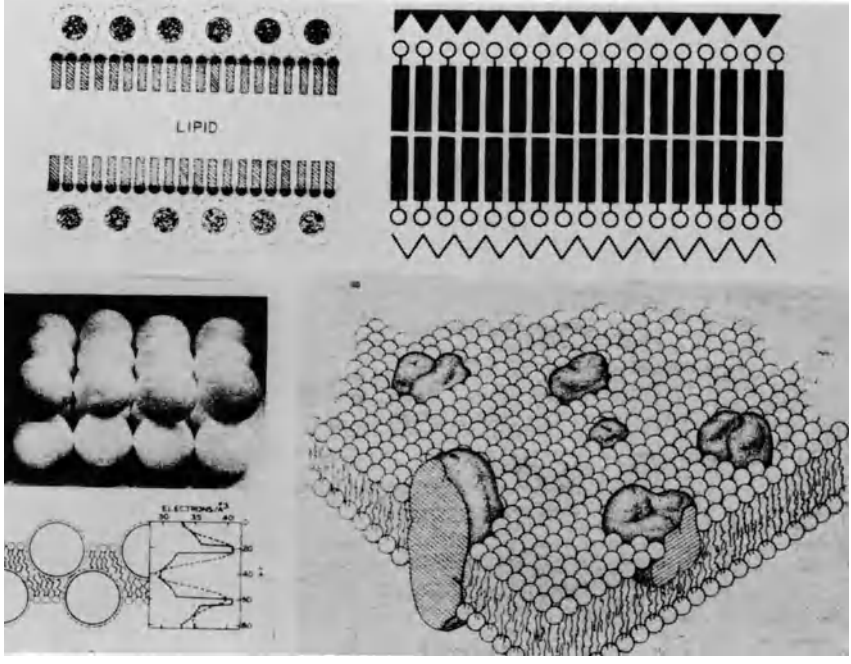


Fig. 10. Models of membrane organization. *Upper left*, Danielli and Davson (1935) model of membrane structure. Phospholipids are coated on each surface by globular proteins. *Upper right*, Robertson (1960, 1966) unit membrane hypothesis. Circles represent polar end and bars the nonpolar carbon chains of phospholipids. Polar surfaces of the lipid layer are covered by monolayers of proteins or other nonlipids which may be asymmetrically distributed. *Lower left*, protein crystal model of membranes. Large circles represent proteins, small circles are the polar lipid heads and the wavy lines are the nonpolar lipid tails (Green and Perdue, 1966; Vanderkooi and Green, 1970). *Lower right*, fluid mosaic model. Solid globular bodies represent proteins. Circles represent ionic and polar head groups of the phospholipids and wavy lines represent the fatty acid chains (Glaser *et al.*, 1970; Singer and Nicolson, 1972).

relation with the fatty-acid chains of the phospholipids while the polar head groups of the phospholipids are at the surface of the membrane (Green and Perdue, 1966; Vanderkooi and Green, 1970; Glaser *et al.*, 1970; Singer and Nicolson, 1972). In such an arrangement hydrophobic or nonpolar interactions would be expected to be of major importance. It would seem that our results favor such a structure. The rapid loss of polar head groups into the incubation media by exposure to PhC, coupled with the maintenance of conduction and normal membrane permeability, would appear to be more compatible with such a structure.

In order to obtain further information concerning the organization of the squid axonal membranes we exposed squid axons for 30 min to PhA, PhC, or

lysolecithin (LL) and then for 30 min to normal sea water (Rosenberg and Khairallah, 1974). Phospholipids and free amino acids were then determined in envelope and axoplasm and the released amino acids in the incubation solution. We wanted to determine the effects of disrupting hydrophobic binding with PhA or hydrophilic interaction with PhC on the structure of the nonlipid portion of the axonal membrane. The percentage of phospholipid hydrolysis is shown on Table 3. Note especially that the highest concentration of PhC caused more hydrolysis than the two lowest concentrations of PhA and about as much as the highest concentrations of PhA. The normal free amino acids of squid envelopes, axoplasm, and incubation solutions were determined. Those present in the greatest amounts were the polar amino acids, taurine, aspartic acid, and glutamic acid. PhA caused a much larger decrease in the free amino acid content of the envelope than PhC or LL, even when used in its lowest concentration, where it caused less phospholipid splitting than PhC. Similar observations were made in the axoplasm. Associated with the decrease in free amino acids of the axoplasm and envelope caused by PhA, there was a huge increase in the free amino acid content of the two incubation solutions, much greater than that caused by PhC or LL. A summary of these results is shown in Table 4. You will note the much greater decrease in envelope and axoplasmic free amino acids with PhA than is observed with PhC or LL, whereas the increase in incubation solution free amino acids is also greater with PhA. The effects of PhA are not due to production of lysophosphatides since LL had much less effect. It would thus appear that electrostatic or hydrophilic interactions are of much less importance in binding between phospholipids and amino acids than are hydrophobic interactions. The increase in released amino acids in the incubation solutions is greater than the decrease in amino acids in the axon, which could imply that hydrolysis of phospholipids exposes protein to endogenous proteases. The enzymes used, PhA and PhC, have no proteolytic activity themselves.

Table 3. Splitting of Phospholipids in Envelope and Axoplasm of Squid Giant Axon by Phospholipases A and C[a]

	Concentration, mg/ml	% Hydrolysis					
		Envelope			Axoplasm		
		PC	PE	PS	SM	PC	PE
PhA	1.0	87	70	30	0	61	70
	0.2	59	45	10	0	35	53
	0.025	35	20	5	0	20	25
PhC	10.0	80	58	11	87	60	45
	0.5	30	25	6	31	18	16

[a]Modified from Rosenberg and Khairallah, 1974.

Table 4. Total Free Amino Acids (FAA) in Envelope, Axoplasm, and Incubation Solutions of Squid Axon in Control, PhA, PhC, and LL Exposed Axons[a]

	Concentration, mg/ml	Envelope, nmol/0.8 mg	Axoplasm, nmol/0.2 mg	Incubation solutions, nmol/mg of axon exposed		Total FAA
				1	2	
Control		63	34	14	16	127
PhA	1	7	5	139	119	270
	0.2	16	6	56	106	184
	0.025	29	11	29	57	126
PhC	10	47	27	30	64	168
	0.5	68	37	9	12	126
LL	1	62	17	34	33	146
	0.2	58	37	17	20	132

[a]These results are based upon the estimation that axons as dissected consist of 20% axoplasm and 80% envelope (Rosenberg and Khairallah, 1974).

In conclusion, our studies on the squid giant axon have not demonstrated that phospholipids are essential for conduction or maintenance of membrane permeability. We have, however, been able to tentatively conclude that hydrophobic forces are of major importance in lipid-protein binding in the squid giant axon and that the polar head groups appear to be predominantly at the surface of the membrane.

EFFECT OF PHOSPHOLIPASES ON THE EEL ELECTROPLAX: A SYNAPTIC CONTAINING PREPARATION

Are phospholipids essential for conduction at the synapse? Here is where most of Hokins' and Larrabees' studies seemed to show the function of phospholipids. I decided to use the electroplax of the electric eel (Fig. 11) as an example of a synaptic-containing preparation (Bartels and Rosenberg, 1972). The muscles used in this study run on either side of the vertebrae, directly above the electric tissue. The electric organ is below the swim bladder, there being about 6000 cells in the anterior-posterior direction. Only in the Sachs organ are the cells far enough apart so that a single cell can be dissected. The cells are about 0.2 mm thick, 7-15 mm high, and 3-5 mm wide. The connective tissue includes the gelatinous material between the cells plus the cell compartment membrane. We also separated the innervated membrane, containing 25,000-50,000 synapses which cover 3-6% of the total surface area, from the noninnervated membrane.

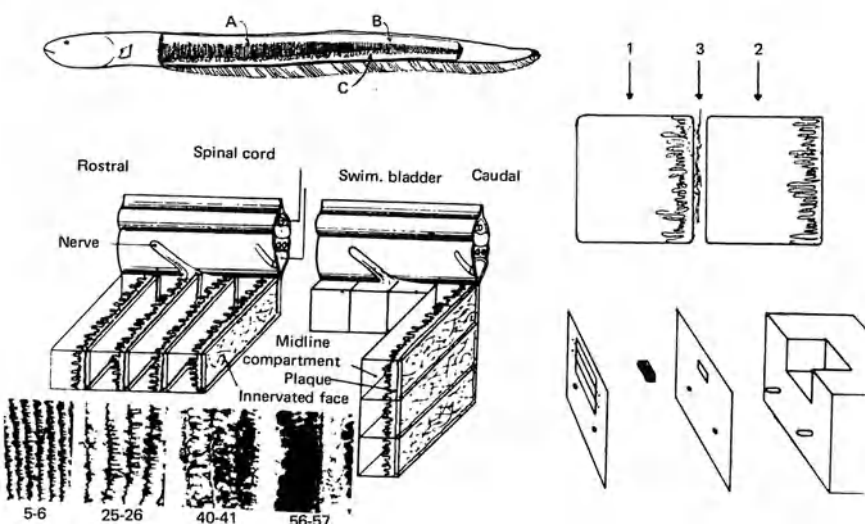


Fig. 11. Tissues of *Electrophorus electricus. Upper left*: position of Main (A), Sachs (B), and Hunter (C) electric organs. *Middle left*: fragment of electric organ showing relationships between rows of electroplax, and other organs of electric eel. *Lower left*: changes in frequency of electroplax/cm of Main organ. Numbers indicate distance in cm from the anterior end of the organ. *Upper right*: method of dissection of single electroplax. Cutting at 1 and 3 isolates a single electroplax. Noninnervated, nonconducting, membrane is indicated by deep invaginations. The conducting, synaptic-containing membrane is represented by straight line to the left of 3. *Lower right*: arrangement for mounting a single isolated electroplax for electrical recording. Shown are one chamber for a pool of fluid (other chamber not shown), the sheet of nylon containing a window, the single electroplax, and the grid used for pressing the cell against the window (modified from Nachmansohn, 1959).

Since so little was known about the lipids of the electric eel we first did a complete phospholipid and neutral lipid analysis. For example, Table 5 shows the total lipid phosphorus (TLP) values obtained on lipid extracts of these tissues. Note the much higher values in the innervated membrane where the synapses are, as compared to the noninnervated membranes and the connective tissue. This may reflect a real difference in membrane structure or may be due to the presence of small nerve endings on the innervated membrane. I, therefore, denervated an electric eel by destroying the spinal cord and did the analyses again. Even after all of the nerve endings degenerated, the innervated membrane had significantly more phospholipids (higher TLP values), than the noninnervated membrane (Rosenberg, unpublished observations). We also determined cholesterol, cholesterol esters, diglycerides, triglycerides, and the distribution of individual phospholipids in each of these tissues.

We then tested the effects of PhA on the resting and action potential of the electroplax. We used acid-boiled cottonmouth moccasin venom, a preparation

Table 5. Total Lipid Phosphorus (TLP) in Tissues of the Electric Eel[a]

Tissues	TLP, μg/g	Number of experiments
Sachs organ	33 ± 2	8
Single cells	20 ± 1	13
Innervated membrane	59 ± 3	10
Noninnervated membrane	11 ± 2	10
Connective tissue	3.0 ± 0.2	4
No membrane	2.5 ± 0.2	4
Main organ	107 ± 4	20
Muscle	135 ± 30	4

[a]See Fig. 11 and text for description of tissues used. TLP values are expressed as μg per gram wet weight (Bartels and Rosenberg, 1972).

free of all enzymes except PhA, as our source of PhA. You see in Fig. 12 that 1 mg/ml of this preparation of PhA depolarized and irreversibly blocked conduction. These effects were not mimicked by neurotoxic or direct lytic components which may still have been present in the acid-boiled preparation. Phospholipase C had an identical effect at the same concentration as the acid-heated venom PhA. Boiling cottonmouth venom at an alkaline pH destroys its PhA activity and also destroys its effects on the action and resting potential.

In our next series of experiments we determined the percentage of phospholipid hydrolysis associated with exposure to the acid-heated venom (Table 6).

Table 6. Hydrolysis of Phospholipids in Tissues of the Electric Eel by Acid-Boiled Cottonmouth Moccasin Venom (CMV)[a]

Tissue	CMV, mg/ml	% Hydrolysis		
		PC	PE	PS
Sachs single cells	0.001	10 ± 1	7 ± 2	5 ± 2
	0.01	31 ± 2	44 ± 3	45 ± 18
	0.1	37 ± 1	75 ± 4	89 ± 5
	1.0	89 ± 2	76 ± 13	98 ± 2
Innervated membrane	0.005	21 ± 1	16 ± 2	79 ± 4
	1.0	88 ± 2	61 ± 10	100 ± 0
Noninnervated membrane	0.005	17 ± 1	5 ± 5	92 ± 8
	1.0	95 ± 2	78 ± 3	100 ± 0

[a]Tissues were exposed to SV for 20 min prior to lipid extraction. To determine splitting in membranes, rows of cells (one-cell thick) were exposed to solutions of SV for 20 min after which rows were extensively rinsed with eel Ringers and the separated membranes were dissected. Each value represents mean ± S.E. based on 3 experiments with single cells and two with membranes (Bartels and Rosenberg, 1972).

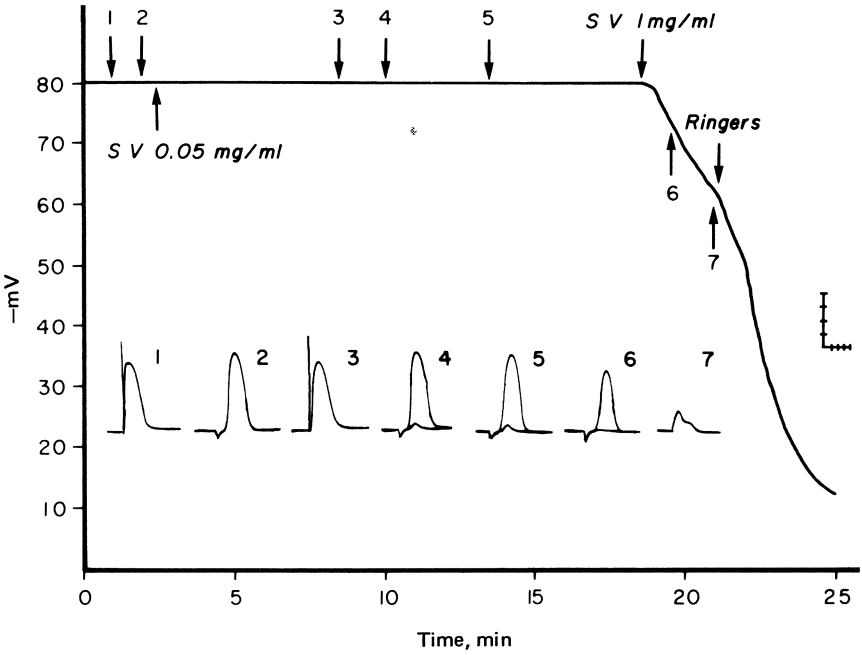


Fig. 12. Effect of acid-boiled cottonmouth moccasin venom (SV) on the resting and action potential of a single isolated electroplax from the Sachs organ of *Electrophorus electricus*. The –mV values represent the resting potential of the cell (inside relative to outside). The numbers denote the action potentials with the time of elicitation indicated on the resting potential tracing; 1, 3, and 7 were directly evoked action potentials 2, 4, 5, and 6 were neurally evoked. The lower traces in 4 and 5 show the postsynaptic potential observed at a lower voltage which did not trigger a neurally evoked apike. No postsynaptic potential was observed in 6. Calibration for action potential: 25 mV (vertical scale) and 2 msec (horizontal scale) for each small division. (Bartels and Rosenberg, 1972).

0.10 mg/ml of acid-boiled venom split most of the PE and PS, but only a third of the lecithin. A concentration (1 mg/ml) which hydrolyzed most of the lecithin, also blocked conduction. The results in the separated innervated and noninnervated fractions were similar to that in the intact cell. Since ACh is the normal transmitter in the synapse of this preparation the results with lecithin might be of interest in relationship to Watkins (1965) proposal that ACh competes with lecithin for binding to protein.

In order to further evaluate the possible function of phospholipids in synaptic transmission, single electroplax were incubated in ^{14}C glucose and ^{32}P inorganic phosphate. The effects of stimulation and ACh on radioactive incorporation into phospholipids were measured in dual label experiments (Rosenberg, 1973). The amounts of each phospholipid were not altered by these treatments, however both stimulation and ACh increased radioactive incorporation of ^{14}C and ^{32}P

Table 7. ^{14}C and ^{32}P Incorporation into PC and PI of the Sachs Organ of the Electric Eel[a]

Tissue condition	^{32}P, cpm (hundreds)/μmol P		^{14}C, cpm (hundreds)/μmol P	
	PC	PI	PC	PI
Single cells				
Control	4.5 ± 0.2	43 ± 6	46 ± 4	130 ± 20
ACh[b] + Physo[c]	8.7 ± 0.4[f]	164 ± 9[f]	132 ± 11[f]	840 ± 50[f]
Physo	5.8 ± 0.5[g]	83 ± 4[f]	61 ± 5[g]	370 ± 90[g]
Rows of cells				
Control	5.2 ± 0.7	76 ± 10	24 ± 10	110 ± 20
DS[d]	21 ± 3[f]	190 ± 19[f]	202 ± 23[f]	520 ± 40[f]
IS[e]	29 ± 2[f]	217 ± 17[f]	111 ± 19[f]	320 ± 10[f]

[a] Single cells were exposed to 25 μCi ^{32}P-H_3PO_4 and 2.5 μCi U-^{14}C glucose per ml for 6 hr, while rows of cells were exposed for 3 hr. Each value (Mean ± S.E.) is based upon three experiments.
[b] ACh is 10^{-4} M acetylcholine.
[c] Physo is 10^{-4} M physostigmine.
[d] DS is direct stimulation.
[e] IS is indirect stimulation.
[f] $p < 0.01$ compared with controls (Rosenberg, 1973).
[g] $p < 0.05$ compared with controls (Rosenberg, 1973).

into PC and PI (Table 7). Incorporation into SM, PS, PE, and phosphatidic acid was unaffected. The control rate of ^{32}P incorporation was quite low, probably because of the relative impermeability of the electroplax cell to phosphorus. These results are of interest in relationship to the theories of Hawthorne and Kai (1970), Durrell *et al.*, (1969), Watkins (1965), Larrabee (Larrabee, 1968; Larrabee and Leicht, 1965; Larrabee *et al.*, 1963), and others suggesting an association between phospholipid turnover and synaptic activation.

Our studies on the squid axon (Rosenberg, 1970; Rosenberg and Khairallah, 1974) indicated that hydrophobic forces were of greater importance in protein-lipid binding in the membrane than were hydrophilic interactions. We had, for example, found that removal of a fatty acid from phospholipids by PhA caused a much greater release of free animo acids, than did removal of the polar phosphorylated base by PhC. In addition the axon maintained apparently normal conduction and permeability properties even after extensive loss, from the membrane, of phospholipid polar head groups by hydrolysis with PhC. We were interested in determining, also in a synaptic containing preparation, the relative effects of PhA and PhC on membrane structure. I, therefore, exposed isolated single electroplax cells for 30 min to either eel Ringers, PhA, PhC, or LL (2 and 0.2 mg/ml), after which the cells were prepared for electron microscopy (Rosenberg, 1976). Acid-boiled cottonmouth moccasin snake venom was used as the source of PhA.

PhA had a much greater disruptive effect on the electroplax membranes than PhC or LL. The innervated membranes of control and PhA (2 mg/ml) treated cells are shown in Figs. 13 and 14. PhA caused inpocketings of membranes leading to vesicle formation and apparent reformation of membranes around clusters of vesicles. Mitochondrial swelling was also observed with PhA. Similar effects by PhA were also found on the noninnervated membrane. PhC caused some mitochondrial swelling but little if any vesiculation of the plasma membrane. LL caused only slight clustering of vesicles, not nearly as severe as that observed with PhA. These results indicate that disruption of hydrophobic binding forces has a much more deleterious effect on the membrane structure of the electro-

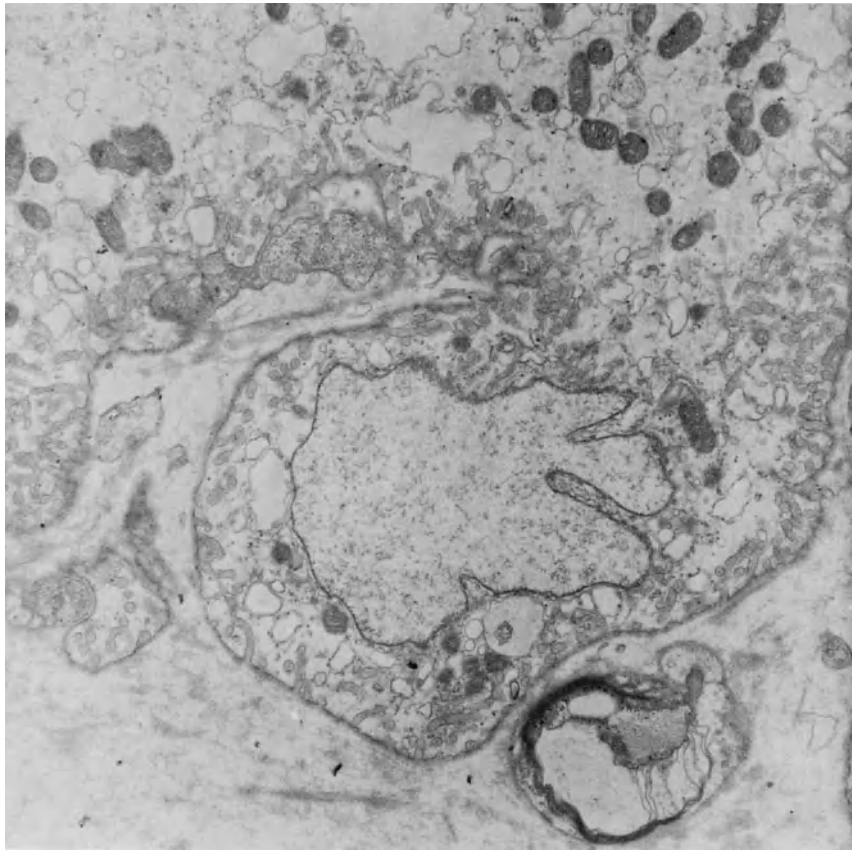


Fig. 13. Innervated membrane of control isolated single electroplax from Sachs organ of the electric eel. Tissues were fixed in glutaraldehyde and osmium, stained with uranyl acetate and examined under a Phillips EM-300 electron microscope. A large nucleus within an evagination of the innervated membrane may be observed. A nerve is near lower right hand corner. Note numerous mitochondria (Rosenberg, 1976).

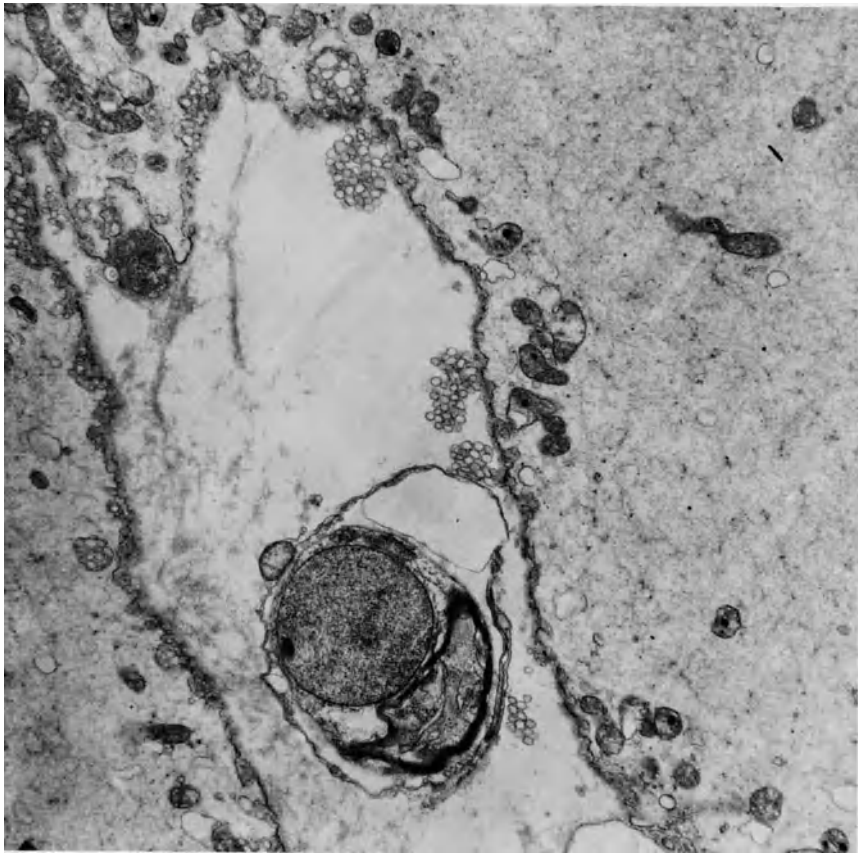


Fig. 14. Innervated membrane of PhA (2 mg acid-boiled cottonmouth moccasin venom per ml) treated isolated single electroplax from Sachs organ of the electric eel. Cells were exposed to PhA in eel Ringers for 30 min. Other conditions are the same as in Fig. 13. Note formation of clusters of vesicles and swelling of some of the mitochondria (Rosenberg, 1976).

plax than does disruption of hydrophilic bonding between protein and phospholipid.

EFFECTS OF PHOSPHOLIPASES ON THE CENTRAL NERVOUS SYSTEM

We have now begun to extend our studies from the peripheral to the central nervous system. This was prompted by our observation that crude cobra venom caused convulsions following intraventricular injection into rats. Russell and Bohr (1962) had observed various effects ranging from sedation to convulsions

following the intraventricular injection of several venoms. As noted in Table 8, crude and acid-boiled cobra (*N. naja*) venom caused convulsions, while purified cobrotoxin, cardiotoxin (similar to direct lytic factor), and alkaline-boiled venom did not cause convulsions (Lysz and Rosenberg, 1974). A purified PhA preparation isolated from cobra venom also caused convulsions, although slower in onset than observed with crude or acid-boiled venom. Cardiotoxin, although not

Table 8. Convulsant and Lethal Effects of *Naja naja* Venom and Its Components Following Intraventricular Injection[a]

Preparation	Dose, μg/rat	Convulsions		Death	
		Number of rats	Onset, min	Number of rats	Min
Crude venom	2.5	0/6[b]	–	0/6	–
	5	3/6	64 ± 12	1/6	176
	10;50	4/4	7–22	4/4	35–137
Acid-boiled venom	2.5	0/6	–	0/6	–
	5	2/6	31, 150	0/6	–
	10	2/2	24, 51	2/2	105, 54
	25;50	4/4	8–16	4/4	38–88
Alkaline-boiled venom	50;100	0/7	–	0/7	–
	250	0/6	–	0/6	–
Cobrotoxin	5	0/4	–	0/4	–
	10	0/4	–	3/4	230 ± 78
	25;50	0/7	–	7/7	27–60
Cardiotoxin	10	0/3	–	0/3	–
	25	0/5	–	3/5	1140–1400
	50	0/5	–	2/5	155, 1200
	75;125	0/3	–	3/3	59–119
PhA	1	0/4	–	0/4	–
	2.5	4/4	380 ± 50	0/4	–
	5	4/4	207 ± 28	4/4	431 ± 107
	10	2/2	175, 165	1/2	475
	30;80	4/4	81–167	4/4	190–240
	130;180	4/4	40–122	4/4	179–206
	280;330	5/5	64–95	4/5	132–205

[a] Acid and alkaline heated venom solutions were boiled at 100°C for 10 min at pH 5.5 or 9.5, respectively, with the pH being readjusted to 7.0–7.5 before injection. PhA was purified from cobra venom by the method of Braganca *et al.* (1969). Cobrotoxin and cardiotoxin were gifts from Dr. C. C. Yang and Dr. W. Vogt, respectively (Lysz and Rosenberg, 1974).

[b] Number of rats which had convulsions/Number of rats.

causing convulsions by itself, markedly potentiated the convulsant action of PhA. Cardiotoxin (5 μg/rat) decreased the minimally effective convulsant dose of PhA from 2.5 to 0.5 μg/rat and decreased the time until onset of convulsions after PhA to that observed after the injection of crude venom. PhC, in doses as low as 1 μg, also caused convulsions. Following intraventricular injection of high doses of PhA and PhC, hydrolysis was observed in several different brain areas. The mechanism of PhA-induced convulsions is now under investigation. It must be considered, for example, whether the hydrolysis of phospholipids by PhA is directly causing the convulsions, or whether the convulsions are secondary to an increase in the permeability of cells or subcellular structures, such as lysosomes, releasing components, possibly catabolic enzymes, thereby causing the convulsive episodes.

SUMMARY

PhA and PhC, derived from snake venoms and bacterial toxins, respectively, have proven to be useful tools for analyzing structure and function in bioelectrically excitable tissues. Several investigators have suggested specific functions in nerve and synapse for membranal phospholipids, however none of these are proven. The squid giant axon can maintain apparently normal conduction and permeability properties even after extensive phospholipid hydrolysis by PhA or PhC and loss of phosphorylated polar head groups (bases) from the membrane after exposure to PhC. Effects apparently due to PhA were actually caused by detergent action of lysophosphatides liberated as a result of PhA action on phospholipids.

In contrast, we found evidence for the specific functioning of phospholipids in a synaptic-containing preparation, the isolated single electroplax. Electrical activity was blocked by both PhA and PhC when more than about one third of the membranal phospholipid lecithin was hydrolyzed. Application of electrical stimuli or ACh also increased the metabolic turnover of phospholipids in the eel electroplax. PhA and PhC caused convulsions when intraventricularly injected into rats, and PhA appears to be the component responsible for convulsions produced by crude snake venom. This convulsant activity appears to be correlated with phospholipid hydrolysis.

At equivalent extents of phospholipid hydrolysis, PhA was much more potent than PhC in disrupting protein-phospholipid binding, thereby altering membrane structure. For example, PhA was much more potent than PhC in causing release of free amino acids from the squid giant axon and in causing vesicle formation and disruption of membranes in the eel electroplax. Our results suggest that binding between phospholipids and proteins is primarily hydrophobic, rather than hydrophilic in nature and that the phosphorylated bases (polar head groups)

of the phospholipids are mostly at the surface of the membrane rather than internally buried.

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Pharmacological Studies of the Hemorrhagic Principles Isolated from the Venom of *Trimeresurus flavoviridis*, a Crotalid

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INTRODUCTION

Hemorrhage is one of the most prominent local symptoms evoked by crotalid and viperid venoms (Boquet, 1964; Ohsaka *et al.*, 1966; Jiménez-Porras, 1968). Recently, Ohsaka *et al.* succeeded in isolating two immunologically distinct hemorrhagic principles, HR1 and HR2, from the venom of *Trimeresurus flavoviridis*, a crotalid (Omori-Satoh and Ohsaka, 1970; Takahashi and Ohsaka, 1970a; Ohsaka *et al.*, 1971a). Cinematographic observations on a microscopic level demonstrated that the early effects of crude venom or venom hemorrhagic principles on the microcirculatory system are characterized by severe vasoconstriction followed by vasodilatation of larger vessels, especially of arterioles, and subsequent hemorrhage in the capillary bed (Ohsaka *et al.*, 1971b); the erythrocytes come out one by one through the pinpoint holes formed through the walls of true capillaries (Ohsaka *et al.*, 1971b; Tsuchiya *et al.*, 1974). Electron-microscopic examination of the vascular endothelial cells treated with the venom

or venom hemorrhagic principle (HR1) revealed that the erythrocytes come out through the junctions of the endothelial cell lining, the adjacent basement membrane being disrupted to permit eventual extravasation of the erythrocytes (Tsuchiya *et al.*, 1974; Ohsaka *et al.*, 1975).

In view of the striking vasomotor changes observed, it is of special interest to study the pharmacological actions of the purified hemorrhagic principles on smooth muscles.

The present paper describes experiments in which the effects of the purified preparations of HR1 and HR2 were tested on the isolated smooth muscles. The results demonstrated the ability of the venom hemorrhagic principles to release some vasoactive mediators from the guinea pig ileum and lung and from the rat peritoneal cells. The significance of these findings will be discussed in relation to the mechanism of hemorrhage. A preliminary report of this work has appeared (Ishida *et al.*, 1975).

MATERIALS AND METHODS

The venom (Batch no. 70B) used (a dried pool of venom taken from specimens of *Habu*, *Trimeresurus flavoviridis*, collected in the Amami Oshima Islands in 1970) was a gift of the Division of Public Health, Kagoshima Prefecture, Japan. Purified HR1 was prepared from crude venom by the method previously described (Omori-Satoh and Ohsaka, 1970) and purified HR2 (a and b) by the method of Takahashi and Ohsaka (1970a). Purified H_2-proteinase, being completely free from hemorrhagic activity, was prepared according to Takahashi and Ohsaka (1970b). Solutions of all these venom proteins were made in 5 mM Borax-HCl buffer (pH 8.0) containing 0.1 M NaCl and 2 mM Ca^{2+}.

Guinea pig ileum (at least 10 cm apart from the ileocaecal junction) was cut into segments, 4-6-cm length, and each segment was suspended in 10 ml of Tyrode's solution maintained at 32°C and constantly aerated. Contraction of the ileum was recorded on a smoked drum with an isotonic lever. The response of the ileum to transmural electrical stimulation was induced by the method of Paton (1955). For the assay of histamine, atropine sulfate (10^{-7} M) was previously added to the organ bath. For estimating slow-reacting substances (SRS), chlorpheniramine maleate (10^{-7} M) and atropine sulfate (10^{-7} M) were previously added to the bath to abolish contraction induced by rapidly released histamine and acetylcholine (ACh), respectively. Purified tetrodotoxin, a kind gift of Sankyo Co., Tokyo, was used to examine the venom hemorrhagic principle for neurotropic action on the smooth muscle.

Ovariectomized rats were pretreated for two days with 30 μg of estradiol benzoate subcutaneously injected. Uterine horns at the oestrus stage were excised and each horn was suspended in 10 ml of a modified physiological saline

(150 mM NaCl, 5.36 mM KCl, 0.36 mM $CaCl_2$, 0.19 mM $MgCl_2$, 0.59 mM KH_2PO_4, 4.76 mM $NaHCO_3$, and 2.78 mM glucose per liter), maintained at 30°C and constantly aerated. Contraction of the horn was recorded on a smoked drum with an isotonic lever. A specific antagonist for 5-hydroxytryptamine (5-HT), S-8 (3-(2′-benzylaminoethyl)-5-methoxyindole hydrochloride) (Takagi *et al.*, 1969), was used for the identification of 5-HT with the above-described uterus preparation.

To demonstrate mediators released from guinea pig lungs, the method of Middleton and Phillips (1964) was used. Finely chopped lungs suspended in Tyrode's solution at 100 mg/ml were incubated with the venom hemorrhagic principle for 20 min at 37°C. The incubation mixture was centrifuged and the supernatant fluid, after filtration through cotton, was heated in a boiling water bath for 15 min and filtered again. The filtrate was then assayed for both histamine and SRS on the isolated guinea pig ileum.

The effect of the hemorrhagic principle on the rat peritoneal cells, containing mast cells at 4%–7%, was tested according to the method of Bloom and Haegermark (1965). A rat, weighing 200–300 g, was bled from the carotid arteries. 10 ml of a buffered saline solution (154 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 4.0 mM Na_2HPO_4, 2.7 mM KH_2PO_4; pH 7.0) containing 1-mg glucose and 10 I.U. heparin per ml was intraperitoneally injected in the rat. After gentle massage for 1–2 min, the peritoneal cavity was opened and the injected fluid was removed with a pipette. The cellular suspension thus prepared was diluted twofold in volume with buffered saline solution (minus heparin) and the diluted suspension was divided into two aliquots which were then centrifuged at 350 *g* for 30 min at 1–2°C. The sediment from one aliquot was suspended in 1.9 ml of buffered saline. To this suspension venom protein was added to a total volume of 2 ml. The sediment from the other aliquot was suspended in 2 ml of buffered saline alone. These two suspensions were then incubated at 37°C for 10 min and centrifuged at 350 *g* for 5 min at 1–2°C. Each of the supernatant fluids collected by decantation was heated in a boiling water bath for 15 min and assayed for histamine on the atropinized guinea pig ileum. The remaining sediments were resuspended in 0.1 N HCl, boiled, neutralized with NaOH, and then centrifuged; the supernatant fluids were assayed for histamine. The amounts of histamine released from the peritoneal cells were calculated in percent of the total histamine content, which is the sum of the amount of histamine released by the venom and that extracted from the remaining sediment.

RESULTS

Actions of Crude Venom, HR1, HR2 and H_2-Proteinase on the Isolated Smooth Muscle Preparations. The administration of crude venom in concen-

trations greater than 10^{-5} g/ml induced contraction (about 50% height) of the isolated guinea pig ileum, but repeated administration produced desensitization (Fig. 1). After the administered crude venom had been washed out thoroughly, the gut showed gradually developing contraction, which was abolished with an antihistamine drug, chlorpheniramine. HR2, depending on its concentration in a range from 10^{-6} to 10^{-5} g/ml, induced contraction of the gut to graded extents, and the response was not diminished after repetition; whereas neither HR1 nor H_2-proteinase, at concentrations up to 10^{-4} g/ml, produced any contraction of the gut.

The contractile response of the gut to HR2 is largely due to ACh released from the parasympathetic nerves by neurotropic action of this venom principle, because the contraction induced by HR2 was inhibited in the presence of atropine or tetrodotoxin and potentiated in the presence of eserine (Fig. 2). HR2 added to the bath, after the response of the gut to transmural electrical stimulation had almost disappeared with tetrodotoxin, still produced small and gradual contraction, which could be inhibited by chlorpheniramine (Fig. 3), indicating that such slow contraction is due to histamine release following ACh release from the gut organ. In a few experiments some component in the contractile response to HR2 could not be inhibited by administration with both tetrodotoxin and chlorpheniramine, suggesting that the release of a slow-reacting

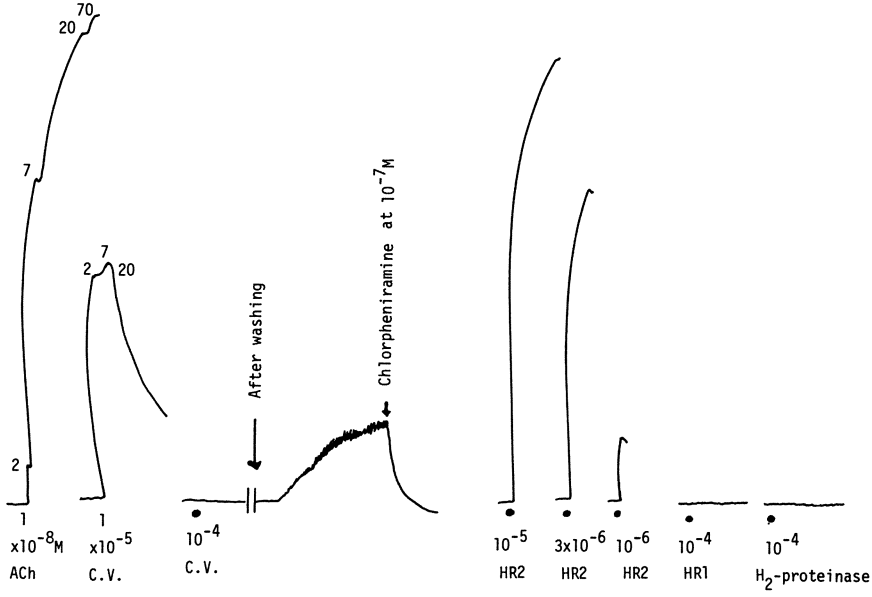

Fig. 1. Actions of crude venom (C.V.), HR1, HR2, and H_2-proteinase (g/ml) on the isolated guinea pig ileum.

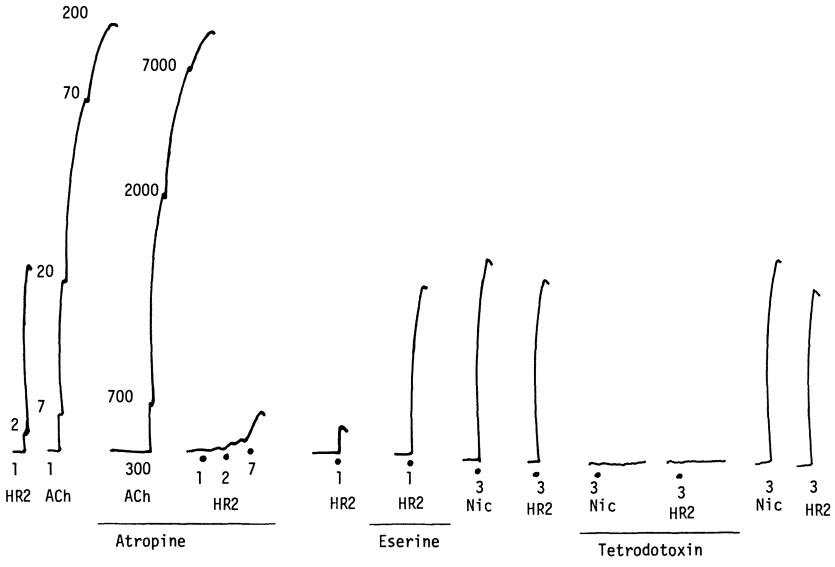

Fig. 2. Contraction of the isolated guinea pig ileum induced by HR2 in the presence of atropine at 10^{-8} M, eserine at 10^{-8} M, and tetrodotoxin at 3×10^{-8} g/ml. Numbers indicate the times of concentration of $\times 10^{-6}$ g/ml for HR2, $\times 10^{-9}$ M for ACh, and $\times 10^{-6}$ M for nicotine (Nic).

substance should also be involved in the contractile response (Fig. 4). Incidentally, HR1 inhibited the contraction of the gut induced by transmural electrical stimulation in the presence of chlorpheniramine (Fig. 4); the mechanism of this inhibition remains to be elucidated.

From these results we may conclude that the contractile response of the guinea pig ileum to HR2 is due to ACh release from the nervous elements in the gut, followed by histamine or SRS release.

With preparations of other smooth muscles such as isolated rat uterus, rabbit aortic strip, isolated guinea pig auricles, phrenic nerve-diaphragm of the rat, and frog rectus abdominis, none of the venom principles tested showed any remarkable responses except transient contraction of the isolated rat uterus induced by crude venom.

Mediators Released from the Guinea Pig Lungs and from the Rat Peritoneal Cells by the Action of Venom Hemorrhagic Principles

Guinea Pig Lungs. The media in which the finely chopped guinea pig lungs were previously incubated with venom proteins contained different amounts of histamine released from the organ. The amounts of histamine released by venom proteins, calculated as histamine base per gram of the chopped lungs, were as

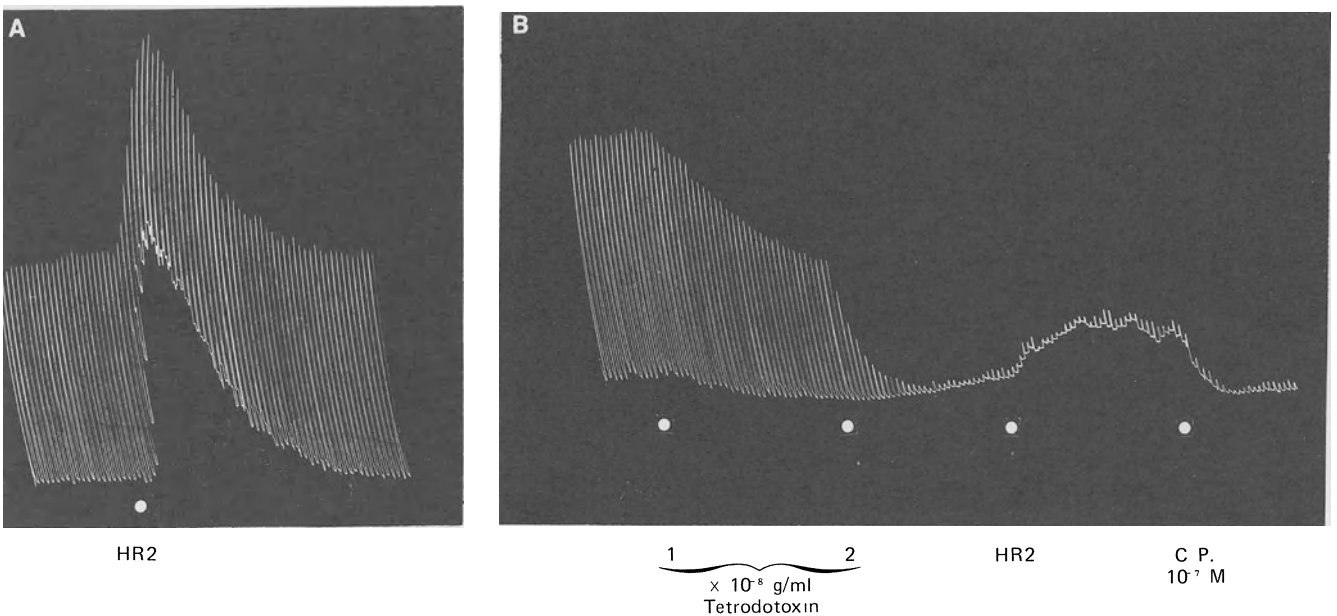

Fig. 3. Effects of HR2 of 10^{-6} g/ml on the contraction induced by transmural electrical stimulation (1 msec, 25 V, 0.1 cps) in the isolated guinea pig ileum. (A) HR2 alone; (B) the contraction induced by HR2 after treatment with tetrodotoxin (3×10^{-8} g/ml) is inhibited by chlorpheniramine (C.P.).

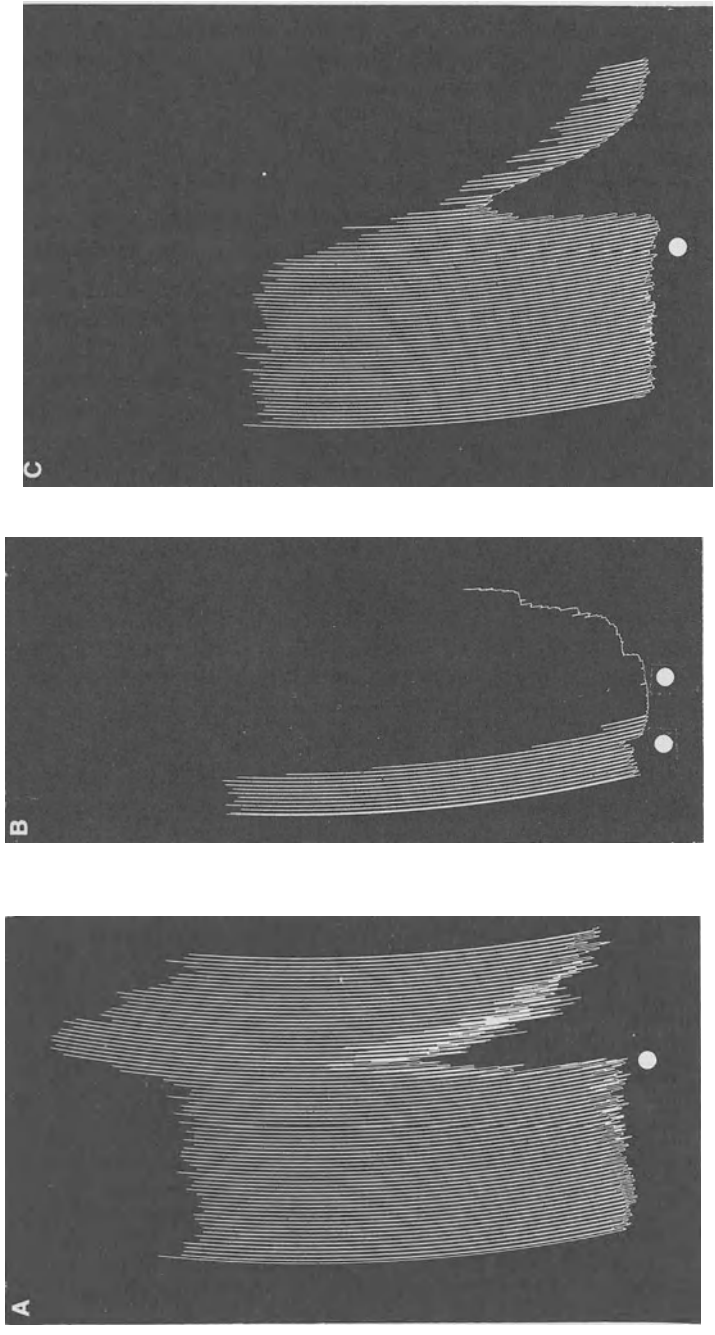

Fig. 4. Effects of HR1 and HR2 in the presence of chlorpheniramine at 10^{-7} M on the contraction induced by transmural electrical stimulation (1 msec, 25 V, 0.1 cps) in the isolated guinea pig ileum. (A) 10^{-6} g/ml HR2; (B) 10^{-6} g/ml HR2 after tetrodotoxin (TT: 3×10^{-8} g/ml); (C) 10^{-6} g/ml HR1.

Table 1. Release of Histamine from the Rat Peritoneal Cells Induced by Crude Venom, HR1, and HR2

Venom proteins, g/ml	Released histamine,[a] %	Control,[a] %
Crude venom of 10^{-4}	59.90 ± 13.43 (4)	3.26 ± 0.22 (4)
HR2 of 3×10^{-5}	52.70 ± 8.25 (5)	7.58 ± 1.26 (4)
HR1 of 3×10^{-5}	12.42 ± 3.18 (5)	5.06 ± 1.08 (5)

[a]Mean ± S.E. Number of experiments in parentheses.

follows: 2.0-2.8 μg by crude venom (10^{-5} g/ml); 1.1-3.0 μg by HR2 (10^{-5} g/ml); 0.32 μg by HR1 (10^{-5} g/ml); and 0.75 μg by H_2-proteinase (10^{-5} g/ml). The control medium gave a value smaller than 0.56 μg. The total histamine content of the lungs, as determined after extraction by heating the lungs at 100°C for 15 min, was 9.2-19.0 μg. These results demonstrated that the histamine-releasing activities of crude venom and HR2 are much more potent than those of HR1 and H_2-proteinase.

The media in which the lungs were incubated with venom proteins contained, besides histamine, ACh and SRS in small amounts, as demonstrated by the fact that the contractile response of the ileum to the medium in which the organ had been incubated with crude venom was not completely inhibited by either chlorpheniramine alone or chlorpheniramine in combination with atropine.

The Rat Peritoneal Cell Suspension. The amounts of histamine released from the rat peritoneal cells by the action of venom proteins were determined on the guinea pig ileum with the results shown in Table 1. The histamine-releasing activity of HR2 or crude venom is much more potent than that of HR1; the latter, though very weak, is still significant.

The midium in which the peritoneal cells had been incubated with crude venom or HR2 at a concentration of 10^{-4} g/ml induced contraction of the isolated rat uterus; while the control medium, as well as the medium in which the cells had been incubated with HR1, did not cause any contraction. The contraction of the rat uterus was inhibited by a specific 5-HT antagonist (S-8) indicating the involvement of 5-HT released from the cells. The amount of 5-HT released into the incubation medium was 0.5 μg with crude venom and 0.13 μg with HR2.

DISCUSSION

Our previous report demonstrated that the early effects of the venom of *T. flavoviridis* or venom hemorrhagic principles, HR1 and HR2, on the microcirculatory system are characterized by severe vasoconstriction, followed by

vasodilatation of larger vessels especially of arterioles, and subsequent hemorrhage in the capillary bed (Ohsaka *et al.*, 1971b).

The observation of the striking vasomotor changes caused by the venom principles, together with the availability of highly purified preparations of HR1 and HR2 (Omori-Satoh and Ohsaka, 1970; Takahashi and Ohsaka, 1970a), prompted us to investigate the pharmacological actions of these purified principles on smooth muscles.

Of the two hemorrhagic principles, only HR2 produced contraction of the isolated guinea pig ileum at concentrations greater than 10^{-7} g/ml. The contraction of the gut induced by HR2 was inhibited to a great extent by atropine or tetrodotoxin and enhanced by eserine, demonstrating the involvement of nervous functions of the gut, e.g., ACh release. The release of histamine from the organ was also involved in this contraction, as evidenced by the fact that the gradual contraction of the gut occurring after pretreatment with tetrodotoxin was completely inhibited by an antihistamine drug, chlorpheniramine. From these results it is apparent that the stimulating effects of HR2 on the smooth muscle are the results of the autopharmacological actions (Beraldo and Dias da Silva, 1966; Jiménez-Porras, 1968) of this hemorrhagic principle. Incidentally, HR2 per se did not exert any effect on the isolated rat uterus, the rabbit aortic strip, or preparations of other smooth muscles.

HR1 and H_2-proteinase, the latter being the major proteinase in this venom, exerted no stimulating effect on the guinea pig ileum or preparations of other smooth muscles. Crude venom at a concentration of 10^{-5} g/ml produced contraction of the gut, which showed tachyphylaxis on repetition.

The crude venom and HR2 induced the release of mediators such as histamine, 5-HT and slow-reacting substances from the chopped guinea pig lung and rat peritoneal cells; while HR1 released these mediators to a less extent. These mediators, if released *in vivo*, may well open the endothelial cell junctions causing the erythrocytes to come out across the endothelium (Ohsaka, 1976).

The apparent contradiction that HR1, possessing hemorrhagic activity higher than that of HR2, exerted a weaker effect on the smooth muscle and induced the release of the mediators to a less extent than did HR2 may well be explained by our hypothesis (Ohsaka, 1976) that these effects, together with other effects of venom principles such as disruption of the basement membrane and inhibition of platelet aggregation, must be involved in the causation of hemorrhage. The mechanism of hemorrhage as elucidated by the use of venom hemorrhagic principles has recently been reviewed (Ohsaka, 1976).

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Studies on the Mechanism of Action of Tityustoxin

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INTRODUCTION

The contraction of the isolated rat ileum produced by purified scorpion toxin (tityustoxin or TsTX) was explained by the release of acetylcholine and probably substance P, whereas the relaxation of the atropine-treated rat duodenum depended on an adrenergic mechanism (Cunha-Melo *et al.*, 1973). As our understanding of the toxin actions remains incomplete, it was decided to reinvestigate the subject. Preliminary reports have been presented elsewhere (Calixto *et al.*, 1971; Calixto and Freire-Maia, 1972; Freire-Maia *et al.*, 1973; Freire-Maia *et al.*, 1974; Futuro-Neto *et al.*, 1974).

MATERIALS AND METHODS

Isolated Rat and Guinea Pig Intestines. Pieces of the ileum or duodenum were suspended in 10-ml aerated Jalon's or Tyrode's solution at 35°C. The contractions or relaxations were recorded on smoked paper on a kymograph by means of a frontal writing lever. The agonistic substances were added to the organ bath in a submaximal dosage, allowed to contact the muscle for 30–60 sec and then washed out. Antagonists and toxin were allowed to act for periods of up to 5 min.

Isolated Rat and Cat Spleen Strips. The method used to isolate rat and cat spleen strips was that described by Bickerton (1963). The strips were immersed in 10-ml Tyrode's solution, at 38°C. The fluid was aerated with a mixture of 95% O_2 and 5% CO_2. The contractions of the strips were registered by means of a photoelectric transducer coupled to a recorder (model PRR, range 0-1 mA, Texas Instruments, Inc., Houston, U.S.A.). With this method, the sensitivity of the recording was increased 5 times (A. D. Azevedo, personal communication).

Incubation Experiments. Slices of rat and guinea pig ilea were incubated for 30 min with tityustoxin and the amount of free acetylcholine present in the incubating fluid was biologically determined. The methods used in these experiments were similar to those described by Gomez *et al.* (1973) using rat brain slices.

Solutions Used. The Jalon's solution had the following composition: 154 mM NaCl; 5.6 mM KCl; 0.4 mM $CaCl_2$; 6.0 mM $NaHCO_3$; 2.8 mM glucose; and sufficient deionized water to make one liter (Burn *et al.*, 1950). Tyrode's solution: 136.8 mM NaCl; 2.7 mM KCl; 1.4 mM $CaCl_2$; 12.0 mM $NaHCO_3$; 5.5 mM glucose; 1.0 mM $MgCl_2$; 0.4 mM NaH_2PO_4.

Drugs Used. Bradykinin (Sandoz, BRS-640, Batch no. 69055), acetylcholine chloride (Roche), adrenaline hydrochloride (Parke-Davis), noradrenaline bitartrate (Sigma), atropine sulphate (Sigma), pentolinium tartrate (Wieth), phentolamine methylate (Ciba), phenoxybenzamine (Smith, Kline, and French Laboratory), nicotine (Sigma), pitressin (Parke-Davis), methysergide maleate (Sandoz), guanethidine (Ciba), reserpine (Ciba), tyramine (Sigma), propranolol (Ayerst), adenosine triphosphate (ATP, Sigma), and substance P (kindly provided by Professor F. Lembeck, Institute of Pharmacology, University of Graz, Universitats platz 4, A-8010 Graz, Austria).

Tetrodotoxin and Tityustoxin. Tetrodotoxin A grade (TTX) was obtained from Calbiochem, La Jolla, California, U.S.A. Tityustoxin (TsTX) used in this paper was obtained from *Tityus serrulatus* venom (kindly provided by Dr. Jandyra Planet do Amaral, Director of Instituto Butantan, São Paulo, Brazil) by a combination of extraction and chromatographic techniques (Gomez and Diniz, 1966). The toxin concentration was calculated by using a Beckman spectrophotometer fitted with silica cells of 1.0-cm pathway. An extinction coefficient of 1.0 O.D. unit mg · ml at 280 nm was assumed. In this paper, the concentration of protein is expressed in μg.

Statistical analysis of the data was performed by using the Student's t test, with $p < 0.05$ indicating significance.

RESULTS

Reversal of Tachyphylaxis Produced by Tityustoxin in the Rat Ileum. When tityustoxin (1-5 μg/ml) was added to the bath containing a piece of rat ileum

immersed in Jalon's solution, the muscle contracted 3–5 cm (Cunha-Melo *et al.*, 1973). However, subsequent doses of toxin produced responses of a smaller magnitude or no response at all (tachyphylaxis). The ability of TsTX to produce contraction was restored by changing Jalon's to Tyrode's solution. Under these circumstances, additions of at least three doses of TsTX at 1-hr intervals did not evoke tachyphylaxis (7 experiments). Figure 1A and B show one typical experiment. Therefore, the effect of TsTX depends, at least in part, on the composition of the perfusion fluid. For this reason, experiments were performed to study the possible effects of calcium on the contraction produced by TsTX, since the concentration of this cation is much lower in Jalon's than in Tyrode's solution. By increasing the $CaCl_2$ concentration of Jalon's solution from 0.4 to 1.4 mM, the tachyphylaxis phenomenon was prevented (Fig. 1C).

Effect of TsTX on the Guinea Pig Ileum. The addition of TsTX (5 μg/ml) to an isolated bath containing a piece of guinea pig ileum immersed in Jalon's solution produced contraction of 2–3 cm, confirming the previous results of Diniz and Gonçalves (1956) using Tyrode's solution. The tachyphylaxis phenomenon was observed only after 3 or 4 doses, given at 20-min intervals (5 experi-

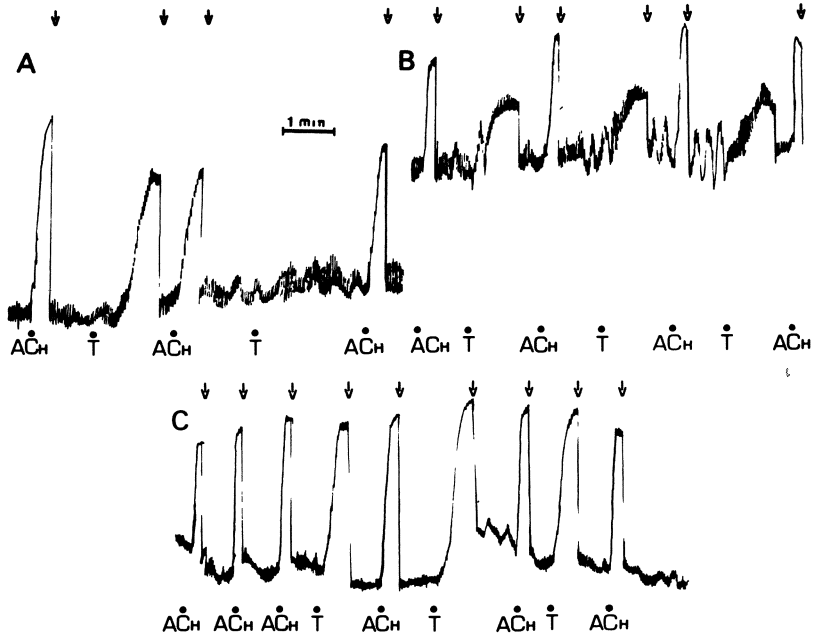

Fig. 1. Rat ileum preparations suspended in a 10-ml aerated perfusion bath at 35°C. At A, Jalon's solution was used. Between A and B, Jalon's was changed to Tyrode's solution. At lower tracing (C), Jalon's solution with a higher calcium concentration (1.4 mM) was used in another ileum preparation. Acetylcholine (ACh) 1.6×10^{-7} M; tityustoxin (T) 2.5 μg/ml.

ments). However, in the experiments with Tyrode's solution, tachyphylaxis was not recorded, even after 10 doses of toxin, given at 20-min intervals (5 experiments).

Comparison between the Effects of Tityustoxin on the Rat and Guinea Pig Ilea. A comparative study on the effects of TsTX (3 μg/ml) and acetylcholine (2.5×10^{-8} M) on the isolated rat and guinea pig ilea was conducted. As shown in Fig. 2, the rat ileum is 7.8 times less sensitive to ACh than the guinea pig ileum; however, the rat ileum is 2.1 times more sensitive to TsTX than the guinea pig ileum.

Blockade by Tetrodotoxin (TTX) of TsTX Effects on the Isolated Ileum. Tityustoxin (5 μg/ml) was without an effect when added to the organ bath with a piece of rat ileum immersed in Tyrode's solution containing TTX (2×10^{-7} M). However, after the Tyrode's solution (plus TTX) was washed out and replaced by a solution without TTX, a strong contraction of the preparation was recorded. Addition of TTX to the bath brought the muscle back to its original tone. The contraction recorded after the second washing was abolished, in part, by atropine (Fig. 3). Experiments were then conducted in order to study

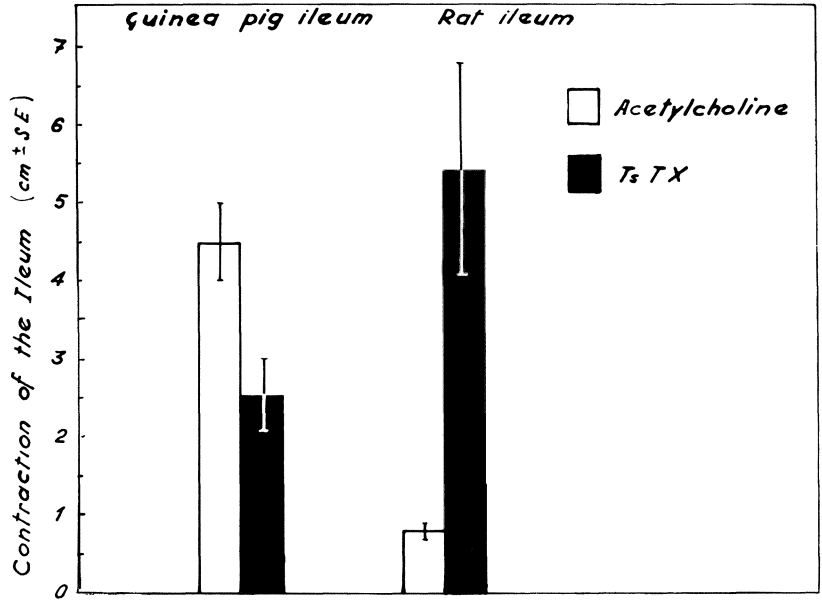

Fig. 2. Contractions induced by acetylcholine (2.5×10^{-8} M) and tityustoxin (TsTX, 3 μg/ml) on the guinea pig and rat ilea immersed in 10-ml, aerated Tyrode's solution at 35°C (N = 7).

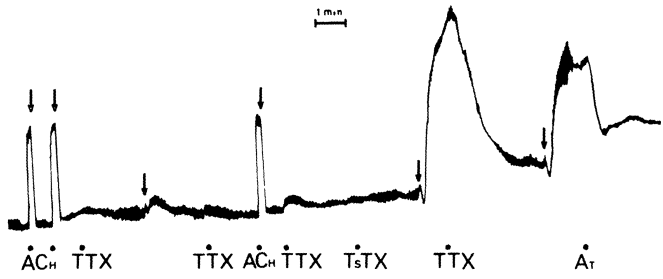

Fig. 3. Rat ileum preparation suspended in 10-ml aerated Tyrode's solution at 35° C. Acetylcholine (ACh), 1×10^{-7} M; tetrodotoxin (TTX), 2×10^{-7} M; tityustoxin (TsTX), 5 μg/ml; atropine (At), 1×10^{-7} M. At arrows, the preparation was rinsed once.

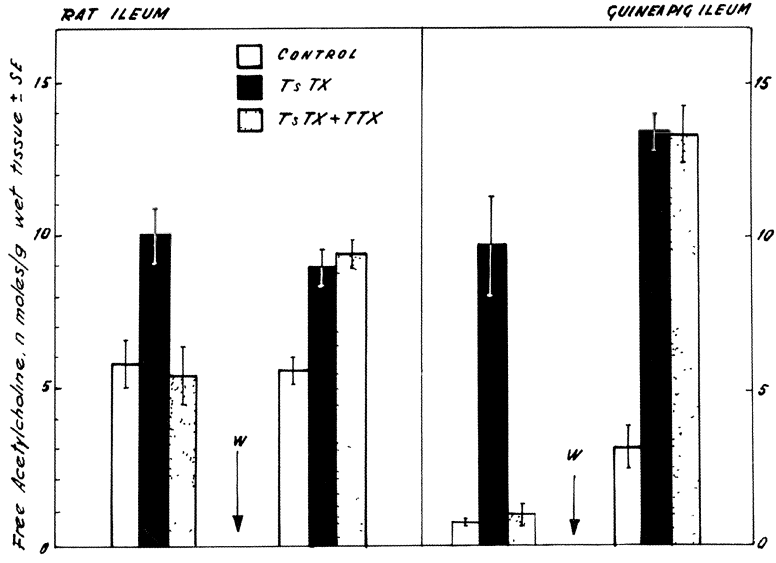

Fig. 4. Effect of tetrodotoxin (TTX, 5×10^{-7} M) on the release of acetylcholine from rat and guinea pig ilea induced by tityustoxin (TsTX, 2×10^{-6} M). The mean values ± S.E. for six experiments are presented. The incubation was carried out for 30 min at 37° C in a medium containing (mM); Na Cl 136; KCl, 2.7; $CaCl_2$, 1.35; NaH_2PO_4, 0.36; $NaHCO_3$, 12; Glucose, 5.5; eserine, 0.01; brought to pH 7.4 with 1 N HCl. The arrows indicate that the slices were washed three times with the incubation fluid and reincubated for 30 min, without further addition of TsTX or TTX.

the effect of TTX on the release of ACh from slices of rat and guinea pig ilea incubated with TsTX. The amount of acetylcholine spontaneously released or released by the action of TsTX (with or without addition of TTX) was bioassayed according to the method described by Diniz and Torres (1968) and Gomez *et al.* (1973). These experiments showed that TTX prevented the release of ACh produced by TsTX; however, after washing the slices with the incubation fluid and reincubation (without further addition of TsTX or TTX) a large increase in the amount of released ACh was measured (Fig. 4). Addition of TTX (5×10^{-7} M) to a medium containing slices of rat or guinea pig ilea did not change the amount of ACh spontaneously released after 30 min of incubation.

Effect of TsTX on the Atropine-Treated Rat Duodenum. The relaxation produced by tityustoxin on the atropine-treated rat duodenum is adrenergic in nature (Cunha-Melo *et al.*, 1973). If the experiments are made with Jalon's solution, only the first dose evokes relaxation; the other doses, even when added at 1-hr intervals, do not relax the preparation. By using Tyrode's solution, the addition of TsTX (5 μg/ml) produced a relaxation equivalent to that produced by bradykinin (9.4×10^{-9} M) or adrenaline (2.3×10^{-7} M). If successive doses of toxin were added with 20-min intervals, the tachyphylaxis phenomenon was recorded (7 experiments, Fig. 5). Therefore, in order to study the mechanism of

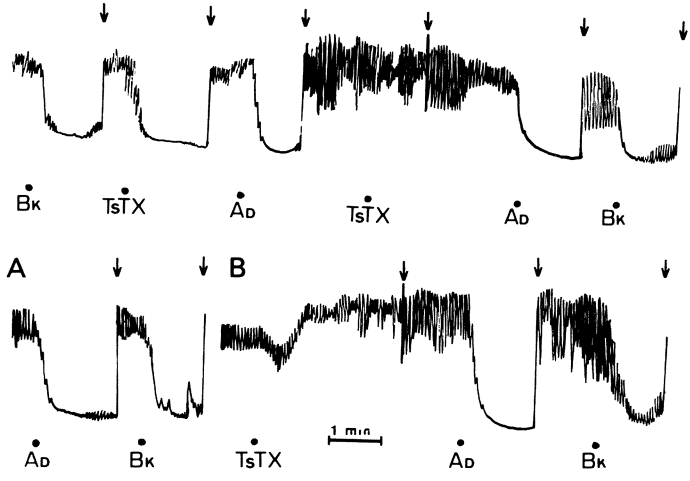

Fig. 5. Rat duodenum preparations suspended in 10 ml of Tyrode's solution. Atropine (1.5×10^{-7} M) was maintained in the bath throughout the experiments. Upper and lower tracings: bradykinin (Bk), 9.4×10^{-9} M; tityustoxin (TsTX), 5 μg/ml; adrenaline (Ad) 2.3×10^{-7} M. After each addition to the bath, the preparation was rinsed once or twice (small arrows). Upper tracing: the second dose of TsTX was added to the bath 20 min after the first. Lower tracings: between A and B guanethidine (2×10^{-4} M) was maintained in the bath for 20 min and then washed out.

action of TsTX on the duodenum only one dose of TsTX was added in each experiment.

Mode of Action of TsTX on the Rat Duodenum (Tyrode's Solution)

Phentolamine and Propranolol. Blockade of alpha and beta adrenergic receptors with phentolamine and propranolol prevented the relaxation produced by TsTX (Table 1).

Guanethidine. In pieces of rat duodenum previously treated with guanethidine, TsTX produced a small relaxation (Table 1) followed by a contraction (16.5 ± 4.2 mm). Figure 5 shows one of these experiments. Under these circumstances, both ATP (2×10^{-4} M) and substance P (1–2 U/ml) produced similar effects.

Reserpine. Five rats were intraperitoneally injected with 5 mg/kg of reserpine. The animals were sacrificed 24 hr later, and the ilea were taken out for the experiments. Tyramine did not produce relaxation of the ilea, whereas in control experiments it relaxed the preparation. In the reserpine-treated animals, TsTX produced a small relaxation (Table 1) followed by a contraction (15.2 ± 4.8 mm). A second dose of TsTX, added 1–3 hr after the first one, did not produce relaxation of the duodenum, but a contraction was recorded (22.0 ± 8.0 mm).

Pentolinium. The ganglionar blocking agent, pentolinium, prevented the

Table 1. Effects of Drugs on the Relaxation Produced by Tityustoxin on the Rat Duodenum Immersed in Tyrode's Solution (with Atropine)[a]

Number of experiments	Treatment	Relaxation produced, mm			
		Tityustoxin, 5 μg/ml	Adrenaline, 2.3×10^{-7} M	Tyramine, 2.2×10^{-3} M	Bradykinin, 9.4×10^{-9} M
7	None	26.4 ± 3.9	24.8 ± 4.7	22.0 ± 1.0	27.1 ± 4.6
5	Phentolamine, 2.6×10^{-5} M and propranolol, 1.7×10^{-5} M	1.6 ± 1.1	0.0	–	16.4 ± 2.0[e]
9	Guanethidine,[b] 2×10^{-4} M	12.5 ± 2.0[d]	29.5 ± 2.8[e]	–	27.0 ± 3.7[e]
5	Reserpine,[c] 5 mg/kg	10.4 ± 1.7[d]	–	3.4 ± 1.0[d]	21.0 ± 2.5[e]
5	Pentolinium, 1.8×10^{-5} M	17.6 ± 1.5[e]	27.0 ± 4.5[e]	–	–

[a] Values are mean ± S.E.
[b] 20 min prior to addition of TsTX.
[c] Intraperitoneal administration, 24 hr before experiment.
[d] Significantly different from control ($p < 0.05$).
[e] Not significantly different from control ($p < 0.05$).

relaxation produced by nicotine (6.2×10^{-6}–3.0×10^{-5} M), but not the effect of TsTX or adrenaline (Table 1).

Tetrodotoxin. The duodenum relaxations produced by tityustoxin (5 μg/ml) and nicotine (6.2×10^{-6}–3.0×10^{-5} M) were prevented by tetrodotoxin (2×10^{-7} M). However, TTX had no effect on the relaxation produced by noradrenaline (5×10^{-7} M).

Effects of Tityustoxin on the Isolated Spleen Strips. Additions of TsTX, adrenaline, and noradrenaline to the isolated bath containing rat spleen strips produced contractions of the preparations. The contraction induced by TsTX is of a slow type, starting after a latent interval ranging from 30 to 60 sec, thereby differing from that produced by catecholamines. Following the responses to TsTX and to catecholamines, the tone of the preparation remained in a high position. But, after washing, the sustained contraction was abolished (Fig. 6A). Successive additions of TsTX at 1-hr intervals produced smaller contractions. Simultaneously, the adrenaline effect was unchanged or potentiated (Fig. 6B). Phenoxybenzamine prevented the contraction produced by TsTX and noradrenaline, but not the effect of pitressin. Reserpine (3 mg/kg, ip, 24 hr before the experiment) prevented the contraction evoked by TsTX and tyramine but not the effect of noradrenaline. Methysergide did not prevent the contractions produced by TsTX and noradrenaline. On the other hand, previous additions of TTX to the bath did not change the effect of noradrenaline, but prevented the TsTX contraction. Table 2 summarizes these results. Moreover, TTX added to the bath during the contraction produced by TsTX brought the preparation close to the

Table 2. Effects of Drugs on the Contraction Produced by Tityustoxin on the Isolated Rat Spleen Strips Immersed in Tyrode's Solution[a]

Number of experiments	Treatment	Contractions produced, mm			
		Tityustoxin, 13.0 μg/ml	Noradrenaline, 1.5×10^{-6} M	Tyramine, 8.1×10^{-5} M	Pitressin, 0.01 u/ml
6	None	33.2 ± 5.6	30.0 ± 0.7	22.3 ± 2.5	19.0 ± 0.9
5	Phenoxybenzamine, 6.9×10^{-6} M	2.2 ± 1.0[c]	0.0	–	19.2 ± 1.5[d]
5	Reserpine,[b] 3 mg/ml	7.0 ± 4.0[c]	29.0 ± 2.0[d]	3.0 ± 2.0[c]	–
5	Methysergide, 1.1×10^{-6} M	32.6 ± 3.9[d]	32.6 ± 4.5[d]	–	–
5	Tetrodotoxin, 2×10^{-7} M	0.0	30.8 ± 3.6[d]	–	–

[a] Values are mean ± S.E.
[b] Intraperitoneal administration, 24 hr before experiment.
[c] Significantly different from control ($p < 0.05$).
[d] Not significantly different from control ($p > 0.05$).

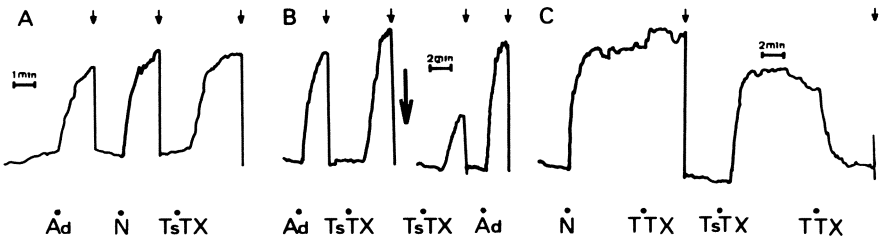

Fig. 6. Rat spleen strip preparations immersed in Tyrode's solution containing 0.11 mM ascorbic acid and 0.04 mM sodium edetate at 38°C. Adrenaline (Ad), 1.4×10^{-6} M; noradrenaline (N), 1.5×10^{-6} M; tityustoxin (TsTX), 13.0 μg/ml; tetrodotoxin (TTX), 2×10^{-7} M. Experiments were conducted with three spleen strips (A, B, and C). At B, five doses of TsTX were added to the bath, with 1-hr intervals. The larger arrow separates the first from the fifth dose. At small arrows, the preparations were rinsed once or twice.

control level, whereas it had no effect on the contraction produced by noradrenaline (Fig. 6-C). TsTX (1.0–3.0 μg/ml) also contracted the isolated cat spleen strip.

DISCUSSION

The tachyphylaxis observed after addition of tityustoxin (TsTX) to a bath containing rat ileum could be due to an inability of the ileum to release active substance(s), as was suggested by Cunha-Melo *et al.* (1973). In the present experiments, it was shown that changing Jalon's for Tyrode's solution, the rat ileum recovered its ability to contract after addition of TsTX. The tachyphylaxis phenomenon was also abolished by increasing the calcium concentration of Jalon's solution. Other investigators (Gomes *et al.*, 1973; Henriques *et al.*, 1973) have shown that the calcium ions play an important role in the release of acetylcholine (ACh) induced by tityustoxin from ileum or brain slices. It seems likely, therefore, that the tachyphylaxis phenomenon, as described in this paper, could be due to the inability of the rat ileum to release chemical mediators, when the perfusion fluid has a low calcium concentration.

As atropine prevented the contractions produced by TsTX in the guinea pig ileum (Diniz and Gonçalves, 1956) but not in the rat ileum (Cunha-Melo *et al.*, 1973), a comparative study of the effects of TsTX on these preparations was conducted. The data have shown that the rat ileum is much more sensitive to TsTX than is the guinea pig ileum. This difference could be due to an increased sensitivity of the rat ileum to acetylcholine: however, the results showed the contrary, that is, the rat ileum is less sensitive to ACh than is the guinea pig ileum. Moreover, TsTX releases less ACh from the rat ileum than from the guinea pig ileum. These data reinforce our previous hypothesis (Cunha-Melo *et*

al., 1973) that the contraction produced by TsTX in the rat ileum could be due to release of more than one mediator, one of which is acetylcholine.

Previous experiments have shown that scorpion venom produces depolarization of the axonal membrane (Adam *et al.*, 1966; Koppenhofer and Schmidt, 1968; Narahashi *et al.*, 1972), whereas tetrodotoxin (TTX) blocks the sodium channel of the nerve membrane (Narahashi *et al.*, 1964; see review in Narahashi, 1972). In the present paper, it was demonstrated that TTX prevented the release of ACh and the contraction of the rat ileum produced by TsTX. Similar results were obtained by other investigators in the guinea pig ileum (Henriques *et al.*, 1971, 1973). TTX also blocked the release of ACh produced by incubation of TsTX with rat brain slices (Gomez *et al.*, 1973). In the doses used by our group, TTX does not block the smooth muscle (Ogura *et al.*, 1966). It seems likely, therefore, that absence of a contraction of the rat ileum after addition of TsTX to a bath containing TTX could be due to a blockade of the depolarization of the nerve membrane in the intestine. Our findings also seem to indicate that TTX links weakly to its receptors in the nerve membrane and can easily be removed by washing, whereas the binding of TsTX is stronger and resists washing. Therefore, TTX prevents the effects of TsTX, but does not prevent its binding to the nerve membrane. Based on these results, we postulate that TsTX acts in at least two points of the nerve membrane, one of which is presumably the sodium channel.

If the contraction produced by TsTX in the atropine-treated rat ileum is due to the release of a second mediator, then its release is also prevented by TTX. Indirect evidences presented in this paper and by other investigators (Hial and Diniz, 1971, Cunha-Melo *et al.*, 1973; Tafuri *et al.*, 1974b) seem to indicate that substance P could be this mediator.

Our results have shown that the relaxation of the atropine-treated rat duodenum produced by TsTX is due, at least in part, to the release of catecholamines from postganglionic nerve endings. In the reserpine- or guanethidine-treated animals, the TsTX produced a small relaxation followed by a contraction. It seems likely that acetylcholine and catecholamines are not involved in these effects. The release of substance P and/or ATP by TsTX could explain the production of the biphasic effect on the duodenum.

Based on our data we think that TsTX releases acetylcholine, catecholamines, and probably substance P from the rat intestine. But, where did these mediators come from? Experiments of our group (Tafuri *et al.*, 1971, 1974a) have shown that TsTX produces a decrease in the number of the granular vesicles and an increase in the agranular ones, in the myenteric plexus of the rat. The nature of the chemical mediators present in the granular vesicles of Auerbach's plexus is not well known (Baumgarten *et al.*, 1970; Brunstock, 1972). We postulate that these vesicles could store catecholamines and probably substance P, and that TsTX, by producing depolarization of the nerve membrane, releases these mediators from the granular depots.

As the spleen has a totally sympathetic inervation (Fillenz, 1970), we studied the effect of TsTX on the isolated rat spleen strips. As the contraction produced by TsTX is prevented by tetrodotoxin, phenoxybenzamine, and reserpine, and is not abolished by methisergide, we assume that the effect of TsTX is due to the release of catecholamines from the sympathetic nerve endings. The sustained contraction evoked by TsTX seems to be due to a continuous release of catecholamines, since the effect is abolished by tetrodotoxin.

SUMMARY

The effects of purified scorpion toxin (tityustoxin or TsTX) were examined on the isolated rat and guinea pig ilea, on the isolated spleen strips and on the release of acetylcholine (ACh) from slices of ileum. Tachyphylaxis recorded after addition of TsTX to a Jalon's bath containing a piece of rat ileum was abolished by using Tyrode's solution or by increasing the calcium concentration of Jalon's solution.

The rat ileum is more sensitive to TsTX and less sensitive to ACh than is the guinea pig ileum. Incubation experiments have shown that TsTX releases less ACh from the rat ileum than from the guinea pig ileum. Moreover, TsTX still contracted the atropine-treated rat ileum. The contraction of the rat ileum seems, therefore, to be due to the release of more than one mediator. It is assumed that substance P could be one of these mediators.

The relaxation produced by TsTX on the atropine-treated rat duodenum is due, at least in part, to the release of catecholamines. The TsTX contraction of the rat spleen strip seems to be due to the release of catecholamines from the sympathetic nerve endings.

Tetrodotoxin (TTX) prevents the TsTX contraction of the rat ileum. However, after washing the ileum with the perfusion fluid, a strong contraction was recorded. The release of ACh produced by incubation of TsTX with slices of rat ileum was also prevented by TTX. However, after washing the slices with the incubation fluid and reincubation, the ACh release was increased.

The results seem to indicate that TTX prevents the effects of TsTX, but does not prevent its binding to the nerve membrane. It is postulated that TsTX acts in at least two points of the nerve membrane, one of which is presumably the sodium channel.

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Pharmacological Blockade of the Cardiovascular and Respiratory Effects Produced by Tityustoxin in the Rat

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INTRODUCTION

The intravenous injection of the purified toxin (tityustoxin or TsTX) obtained from the venom of the Brazilian scorpion *Tityus serrulatus* in rats produces complex effects on the cardiovascular and respiratory systems (Freire-Maia and Diniz, 1970; Freire-Maia *et al.*, 1970; Freire-Maia *et al.*, 1973; Freire-Maia *et al.*, 1974b). For these reasons experiments were conducted in order to block, with drugs, the TsTX effects. Preliminary data were published elsewhere (Azevedo and Freire-Maia, 1974; Lima and Freire-Maia, 1974).

MATERIALS AND METHODS

Albino rats of either sex, weighing 150–250 g each, were used. The animals were anesthetized with urethane (140 mg/100 g, i.p.) and a cannula was inserted in the trachea. All solutions were injected through a cannula in the femoral vein, with the exceptions of the experiments in which cannulas were implanted into the cerebral ventricles. Heparin (100 I.U./100 g body weight) was injected before blood pressure recordings were obtained. The experiments were conducted at room temperature (15–30°C).

Electrocardiogram. The electrical activity of the heart was recorded by a Cardiofax electrocardiograph (Nihon Kohden, Tokyo, Japan). A lead corre-

sponding to Einthoven D_{II} was used throughout the experiments. In the tracings, a height of 2 cm corresponds to 1 mV; paper speed was 25 mm/sec.

Blood Pressure. Femoral arterial pressure was recorded with a pressure transducer and an E & M Physiograph.

Respiration. The respiratory movements were recorded with an impedance pneumograph and an E & M Physiograph.

Artificial Ventilation. Intermittent positive pressure respiration was delivered with a Palmer miniature Ideal pump.

Intraventricular Injections. For injections into the cerebral ventricles, a stainless steel cannula was implanted in the left lateral ventricle under pentobarbital sodium (40 mg/kg, i.p.) anesthesia, according to the method described by Feldberg and Lotti (1967) and Correa and Graeff (1974). Intracerebroventricular (ICV) injections were made by means of a Hamilton microlite syringe (no. 705), connected to the cannula by means of polythene tubing. The injection volumes were 10 or 20 μl. Experiments were made 3–5 days after the implantations of the cannulas.

Drugs used. Doses of atropine sulfate (Sigma Chemical Company, St. Louis, Missouri) and phenoxybenzamine (Dibenzyline, Smith Kline and French Laboratories, Philadelphia, Pennsylvania) are expressed as weights of the salts. Urethane (Sigma), heparin (Evans, 1 ml = 5000 I.U.), lidocaine (Xylocaine, Astra) and anti-scorpionic serum (Instituto Butantan, São Paulo, Brazil), were used.

Venom Preparation. Tityus serrulatus venom was obtained from the Instituto Butantan and the purified toxin (tityustoxin or TsTX) was prepared by a combination of water extractions and column chromatography on Sephadex G-25 (Gomez and Diniz, 1966). The toxin concentration was calculated with a Beckman spectrophotometer fitted with silica cells of 1.0-cm pathway. An extinction coefficient of 1.0 O.D. unit mg · ml at 280 nm was assumed. In this paper the concentration of protein is expressed in μg.

Analysis. Statistical analysis of the data was performed by using the Student's t test, with $p < 0.05$ indicating significance.

RESULTS

Cardiovascular and Respiratory Effects of TsTX. Simultaneous recordings of the electrocardiogram, arterial pressure, and respiratory movements have shown that 50 μg/100 g of TsTX injected intravenously produces S-A block, A-V block, sinus bradycardia, hypotensive and hypertensive effects, and respiratory arrhythmia (tachypnea, hyperpnea, ataxic and gasping breathing, periodic respiration). Figure 1 A and B shows one of these experiments.

Effect of Anti-Scorpionic Serum (A.S.S.). Experiments were conducted in order to prevent or abolish the cardiovascular and respiratory effects of TsTX by the intravenous injections of A.S.S. (0.5 ml/100 g) given in two doses at 30-min

intervals. The injection of A.S.S. 15 min after TsTX, in 5 rats, did not abolish the bradycardiac effect produced by the toxin, since the mean heart rate (± S.E.) was 368 ± 24 before and 127 ± 13 1 hr after the injection of the toxin ($p < 0.05$). In another group of five rats in which the A.S.S. was injected before the toxin, the heart rates were the following: 336 ± 34 before the serum, 327 ± 28 after the serum, and 317 ± 25 1 hr after the injection of TsTX. These differences are not statistically significant ($p > 0.05$).

In five rats in which the mean arterial pressure (MAP) was 87 ± 9-mmHg TsTX produced a pressor effect of 37 ± 5 mmHg; on the other hand, in five other rats treated with A.S.S., in which the MAP was 76 ± 6 mmHg, the pressor effect was reduced to 15 ± 4 mmHg ($p < 0.05$).

The injection of A.S.S. before TsTX prevented respiratory arrhythmia produced by TsTX, whereas after toxin it had no effect, during the time of the experiment (1 hr). Figures 1 and 2 show two typical experiments.

When the serum was injected after TsTX, it did not abolish the salivation produced by toxin, during the period of 1 hr. Therefore, it seemed worthwhile

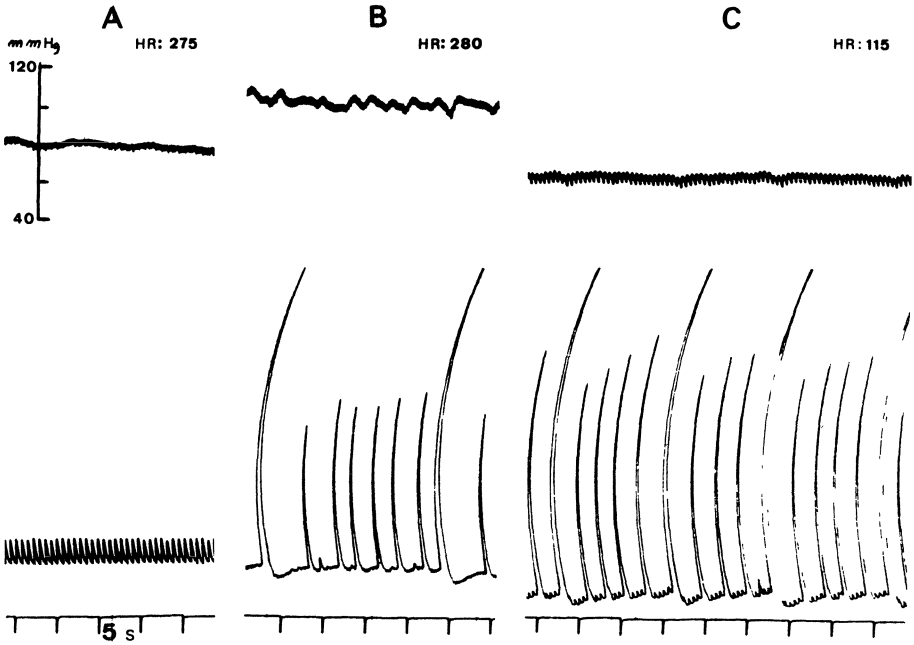

Fig. 1. Mean arterial blood pressure and respiratory movements of a rat. Inspirations indicated by upward deflections. Numbers on the top indicate heart rate (HR). Urethane anesthesia was used. Before (A) and 15 min after i.v. injection of 50-μg tityustoxin per 100 g (B). Between (B) and (C), 0.5-ml anti-scorpionic serum per 100 g was slowly intravenously injected. Record C, 30 min after serum injection.

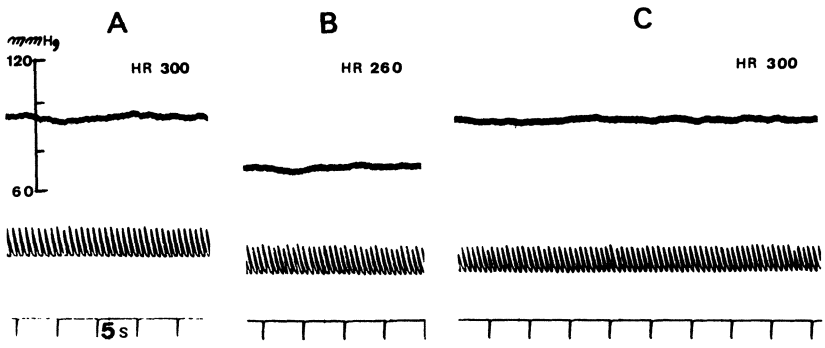

Fig. 2. Mean arterial pressure and respiratory movements of a rat. Inspirations indicated by upward deflections. Numbers on the top indicate mean heart rate (HR). Urethane anesthesia was used. Before (A) and 45 min after (B) i.v. injection of anti-scorpionic serum (0.5-ml/100 g). Between B and C, tityustoxin (50 μg/100 g) was intravenously injected. Record C, 15 min after TsTX.

to find substances which, when injected after the tityustoxin, would block the effects of the toxin on the cardiovascular and respiratory systems.

Effect of Atropine on Cardiac and Respiratory Arrhythmias. The idioventricular rhythm produced by 100 μg/100 g of TsTX in rats under artificial ventilation was abolished by i.v. injection of atropine (50 μg/100 g). The increase in the heart rate after atropine was of long duration, as shown in Table 1.

As atropine also prevented the bradycardia and hypotensive effects which appeared immediately after TsTX injection (Freire-Maia *et al.*, 1974b), we decided to study the effect of atropine on the respiratory arrhythmias (tachypnea, hyperpnea, gasping and ataxic breathing, periodic respiration, and apnea) produced by tityustoxin (80 μg/100 g). As shown in Fig. 3, atropine did not prevent respiratory arrhythmia produced by toxin (10 experiments).

Table 1. Effect of Atropine on the Cardiac Rhythm of Rats Receiving Artificial Ventilation and Injected with Tityustoxin[a]

Body weight, g	Heart rate per minute			
	Control	After tityustoxin, 100 μg/100 g	After atropine, 50 μg/100 g	
			1 min	40 min
217 ± 17	403 ± 3	188 ± 57[b]	395 ± 16[c]	373 ± 22[c]

[a] Values are mean ± S.E. (N = 3).
[b] Idioventricular rhythm.
[c] Sinus rhythm.

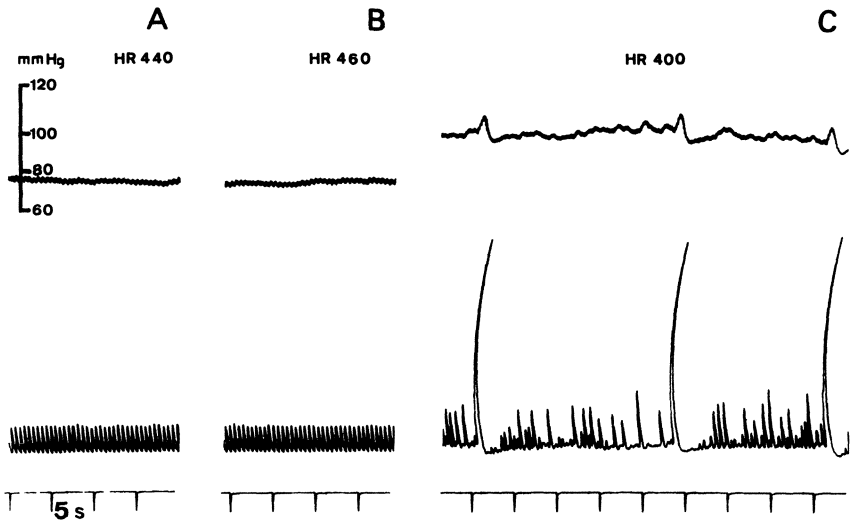

Fig. 3. Mean arterial pressure and respiratory movements of a rat. Inspirations indicated by upward deflections. Numbers on the top indicate mean heart rate (HR). Urethane anesthesia was used. Before (A) and 3 min after 50 μg/100 g atropine, intravenously injected (B). Between (B) and (C) 80 μg/100 g tityustoxin was intravenously injected. Record C, 20 min after (B).

Effect of Atropine and/or Artificial Ventilation on Hypotension and Death Produced by TsTX. Injection of 80 μg/100 g of TsTX in rats produced hypertension followed by hypotension, bradycardia, periodic respiration, apnea, and death. Previous injection of atropine potentiated the pressor response, prevented bradycardia, but did not prevent hypotension, periodic respiration, apnea, or death. In the artificially ventilated animals, TsTX also produced the pressor and depressor effects, bradycardia, and death. In the atropine-treated rats, under artificial ventilation, TsTX still produced hypertension followed by hypotension but death did not occur during the time of the experiment, that was 2 hr 30 min (Fig. 4).

Effect of Lidocaine. As many actions of TsTX in the rat are due to stimulation of peripheral nerve endings (Freire-Maia *et al.*, 1973; Freire-Maia *et al.*, 1974b), it was decided to study the possible effects of local anesthetic (lidocaine) on respiratory arrhythmia and on the cardiovascular effects produced by TsTX. Intravenous injection of 0.5-1.0 mg/100 g of lidocaine abolished the respiratory arrhythmia (Fig. 5), increased the heart rate (atropine-like effect) and decreased the arterial pressure. However, the lidocaine effect was transient. For this reason, continuous infusion of lidocaine (0.3 mg/100 g/min) was performed. These experiments have shown that respiratory arrhythmia was abolished during the time of infusion. However, the arterial pressure and heart

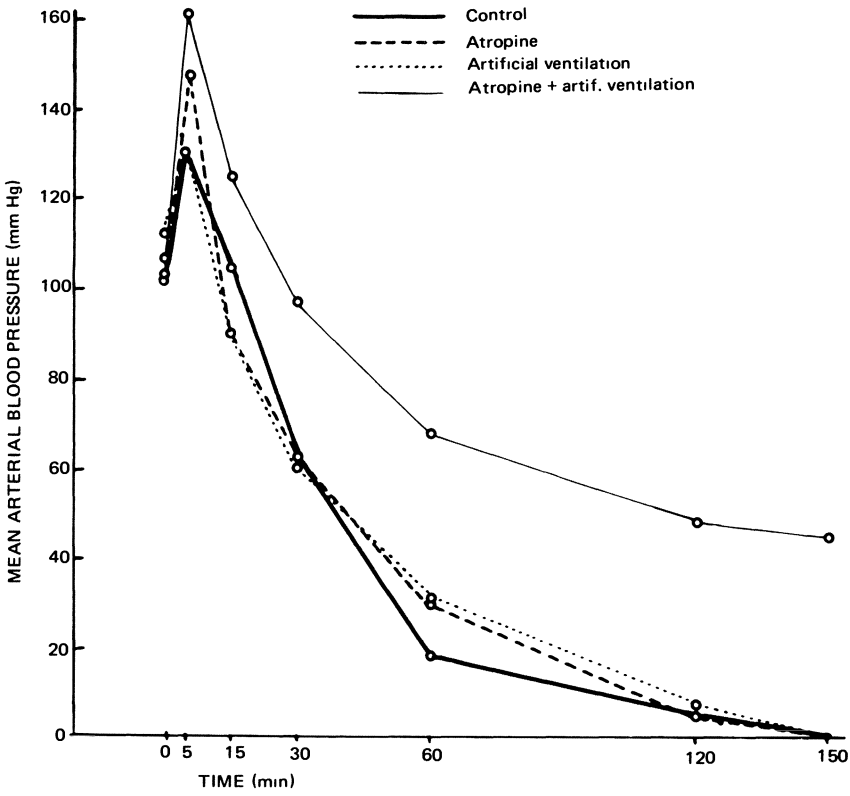

Fig. 4. Effects of i.v. injections of 80 μg/100 g of tityustoxin on the mean arterial pressure of rats. Urethane anesthesia was used. Each point on the curves represents the mean of seven experiments.

heart rate decreased to levels much lower than the controls. Lidocaine also abolished respiratory arrhythmia in the atropine-treated animals.

These experiments did not show, however, the site of action of lidocaine. Experiments were then conducted to observe the participation of the vagi nerves in the effects of TsTX. 10-30 sec after local anesthesia of the cervical vagi nerves with lidocaine, the periodic respiration was abolished and changed into a regular and deep type of breathing, the bradycardiac effect was not changed, whereas the arterial pressure rose to values higher than the controls. Figure 6 shows one of these experiments and Table 2 summarizes the results.

Lidocaine (200 μg/100 g) also abolished the idioventricular rhythm produced by TsTX and increased the rate of the sinus rhythm; however, the latter effect was transient, that is, lasted no more than 2-5 min (5 experiments). In some experiments the ventricular rhythm resumed and a second dose of lidocaine was necessary to abolish it (Fig. 7).

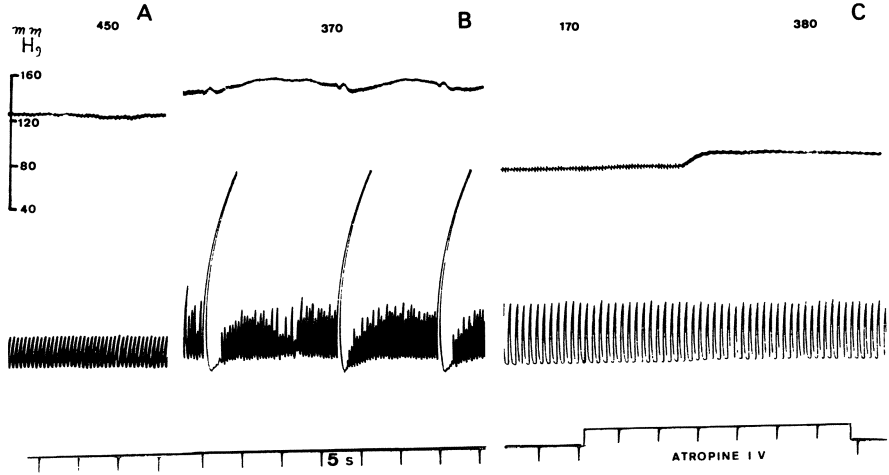

Fig. 5. Mean arterial pressure and respiratory movements of a rat. Inspirations indicated by upward deflections. Numbers on the top indicate mean heart rate. Before (A) and 8 min after i.v. injection of 80 μg/100 g tityustoxin (B). Between (B) and (C), two doses of 500 μg/100 g lidocaine were intravenously injected. Record C, 10 min after lidocaine. Dose of atropine: 50 μg/100 g.

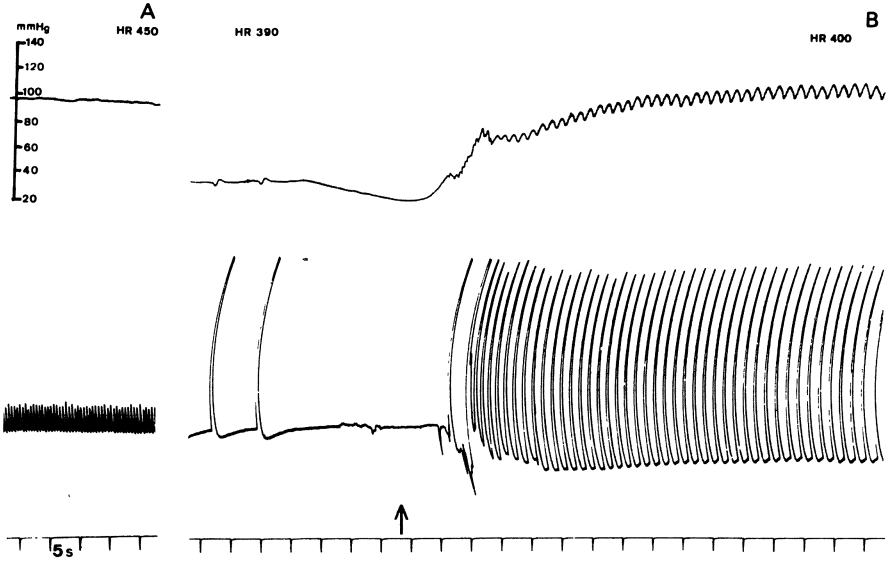

Fig. 6. Mean arterial pressure and respiratory movements of a rat. Inspirations indicated by upward deflection. Numbers on the top indicate mean heart rate (HR). Before (A) and 12 min after i.v. injection of 80 μg/100 g tityustoxin (B). At the arrow, one drop of lidocaine was applied to each of the cervical vagi nerves.

Table 2. Effect of Local Anesthesia of the Cervical Vagi Nerves on the Cardiovascular and Respiratory Effects of Tityustoxin in the Rat[a]

	Control	After TsTX[b]	After lidocaine[c]	
		10-15 min	3 min	10 min
Mean arterial pressure, mmHg	84 ± 6	45 ± 9[d]	101 ± 7[e]	86 ± 7[g]
Heart rate/min	358 ± 23	205 ± 52[d]	216 ± 53[f]	159 ± 26[h]
Respiratory rate/min	97 ± 8	10 ± 2[d]	44 ± 3[e]	50 ± 4[g]

[a]Values are mean ± S. E. (N = 5).
[b]Dose, 80 μg/100 g, iv
[c]One drop of lidocaine (2%) was applied to each cervical vagus nerve.
[d]Significantly different from control ($P < .05$).
[e]Significantly different from the mean after TsTX ($P < .05$).
[f]Not significantly different from the mean after TsTX ($P > .05$).
[g]Not significantly different from the mean 3 min after lidocaine ($P > .05$).
[h]Significantly different from the mean 3 min after lidocaine ($P < .05$).

Intracerebroventricular (ICV) Injections of TsTX. The ICV injections of 5 μg of TsTX in rats produced hypotension, (36 ± 6 mmHg), tachypnea, hyperpnea, ataxic, and gasping movements 10-20 sec after the injection. The hypotensive effect was not related to the decrease in the heart rate. Tachypnea and gasps disappeared 5-10 min later. After this phase, hyperpnea, bradypnea, and hypertension (51 ± 5 mmHg) were recorded. ICV injections of 100 μg of atropine prevented hypotension and initial respiratory arrhythmia, whereas 50 μg of phenoxybenzamine prevented the hypertensive effect (Lima and Freire-Maia, 1974, 1976).

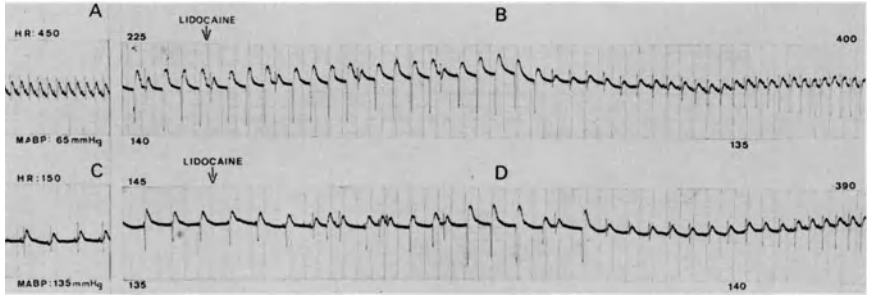

Fig. 7. Electrocardiogram (lead II), mean heart rates (HR) and mean arterial blood pressure (MABP) of rats receiving artificial ventilation. Urethane anesthesia was used. Upper tracing: Between (A) and (B) 100 μg/100 g tityustoxin (TsTX) was intravenously injected. Record B, 4 min after TsTX. Dose of lidocaine intravenously injected: 200 μg/100 g. Lower tracing: Record C, 2 min after lidocaine. Between (C) and (D), 30 sec. Dose of lidocaine injected in D: 200 μg/100 g.

DISCUSSION

The experiments described in this paper have shown that the injection of anti-scorpionic serum after injection of purified scorpion toxin (tityustoxin or TsTX) did not abolish the cardiovascular and respiratory effects produced by TsTX. As the serum is effective when administered prior to the toxin it seems likely that after the phase in which the toxin is bound to the nervous system its neutralization becomes more difficult by the specific serum. For this reason it seemed worthwhile to find such drugs that could block the toxin's effects on the body. In previous papers (Freire-Maia and Diniz, 1970; Freire-Maia *et al.*, 1974b), it was shown that atropine abolished the S-A block, A-V block, and sinus bradycardia; that propranolol abolished the idioventricular rhythm; and that phenoxybenzamine prevented the hypertensive effect. In the present paper we have shown that atropine also abolished the idioventricular rhythm. Since it was assumed that this rhythm was due to stimulation of the beta adrenergic receptors in the heart during the periods of slow sinus rate (Freire-Maia *et al.*, 1974b), it seems likely that the effect of atropine could be explained by an increase in the pacemaker activity of the sinus mode.

We have also shown that atropine abolished the sinus bradycardia occurring 30–60 min after the injection of TsTX without a significant effect on hypotension. It was, therefore, assumed that this late hypotension could not be explained as only a reduction in the heart rate. To test this hypothesis, experiments were made in which atropine was injected before the injection of toxin. Under these circumstances, TsTX did not produce bradycardia, but still produced hypotension, periodic respiration, apnea, and death of the animals. On the other hand, artificial ventilation did not prevent bradycardia, hypotension, or death produced by TsTX. Association of atropine with artificial ventilation did not prevent hypotension, but increased the survival time of the rats. Therefore, it seems likely that apnea and bradycardia would be responsible, at least in part, for the hypotensive effect and death of the animals.

Respiratory arrhythmia produced by tityustoxin seems to be due to stimulation of the peripheral chemoreceptors (Freire-Maia *et al.*, 1973). On the other hand, Eyzaquirre and Zapata (1968) admitted that a chemical mediator, acetylcholine (ACh), is released when the peripheral chemoreceptors are stimulated and that the effect of ACh on the chemoreceptors is decreased by atropine. As it was shown that ACh is released by tityustoxin from other structures such as guinea pig ileum (Diniz and Torres, 1968), rat brain (Gomez *et al.*, 1973), and rat ileum (Freire-Maia *et al.*, 1974a), we assumed that the respiratory effects of tityustoxin could be explained by the release of this mediator. However, atropine did not prevent or abolish respiratory arrhythmias produced by TsTX. Therefore, it seems likely that the respiratory effects of TsTX are not due to the release of ACh. As it was admitted for other substances, such as phenyldiguanide

and NaCN (see review in Paintal, 1973), TsTX could act directly on the regenerative region of the chemoreceptors.

The present experiments have shown that tityustoxin produces periodic respiration (characterized by long periods of apnea intermingled with gasping breathing), sinus bradycardia, and hypotension. The periodic respiration and hypotension were abolished by local anesthesia with lidocaine of the cervical vagi nerves. It seems likely, therefore, that tityustoxin produces these effects through the stimulation of the peripheral receptors. It has been shown that the pulmonary J receptors are stimulated by a variety of substances, such as phenyldiguanide, 5-HT, nicotine, and acetylcholine, producing reflexly apnea, bradycardia, and hypotension (Paintal, 1969; 1973). Based on these data, we present a hypothesis that TsTX, by acting on the pulmonary receptors, produces reflexive apnea and hypotension. Bradycardia was explained mainly by the actions of TsTX on vagal ganglia and postganglionic nerve endings in the heart (Freire-Maia *et al.*, 1974b).

Intravenous injections of lidocaine abolished the idioventricular rhythm produced by TsTX. At least a part of this effect could be due to the well-known atropinelike effect of lidocaine. This hypothesis seems to have been verified by the experiments in which the ventricular rhythm resumed when the sinus rate became slower.

Respiratory arrhythmia produced by TsTX (tachypnea, gasping, and ataxic breathings) were abolished by bilateral vagotomy and denervation of the carotid body regions (Freire-Maia *et al.*, 1973). In the present experiments, we have shown that intravenous injection of lidocaine also abolished these forms of arrhythmia. The lidocaine effect could be due to anesthesia of the peripheral chemoreceptors.

Our experiments have shown actions of TsTX on the peripheral nervous system. As there is the possibility that the toxin, under certain circumstances, e.g., asphyxia, could cross the blood-brain barrier, we injected tityustoxin into the lateral ventricles of the rats. The data have shown that TsTX produced respiratory arrhythmia (tachypnea, hyperpnea, ataxic, and gasping breathing) and hypotension followed by hypertension. As atropine prevented respiratory arrhythmia and hypotension, whereas phenoxybenzamine prevented hypertension, we assumed that these effects could be explained by the release of acetylcholine and catecholamines from the central nervous structures. This hypothesis is supported by the *in vitro* experiments, in which scorpion toxin, incubated with brain slices or with synaptosomes, released acetylcholine and catecholamines (Gomez *et al.*, 1973; Moss *et al.*, 1974).

SUMMARY

Effects of some drugs on the cardiovascular and respiratory effects produced by intravenous injection of purified scorpion toxin (tityustoxin or TsTX) were

examined in anesthetized rats. Injections of anti-scorpionic serum prior to the injection of TsTX prevented the cardiovascular and respiratory effects of the toxin; however, injection of the serum after the toxin had no neutralizing effect.

The idioventricular rhythm produced by TsTX was abolished either by atropine or lidocaine. It is assumed that at least a part of this effect is due to an increase in the rate of the sinus rhythm.

The intravenous injection of TsTX produced hypertension followed by hypotension, bradycardia, periodic respiration, apnea, and death. Injection of atropine in artificially ventilated animals did not prevent hypotension, but increased the survival time of the rats. Atropine did not prevent or abolish respiratory arrhythmia (tachypnea, gasping and ataxic breathing, periodic respiration, and apnea) produced by TsTX. It is assumed that these forms of arrhythmia are not due to the release of acetylcholine. The intravenous injection of lidocaine abolished transiently respiratory arrhythmia produced by TsTX.

The periodic respiration, apnea, and hypotension were abolished by local anesthesia of the cervical vagi nerves with lidocaine. It is assumed that these effects are reflexive in nature and due to stimulation by TsTX of the peripheral receptors. Sinus bradycardia was not abolished by local anesthesia of the vagi nerves, but was abolished by atropine.

Injection of TsTX into the lateral ventricles of rats produced respiratory arrhythmia (tachypnea, hyperpnea, gasping, and ataxic breathing) and hypotension, followed by hyperpnea and hypertension. Intracerebroventricular (ICV) atropine prevented initial respiratory arrhythmia and hypotension, whereas ICV phenoxybenzamine prevented the hypertensive effect produced by intraventricular TsTX.

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Mode of Action and Specificity of *Habrobracon* Venom (Hymenoptera, Braconidae)

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Like many Hymenoptera the females of *Habrobracon* paralyze other insects by injecting a venom into their body before laying eggs on the immobilized prey. The effect of *Habrobracon* venom was studied electrophysiologically by several authors (Beard, 1952; Piek, 1966; Rathmayer, 1966; Piek and Engels, 1970) using nerve-muscle preparations of different Lepidoptera. We have recently continued these investigations both on muscles of a natural prey of *Habrobracon hebetor*, the larvae of the mealmoth *Ephestia kühniella*, and on *Locusta* (Walther and Rathmayer, 1974), because most of the knowledge on neuromuscular transmission in insects comes from studies with this animal.

The investigations were performed on *in vitro* paralyzed nerve-muscle preparations. Paralysis of *Ephestia* was achieved in the course of natural predation through the sting of the wasp (Fig. 1). Paralysis of *Locusta* was obtained by injecting small amounts of secreted venom or gland extract into a hindleg. Sometimes venom was applied to the bath containing the preparation.

The effect of *Habrobracon* venom in *Ephestia* is seen in Fig. 2. Stimulation of the motor axons innervating the ventral longitudinal muscles once every two seconds produced a large action potential overshooting zero potential. After application of venom the amplitude of the electrically excited response declined rapidly, until after 6 min only a small excitatory junction potential (ejp) remained (Fig. 2E), which after some time disappeared totally. This decline is not due to any postsynaptic effects of the venom. Walther and Rathmayer (1974) have shown that neither the resting potential, current-voltage relationships,

Fig. 1. *Habrobracon hebetor* female stinging its prey, a larva of the mealmoth *Ephestia kühniella.* The sting, guided by the sting sheaths, is inserted (arrow) laterally between the second and third pair of legs.

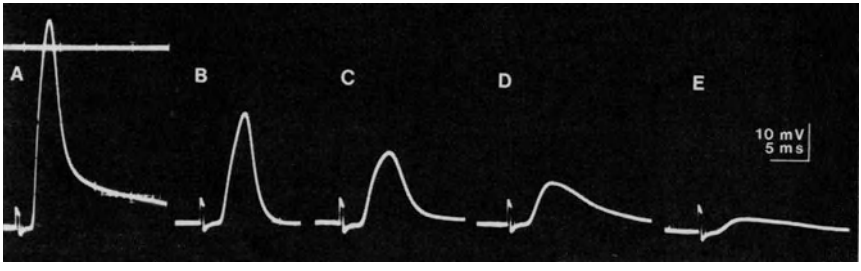

Fig. 2. Effect of *Habrobracon* venom on the excitatory junction potentials of a ventral longitudinal muscle of *Ephestia.* A normal response; B–E progressive paralysis. Decline of the ejp at 3.5, 4, 4.5, 5, and 6 min after venom application.

nor excitation-contraction coupling in the muscle fibers are affected. This confirms the results of the authors cited above. In addition, we found that direct stimulation of these muscle fibers still caused normal graded electrogenesis and that application of glutamate, the most likely candidate for the excitatory transmitter in insect muscles, evoked depolarizations in paralyzed preparations such as those of the controls. Since the postjunctional sensitivity to glutamate was not altered by the venom, the blocking of the ejps must be due to a presynaptic effect.

In cases where single nerve stimulation is no longer effective in eliciting an ejp, repetitive stimulation can often still release small amounts of the transmitter (Fig. 3). In these preparations the spontaneous release of the transmitter continues to result in miniature excitatory junction potentials (mejps; see Fig. 4). Figure 3 shows this effect of repetitive stimulation in partially paralyzed locust extensor tibiae muscle fibers. The normal response of these fibers to nerve stimulation consists of a large ejp, usually more than 10 mV. In Fig. 3A stimulation of a weakly paralyzed preparation with 8/sec still elicited small ejp's; their amplitude was drastically reduced, almost to the size of the mejp preceding the train. In another fiber of the same preparation, only some of the stimuli of a

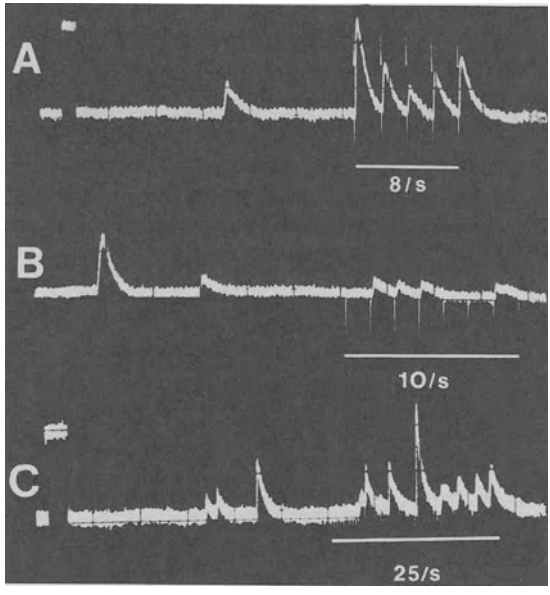

Fig. 3. Spontaneous and evoked postsynaptic responses at partially blocked excitatory neuromuscular junctions of locust extensor tibiae muscle. A and B two different fibers in the same preparation, one day after weak intoxication; C after two days. Calibration pulse: 1 mV, 50 mscc for A and B; 200 mscc for C. The bars indicate period of stimulation.

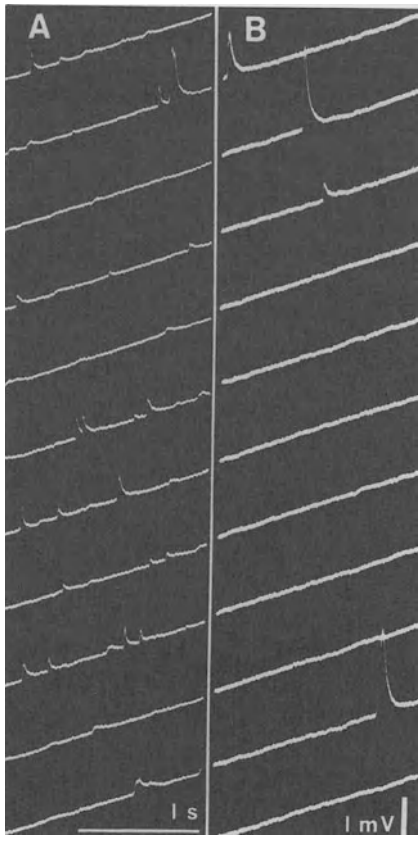

Fig. 4. Miniature excitatory junction potentials in normal (A) and paralyzed (B) locust extensor tibiae muscle.

10/sec train were able to release small amounts of the transmitter, probably only single quanta (Fig. 3B). Figure 3C shows responses from a different preparation two days after paralysis. Many stimuli failed to release the transmitter; yet there is an increase of the mejp frequency for the period of stimulation. Generally, the amplitudes of these ejp's resemble those of mejp's which are caused by spontaneous release of the transmitter (see left halves of Fig. 3A–C). With progressing degrees of paralysis the amount of the transmitter which is available for release upon repetitive stimulation gradually declines. The further paralysis proceeds, the higher the stimulation frequencies must be in order to obtain a rise in transmitter release and the shorter become the periods over which this is possible. Even in preparations like that of Fig. 3A, where every stimulus of the train released one or more quanta, the response vanished after a few minutes of continuous stimulation.

An analysis of spontaneous transmitter release in normal and paralyzed nerve-muscle preparations in the locust showed that *Habrobracon* venom re-

duced the frequency of the mejp's to as little as 1% of the controls (Fig. 4). This is not an artifact due to a reduction of the amplitudes of the mejp's, since amplitude histograms of mejp's (Fig. 5) from completely paralyzed preparations are very similar to those of the controls. This finding is strong evidence for a purely presynaptic site of action of this venom.

We have carried out an electron microscopic investigation of the ultrastructure of neuromuscular junctions of several ventral longitudinal muscles of *Ephestia* larvae at various time intervals after the onset of paralysis. In all cases, the nerve terminals were not obviously different from the controls (Fig. 6). This

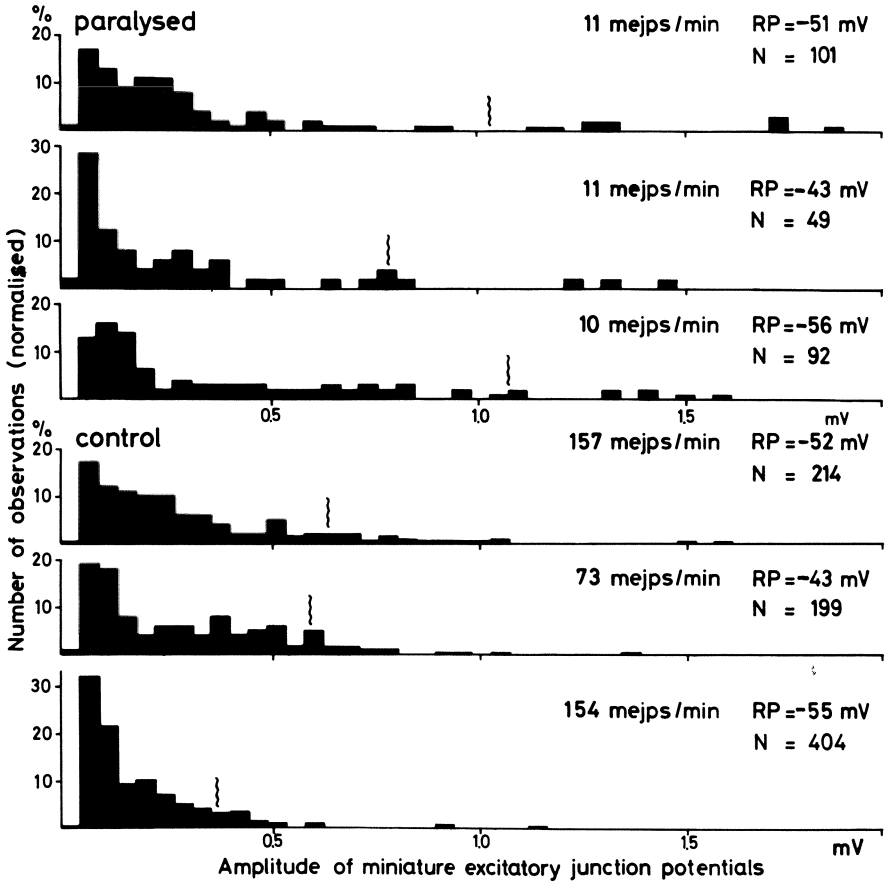

Fig. 5. Amplitude histograms of miniature excitatory junction potentials of locust extensor tibiae muscle. Upper part: responses in a hindleg after venom application. Lower part: responses in the contralateral untreated leg of the same animal. Note the reduction in the frequency of mejp's in the paralyzed muscle (given on the right) Mejp's of very small size were omitted because of the limitations due to the signal to noise ratio. RP is the resting potential; N is the number of observations.

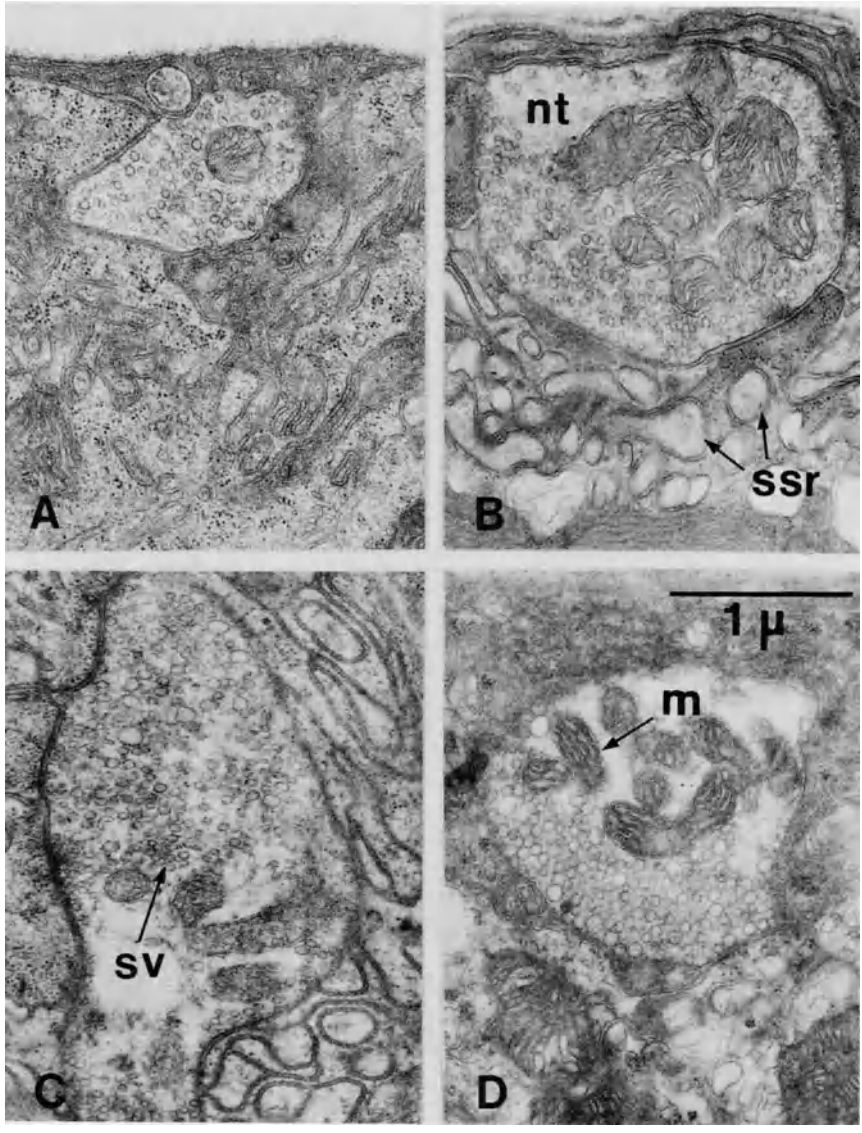

Fig. 6. Electronmicrographs of neuromuscular junctions of ventral longitudinal muscles of normal (A) and completely paralyzed (B–D) *Ephestia* larvae. B, 24 hr after onset of paralysis; C, after three days; D, after four days. Fixation: Glutaraldehyde, OsO_4; m is a mitochondrion; nt is the nerve terminal; ssr is the subsynaptic reticulum; sv are synaptic vesicles.

also applies to the retractor unguis muscle of the locust. The average number of synaptic vesicles per unit area, as well as their size and distribution, seems to be unchanged even after four days of paralysis. Long-term changes in the ultrastructure, which could be detected after several days in, for example, the subsynaptic reticulum, are more likely to be due to the failure of neuromuscular transmission rather than to a direct effect of the venom. This problem, comparable to pharmacological denervation, is under further investigation.

It is of particular interest that the effect of *Habrobracon* venom is confined to the excitatory neuromuscular junction. Inhibitory neuromuscular transmission is not affected (Piek and Mantel, 1970; Walther and Rathmayer, 1974). Figure 7 shows inhibitory junction potentials (ijp's), together with three spontaneous mejp's, from a locust extensor tibiae muscle fiber. The ejp's are completely blocked but the ijp's show normal facilitation and potentiation.

The venom blocks excitatory transmission in a number of nerve-muscle preparations of Lepidoptera and in *Locusta*. Drenth (1974) has shown that injection of extracts from *Habrobracon* venom glands immobilized insect species belonging to the orders Hemiptera, Diptera, and Hymenoptera. Members of several other orders of insects were apparently not affected by the venom (see also Beard, 1952). We have tested the effect of *Habrobracon* venom on a number of noninsect nerve-muscle preparations using electrophysiological techniques. The preparations employed were the levator pretarsi muscle of the spider *Dugesiella hentzi*, and the closer muscle in the walking legs of the crab *Eriphia spinifrons* and of the crayfish *Astacus astacus*. In no case had the venom, if applied as a dialyzed venom gland extract and in doses similar to those producing complete paralysis in the locust, any effect on the ejp's during the 2 h of observation. The quick transient decline of the ejp amplitude produced by venom gland extracts, without previous dialysis, might well be attributed to a partial desensitization of the postsynaptic glutamate receptors by glutamate present in the extracts.

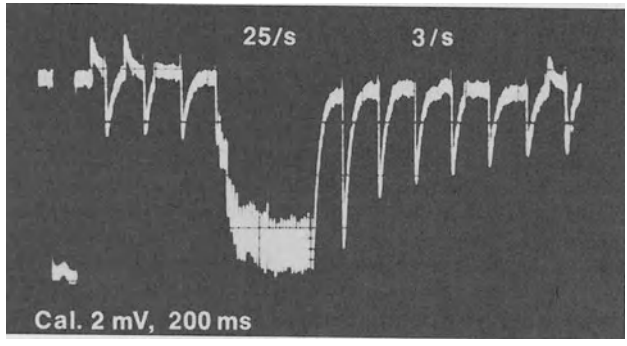

Fig. 7. Inhibitory junction potentials of a paralyzed extensor tibiae muscle in the locust.

No effect of the venom could be seen in two nonarthropod preparations. Application of venom, even at ten times the concentration used with the locust, had no effect in the cholinergic motor endplate of the frog sartorius muscle (Deitmer, 1973). Transmission at the giant synapse in the stellar ganglion of the squid is unimpaired even several hours after venom application to the bath.

In some ways the effects of *Habrobracon* venom are similar to those of other presynaptically acting neurotoxins such as botulinum and tetanus toxins (Mellanby *et al.*, 1973). Like *Habrobracon* venom, both of these toxins show an action on neuromuscular junctions which builds up slowly and which persists over long periods without any apparent changes in the ultrastructure of the terminals. Also, the effects of tetanic nerve stimulation on transmitter release in incompletely blocked preparations are very similar.

The effect of *Habrobracon* venom also resembles the neuromuscular block obtained with high-Mg and low-Ca concentrations in several respects. However, various treatments which lead to a conspicuous increase in the mejp frequency in Mg-treated preparations do not show this effect in muscles paralyzed by *Habrobracon* venom (Walther and Rathmayer, 1974). Also, repetitive stimulation of weakly paralyzed preparations (Fig. 3) results in a release of the transmitter which is sustained for only a few minutes (as opposed to more than half an hour during Mg block). Both of these observations cannot be explained by the assumption that the venom has an exclusive effect on the release mechanism. Rather, it seems that *Habrobracon* venom impairs and eventually stops some mechanism involved in replenishing the pool of transmitter available for release. Combined electronmicroscopic and electrophysiological experiments are in progress to bring this problem to further clarification.

ACKNOWLEDGMENTS

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Effect of Hornet Venom on Crustacean Neuromuscular Junctions

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INTRODUCTION

The specific properties of various animal toxins affecting the nervous tissue have gained interest. For example, a component of snake venom, α-bungarotoxin, proved to bind specifically to cholinergic receptor protein (Changeux *et al.*, 1970; Miledi *et al.*, 1971). The venom of a spider (black widow) accelerates chemical transmitter release resulting in depletion of the transmitter storage (Longenecker *et al.*, 1970; Clark *et al.*, 1972). Action of this spider venom is not limited on cholinergic synapses but is also effective on such aminergic synapses as crustacean neuromuscular junctions (Kawai *et al.*, 1972).

Recently considerable work has been done on venoms of wasps and European hornets, and their pharmacological activities on various tissues have been documented (Piek *et al.*, 1971; Habermann, 1972; Edery *et al.*, 1972; Kaplinsky *et al.*, 1974; Ishay *et al.*, 1974; Walther and Rathmayer, 1974).

In the present paper, we report the effect of Asian hornet venoms on crustacean neuromuscular junctions as determined by intracellular recording. Hornets (*Vespa mandariana, Vespa xanthoptera*, and *Vesta analis*) are prevalent in Asian countries including Japan, and are known to be strongly toxic to insects and even fatal to man. The properties of chemical transmission in the crustacea are very similar to those of the insect (see Gerschenfeld, 1973), which is the common prey of the hornet. Lobster neuromuscular preparations have the further advantage of permitting us the study of both excitatory and inhibitory transmission by isolating the excitatory and inhibitory axons for independent stimulations.

MATERIALS AND METHODS

Lobster Neuromuscular Preparation. Experiments were performed on the stretcher muscle of the walking legs of the lobster (*Panulirus japonica*). The excitatory and inhibitory nerves innervating the stretcher muscle were dissected to single axons and introduced in suction electrodes for stimulation. The bathing solution for the preparations was composed of 468 mM NaCl, 10 mM KCl, 22 mM $CaCl_2$, and 8 mM $MgCl_2$. The pH was adjusted to 7.5 ± 0.5 with a Tris (hydroxymethyl)-aminomethane buffer. A pair of glass microelectrodes filled with 3 M KCl were used for recording; one was inserted in the muscle fiber and the other was placed outside the cell to record the potentials differentially. Another glass microelectrode, filled with 2 M K-citrate, was used for the intracellular passing of currents. The equipment for recordings was conventional. The experiments were done at room temperature (20-25°C).

Hornet Venom. Venoms from three species of hornets (*Vespa mandariana*, *V. xanthoptera*, and *Vespa analis insularis*) were used for the experiments. No qualitative difference appeared among venoms of these three species as to the effect on neuromuscular transmission, though the venom of *V. analis* was less potent than those of the other two species. Nests of live hornets were collected in the western outskirts of Tokyo. The venom sac of each hornet was taken out and deep frozen at -70°C.

In the experiments using crude venom, 2-4 venom sacs were thawed and homogenized with 0.1 ml of the bathing solution immediately before the experiment. In most cases, 2-5 μl of the venom solution was applied to the neuromuscular junction area covered with a thin layer of the bathing solution (approximately 1 ml). The minimal effective dose to cause neuromuscular blockade corresponded to 10%-20% of a venom sac content.

Chromatography. Venom was extracted three times from 30-100 venom sacs by homogenizing with 1.5 ml of 0.1 M ammonium-acetic acid (pH 4.5) followed by centrifugation at 2000*g* for 30 min. The combined supernatant was lyophilized and stored at -70°C until use. The dried material was suspended in 1.0 ml of 0.1 M ammonium-acetic acid (pH 4.5) and the insoluble material was removed by centrifugation at 105,000*g* for 30 min. The clear supernatant was applied to ascending-column chromatography on Sephadex G-50 (0.9 × 60 cm). Fractions of about 2 ml were collected and the elution profile was determined with the phenol reagent according to Lowry *et al.* (1951) and by the fluorometrical method (in 3 N HCl, excitation wave, 300 nm, emission wave, 540 nm). The first six fractions (part A) did not contain any substance detectable by the colorimetrical or fluorometrical method. Subsequent 12 fractions (part B) had four peaks by colorimetrical assay with the phenol reagent but no peak showed fluorescence. Fractions (part C) between the tubes numbered 19 and 25 contained 5-HT like fluorescent substance. Fractions between 26 and 50

were collected as part D, which had no colorimetrically or fluorometrically detectable substance. Each of parts A, B, C, and D was lyophilized and stored at -70°C until use.

RESULTS

Effect of Crude Venom on Neuromuscular Junctions. An example of the effects of crude venom on the excitatory postsynaptic potentials (epsp's) and on the inhibitory postsynaptic potentials (ipsp's) is shown in Fig. 1. Both epsp's and ipsp's were evoked in the stretcher muscle by repetitive (40-120 Hz) stimulation of the excitatory and inhibitory axons, respectively. Spontaneous miniature potentials were also seen in the resting state. Shortly after application of the crude venom, amplitudes of both epsp's and ipsp's began to increase. This augmentation of postsynaptic potentials reached a peak in several minutes, then gradually declined and finally disappeared. Usually, psp's were abolished in

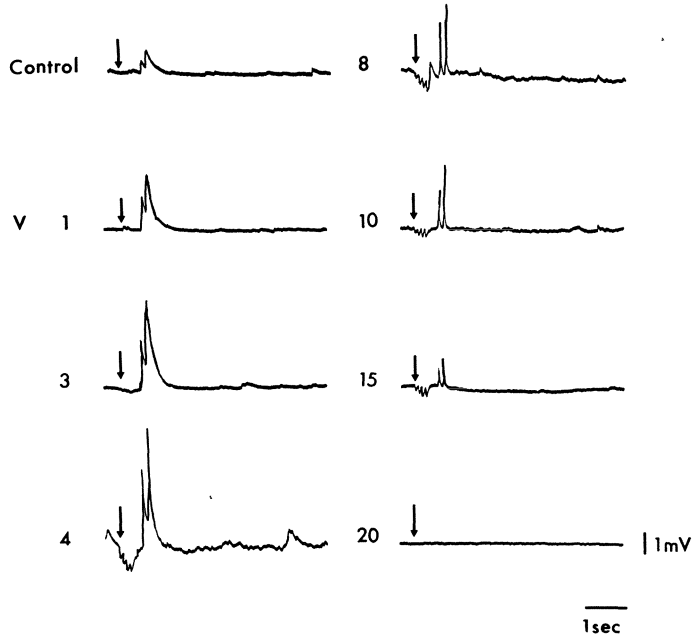

Fig. 1. Effect of crude venom on epsp's and ipsp's evoked in the stretcher muscle of the lobster walking leg. In each record, train pulses of 13 Hz to the inhibitory axon begin at the arrows and the summated ipsp's are followed by epsp's evoked by two stimuli of 8 Hz on the excitatory axon. In this and following figures, numbers in each record indicates time in minutes after applying the venom.

15-40 min after application of the venom. The frequency of miniature potentials appeared to increase temporarily during the period of psp's augmentation, but soon returned to the normal level and abolished almost simultaneously with disappearance of psp's. In Fig. 1, ipsp's in the depolarizing form in the control record were reversed in the polarity to the hyperpolarizing form after the venom application. From this observation, it appeared that membrane depolarization was caused by the crude venom. This membrane depolarization proceeded gradually and it finally amounted to 10-30 mV. In contrast to the changes in the postsynaptic potentials, the antidromically evoked action potentials of the presynaptic axons were unaltered even after abolition of psp's. These results indicate that the disappearance of postsynaptic potentials are not due to blockade of the conductile component of the axon.

Figure 2 shows the changes in the membrane resistance as well as the postsynaptic potentials brought about by the action of the venom. To estimate the membrane resistance, a glass microelectrode for passing current was penetrated into the cell located close to the recording electrode (within 100 μ). In upper two sets of the records of Fig. 2, depolarizing currents were applied for 0.65 sec, during which time epsp's and ipsp's were evoked. In the lower sets of the records, the same intensity of currents but opposite polarity (hyperpolarizing) were applied. In 3 min after application of the venom, epsp's and ipsp's were greatly enhanced but the effective resistance was reduced to about half of the control value (from 80 to 43 kΩ). Thereafter the membrane resistance tended to decrease slightly but maintained a steady level in later stages of the venom action, during which time psp's were abolished. It was noted in this experiment that in 34 min epsp's disappeared, while ipsp's were still seen (this was clearly seen under depolarizing current as shown in Fig. 2). The persistence of ipsp's after disappearance of epsp's was frequently observed though both psp's were finally abolished. This observation may relate the unequal effects of a component of the venom of epsp's and ipsp's, as will be described later.

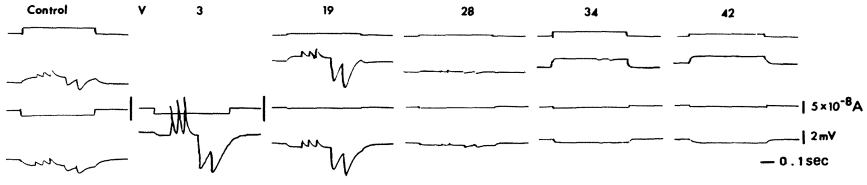

Fig. 2. Changes in membrane resistance and psp's after applying crude venom. In each set of two records, the upper trace monitors duration of the intracellularly applied currents. Upward deflexions indicate outward currents. In the lower trace, epsp's and ipsp's evoked in response to repetitive stimuli under these currents are shown. In the records of the control and those of 3 min after venom application (V-3), the current scale is twice those of other records. Further descriptions are given in the text.

Effects of Histamine and 5-Hydroxytryptamine. It has been known that venoms of hornets of other species, and wasps contain fairly large amounts of such biogenic amines as 5-hydroxytryptamine (5-HT) and histamine (Jaques and Schachter, 1954; Bhoola *et al.*, 1961). In an attempt to analyze the effect of the crude venom used in the present work, histamine and 5-HT were applied to the preparation and the effects of these amines compared with that of the crude venom. In Fig. 3, three consecutive beams were superimposed, during which ipsp's and epsp's were evoked under depolarizing or hyperpolarizing currents. The addition of histamine (1 mM) gave no effect on psp's or on membrane resistance. After washing the preparation with normal solution, application of 5-HT at a concentration of 10^{-6} M produced a great enhancement of both ipsp's and epsp's. The threshold concentration of 5-HT for the augmentation of psp's was approximately 10^{-9} M. The results strongly suggest that the initial augmentation of psp's observed after application of the crude venom can be ascribed to the effect of 5-HT. The presence of 5-HT in the venom was supported by the fluorometrical spectrum. However, it is hard to explain all the effects of the venom by the action of 5-HT, since 5-HT does not depress psp's at all and has little effect on the membrane potential or the membrane resistance.

Effect of Venom Fractions. As described above, the action of crude venom on the crustacean neuromuscular junction was complicated; initial augmentation followed by depression of psp's and decrease in the membrane potential and the membrane resistance. To analyze the effective component of the venom more in detail, chromatography was carried out on Sephadex G-50, as described in Materials and Methods. Of the four fractions of the venom separated by chromatography, parts A or D produced no change on neuromuscular transmission. Part C had the ability to enhance both epsp's and ipsp's but no had effect on the membrane potential or the resistance. The effect was identical to that of pure 5-HT, as described above (Fig. 3). Results obtained in one of the experiments using part B are shown in Fig. 4. Upon application of this fraction, epsp's showed

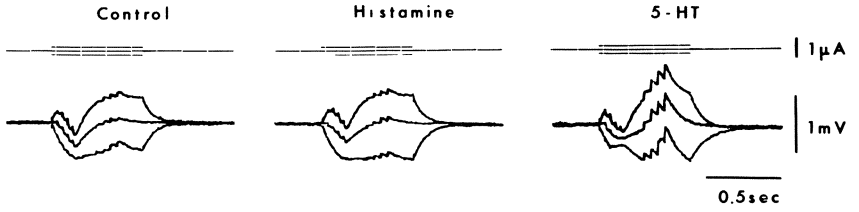

Fig. 3. Effects of histamine and 5-hydroxytryptamine on epsp's and ipsp's. In each record, three consecutive beams were superimposed under depolarizing, zero, and hyperpolarizing current. The upper traces monitored the current; summated ipsp's and those evoked in response to repetitive stimuli are shown in the lower traces. Further descriptions are given in the text.

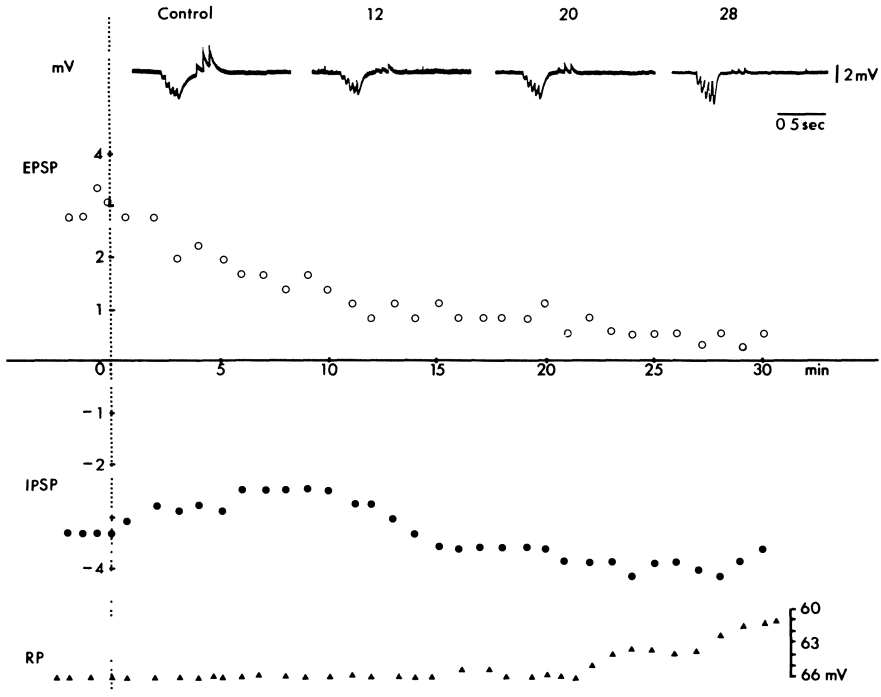

Fig. 4. Time-course of changes in epsp's and isps's and in the membrane potentials after applying the venom part B. In the graph, the ordinate shows peak amplitudes of epsp's (circled) and ipsp's (filled circles) measured from the resting membrane potentials. Triangles are the plots of the resting potentials. Venom was applied at time 0 on the abscissa. Ipsp's and epsp's evoked by repetitive stimuli are shown as specimen records taken at the times indicated in numerals at the top.

a gradual decrement and became almost null in 30 min. Amplitudes of ipsp's were slightly diminished during the first 10 min, but increased again to the same level as that of the control. The resting membrane potential was little affected by the venom up to about 20 min, though later slight depolarization developed. This depolarization may probably be caused by some contaminant directly affecting the muscle membrane.

The effect of part B on the current-voltage relationship of the muscle membrane was examined by passing depolarizing or hyperpolarizing currents through the muscle membrane. Potential changes caused by various intensities of currents before and after venom application were depicted in superimposed records. There appeared to be no significant difference between the two curves representing I-V characteristics in the poisoned and control muscle membrane, as

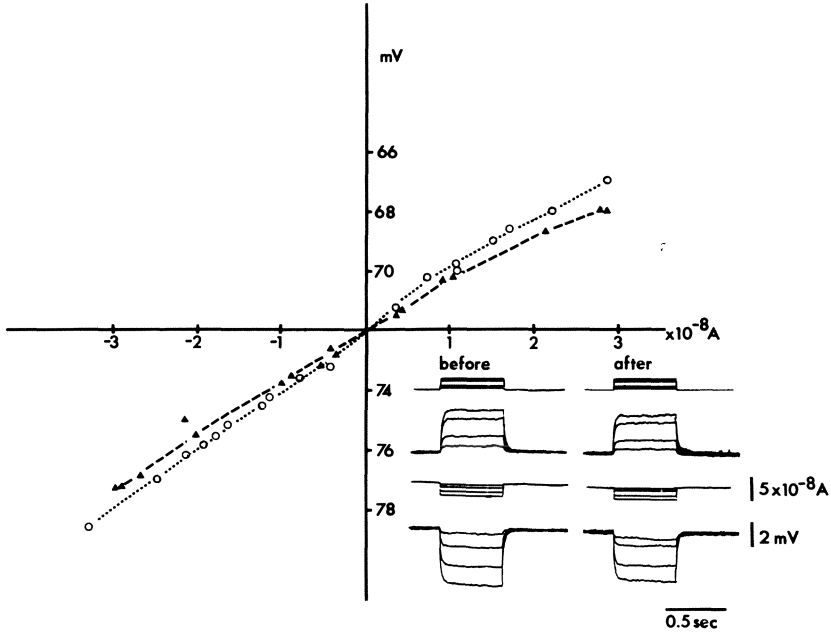

Fig. 5. Current-voltage relationship of the postsynaptic membrane taken before and after applying the venom part B. Inserted records show superimposed beams under depolarizing (upper sets) and hyperpolarizing (lower sets) currents. The graph shows I-V characteristics of the muscle. Circles, control; triangles, 20 min after application of venom. The resting potential was -72 mV.

depicted in Fig. 5. These results suggest that part B of the venom has little or no effect on electrically passive properties of the postsynaptic membrane.

Miniature potentials persisted even after epsp's were depressed (see at 28 min of the specimen record in Fig. 5). The average amplitudes of miniature potentials appeared to be unchanged by the venom.

DISCUSSION

The experimental results revealed that hornet venom is a complex mixture of substances affecting the crustacean neuromuscular junctions and eventually blocking the neuromuscular transmission. Enhancement of both epsp's and ipsp's, initially appearing after application of the crude venom, was attributed to the presence of 5-HT in the venom. Facilitatory effects of 5-HT on epsp's in the crustacean neuromuscular junctions have already been reported (Grundfest and Reuben, 1961; Dudel, 1965). They claimed that the effect of 5-HT was produced by the action on presynaptic nerve terminals. It has been reported that European

hornet venom contains, besides 5-HT, low-molecular histamine and acetylcholine. (Jaques and Schachter, 1954; Bhoola *et al.*, 1961). However, acetylcholine was known to be ineffective on the crustacean muscle (Katz, 1936) and histamine was also shown to be ineffective in the present experiment.

The decrease in the membrane resistance accompanied by membrane depolarization caused by the crude venom may be due to proteolysis. Thus eventual disappearance of both epsp's and ipsp's could be explained by the proteolytic action resulting in depolarization and in conductance increase in the muscle membrane.

Part B gave different effects on epsp's and ipsp's. Upon application, epsp's were greatly suppressed or abolished, while ipsp's remained almost unchanged. No appreciable change was observed in the membrane potential or the membrane resistance. The diminution of epsp's may not be due to reduced sensitivity of the postsynaptic membrane to the excitatory transmitter, because the average amplitudes of miniature potentials were not changed by the venom. Furthermore, when L-glutamate, a strong candidate for the excitatory transmitter in the crustacean neuromuscular junctions was applied on the normal and poisoned muscles, almost the same membrane depolarization and conductance changes took place (unpublished observation). From the data described above, it may be inferred that the action of this venom component is presynaptic. So far we have not elucidated the exact mode of the venom action causing selective blocking of epsp's. The observation that presynaptic action potentials were unimpaired after suppression of epsp's excludes the possibility that venom affects the conductile component of the presynaptic axon. It may be possible that the venom causes extensive release of the excitatory transmitter, resulting in depletion of the transmitter just as the black widow spider venom. However, in the case of spider venom, an enormous increase in frequency of miniature potentials was invariably seen and it lasted for hours before depletion of the transmitter store (Kawai *et al.*, 1972). The behavior of miniature potentials under the action of hornet venom is quite different from that observed with spider venom. As another possibility, it may be conceivable that the venom acts on the nerve terminal, affecting the releasing mechanism of the excitatory transmitter in some subtle way.

Purification of the venom component and tests for the effects on other chemical synapses are left for future work.

SUMMARY

Effects of hornet venom on the crustacean neuromuscular junctions were studied by intracellular recordings. After application of crude venom, both

excitatory and inhibitory postsynaptic potentials (epsp's and ipsp's) were enhanced, and then suppressed. The suppression was accompanied by decrease in the membrane potentials and membrane resistance. The initial enhancement of epsp's and ipsp's can be ascribed to the presence of 5-hydroxytryptamine in the venom. A component of the venom, separated by chromatography on Sephadex G-50, caused depression of epsp's, while ipsp's were unaltered. No appreciable changes were found in the membrane potential, the conductance, or the sensitivity of the postsynaptic membrane.

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New Pharmacobiochemical Data on the Anti-Inflammatory Effect of Bee Venom

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INTRODUCTION

Bee venom has gained popularity in some European countries as an antirheumatic drug. Its healing effect has been known from time immemorial. The encouraging therapeutic results obtained by healers who practiced bee stinging as well as the application of modern pharmaceuticals could hardly be accounted for by the scarce pharmacological data on the mechanism of its action. The venom-induced activation of the hypophysocorticorenal system was believed to be the major phenomenon involved (Shkenderov *et al.*, 1968; Shkenderov, 1971). In line with the concept of its anti-inflammatory effect, were the uncoupling of oxidative phosphorylation and the degranulation of mast cells leading to histamine release (Habermann, 1954; Breithaupt and Habermann, 1968). The radioprotective effect of the venom discovered by Shipman and Cole (1967) aroused deep interest but increased the problems about the mechanism of its action. In the meantime, a number of tests and criteria were elaborated for the evaluation of nonsteroid antirheumatic drugs; some tests are related to the pharmacobiochemical bases of their actions.

Bee venom represents a complex of low-molecular (carbohydrates, lipids, amino acids, and histamine) and high-molecular substances (peptides, proteins, and enzymes). The high biological activity and the elevated level of the latter in the venom suggested its possible anti-inflammatory effect. To this end, we decided to fractionate a large amount of venom (more than 1 kg) and to study the properties of the isolated peptide and protein components from the viewpoint of the criteria for nonsteroid anti-inflammatory drugs. Hanson *et al.*,

(1974) have recently reported interesting data on peptide 401, identical with the MCD peptide of Habermann isolated from bee venom.

The present report summarizes the most substantial results of our recent pharmacobiochemical studies on bee venom. It includes data obtained in collaboration with Gencheva in studying the immunosuppressive action of bee venom; with Koburova in carrying on the protein denaturation tests and the hind paw edemas test; with Gruev on investigating adjuvant arthritis; and with Kosturkov in the study of the macrophage inhibition test. Some of these studies will be published elsewhere in a more detailed form.

MATERIALS AND METHODS

The bee venom was obtained by electric stimulation of the bee families and was lyophilized out of a 10% aqueous solution. The freeze dried venom was stored at -20°C in tightly stoppered containers. The methods of fractionation and purification of the venom were previously described (Shkenderov, 1971, 1973). The fraction Oa was isolated by gel filtration of the lyophilized low-molecular fraction on a Sephadex G-50 column (280 X 5.5 cm). Elution was carried out with 0.1 M ammonium formate buffer, pH 4.8 (Fig. 1). Apamin and phospholipase A were obtained from R. Shipolini. The peptides, melittin and apamin, and the enzyme, phospholipase A, were homogeneous as shown by electrophoresis and N-terminal analysis. The other fractions had various degrees of purity depending on the methods of purification. The components of fraction Oa, designated Oa^{1-8}, were eluted from Sp-Sephadex C-25, equilibrated with 0.1 M ammonium formate buffer, pH 4.8 (Figs. 2 and 3).

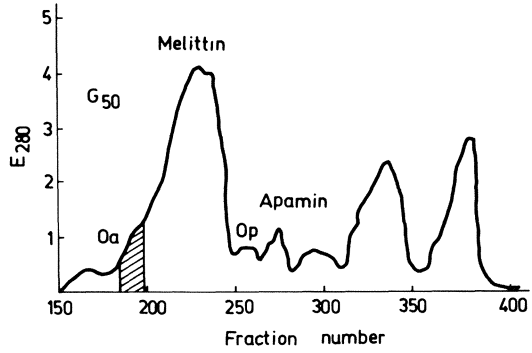

Fig. 1. Isolation of fraction Oa. Gel filtration of 3.6-g freeze dried low-molecular weight (dialyzable) fraction on a 280 X 5 cm Sephadex G-50 column. The column was equilibrated and eluted with 0.1 M ammonium formate buffer, pH 4.8. The hatched fractions were pooled.

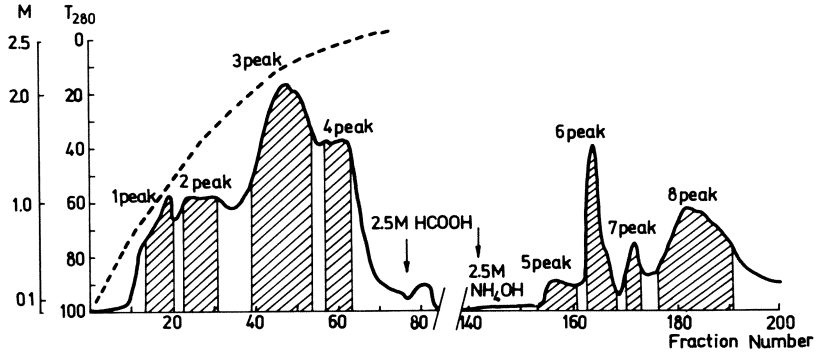

Fig. 2. Chromatography of 0.5-g freeze dried fraction Oa on an SP-Sephadex C-25 column (45 X 2.5 cm). Equilibration was achieved with 0.1 M ammonium formate buffer, pH 4.8. Elution: I, nonlinear gradient of the above buffer ranging from 0.1 to 2.5 M; II, 2.5 M formic acid; III, 2.5 M ammonium hydrate.–optical density at 280 nm;---molarity. The eluates were pooled (hatched area).

Vertical electrophoresis was performed on 2 X 20 X 120-mm 15% polyacrylamide sheets of pH 2.6 for 90 min (U = 8 V/cm, I = 6.8 mA/cm^2) (Ginetis *et al.*, 1968).

Adjuvant Arthritis. Groups of 10 male albino Wistar rats weighing 140–180 g were injected below the aponeurosis of the hind paw with 0.1 ml of Freund's complete adjuvant (6-mg *Mycobacterium butyricum* in 1-ml paraffin oil). The experimental group received daily subcutaneous injections of 100 μg/kg lyophilized venom components (or 200 mU protease inhibitor) starting on the day of

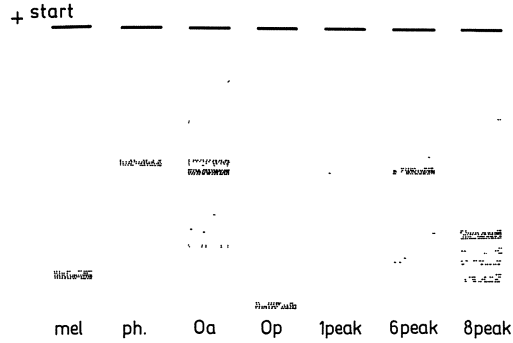

Fig. 3. Vertical plate gel electrophoresis of fraction Oa and its component exhibiting anti-inflammatory effect: peaks 1, 6, and 8 are from the SP-Sephadex column. Melittin (mel), phospholipase A (ph), and fraction Op(Op) served as controls. The electrophoretic separation was performed on 15% polyacrylamide sheets at pH 2.6 for 90 min (U = 8 V/cm; I = 6.8 mA/cm^2).

treatment with the adjuvant. The edema of the paw was measured oncometrically. The degree of polyarthritis which usually developed after day 20 was recorded as a product of the number of the joints affected and the severity of arthritic inflammation expressed by the 4-point system. In order to assay the level of serum haptoglobine and glucoronidase, blood was withdrawn by cardiac puncture.

Tests Based on the Rat Hind Paw Edemas. The following substances were employed as inflammatory agents: carrageenin (Sigma), 1% solution; prostaglandin E_1 (a gift from Upjohn, Calamazoo, U.S.A.), 0.005%; bradykinin triacetate trihydrate (Koch-Light), 0.005%; serotonin creatinine sulfate (Koch-Light), 0.5%; and histamine hydrochloride (Schuhardt), 0.2%.

Half of the experimental animals (groups of 6 rats) were given two subcutaneous injections (10 μg/kg bee venom components given one day and half an hour prior to the administration of the inflammatory agent), whereas the other half received a single injection half an hour before the inflammatory agent. The controls received physiological saline only. The inflammatory agent (0.1 ml) was injected below the aponeurosis of the hind paw. The contralateral paw received an equal volume of the solvent. The volumes of the two paws injected were measured oncometrically 0.5, 1, 2, and 3 hr later. The change in the volume of the paw was related to that of the untreated paw.

Granuloma Pouch of Selye. Thirty-two rats were divided into two groups, experimental and control. Inflammation was provoked by forcing 25-cm^3 air subcutaneously in the back, followed by the injection of 0.5 ml of 3% croton oil. On day 4 an additional 0.5 ml of croton oil was administered. From this day on, the experimental animals were subcutaneously injected with 30 μg/kg apamin everyday for 10 days. The control group received physiological saline. On day 10 the animals were sacrificed and the volume of the exudate and the weight of the pouch were measured. Seromucoid and haptoglobin in the serum were determined as well.

Turpentine Inflammation. In 10 rabbits (five experimental and five control), inflammation was caused by the subcutaneous injection of 1-ml turpentine oil on the back. The experimental animals were injected daily by the same route with 30 μg of apamin/kg in the course of 6 days. The level of haptoglobins in the serum was determined one hour prior to and 3 and 6 days after the application of the agent. The blood was withdrawn by cardiac puncture.

Seromucoid was assayed by the method of Winzler *et al.* (1948). Serum haptoglobin was quantitated by the method of Owen *et al.* (1960). Beta-glucoronidase activity was estimated by the method of Kato *et al.* (1960). Trypsin-inhibitory activity of the serum was assayed by the method of Veremeenko (1971) with casein as substrate. The proteolytic activity of the serum was determined by the method of Veremeenko (1971) with protamin sulfate as substrate. The proteolytic and the trypsin-inhibitory activities of rat sera were measured following subcutaneous injection of 200 mU/kg protease inhibitor (Shkende-

rov, 1973) in Wistar rats for 8 days. Activity of bee venom trypsin inhibitor was assayed by the method of Fritz *et al.* (1970).

Effect on the Spleen Hemolysin-Producing Cells. The number of plaque-forming cells was determined according to the method of Cunningham (1965). The experimental and the control group consisted of 12 albino Swiss mice (16-20 g). The experimental group was treated with 100 μg/kg of the venom component (or 200-mU protease inhibitor/kg) one day before, on the day of immunization (1.5 ml sheep red cell suspension containing 4.10^8 cells/ml, i.p), and on the following day. The control group was immunized with sheep red cells only. The animals were killed 5 days after immunization.

Adrenalectomy of the mice was carried out by bilateral longitudinal section on the back. The operated animals were given a 0.5% solution of sodium chloride *ad libitum.* They were used 10 days after operation.

Test for Inhibition of Denaturation of Serum Proteins. The following proteins were used: human gamma globulin (98.5% purity) and human serum albumin (99% purity) were products of NIEM; bovine serum albumin was obtained from Behringwerke; sheep α and β globulins and bovine fibrinogen (95% clottability) were purchased from Koch-Light. The test was performed as described by Mizushima and Suzuki (1965).

Inhibition of Heat Denaturation. Buffered solutions, 0.067 M, of serum proteins were mixed with various amounts of the fractions to be examined (ranging from 0.3 to 200 μg/ml). The mixtures were left standing for 20 min at room temperature and then exposed to high temperatures as follows: 1% human or bovine albumin at pH 5.3 for 4 min at 67°C; 0.2% human gamma globulin at pH 6.4 for 5 min at 70°C; 0.08% sheep alpha globulin at pH 5 for 1.5 min at 82°C; 0.2% sheep beta globulin at pH 6.8 for 2 min at 85°C; and 0.3% bovine fibrinogen at pH 6.9 for 2 min at 59°C. The controls contained no bee venom and were treated as above.

Protection against Ultraviolet Denaturation. Human or bovine albumin (a 1% solution in 0.067M phosphate buffer, pH 5.3 and 0.2% human gamma globulin in 0.067M phosphate buffer, pH 6.4) with or without a venom fraction was exposed to UV irradiation from a 15-V ultra-violet lamp for 15 min at 15 cm, in a 0.5-cm layer and at a constant temperature of 25 ± 2°C.

Protection against Denaturation on Freezing and Thawing. Fresh human plasma with or without venom components (4-ml plasma in each test tube, 16 by 160 mm) was frozen in an alcohol bath at -40°C and thawed. The procedure was repeated five times.

The degree of denaturation of all samples was read by the O.D. at 660 nm.

***In Vitro* Inhibition of Gelatin-Induced Aggregation of Red Blood Cells (the Method of Görög and Kovacs, 1970).** Blood was taken from the elbow vein of healthy male donors and collected in a centrifuge tube containing 20-U final concentration of heparin. 3-ml samples were pipetted into Wasserman

test tubes. The blood was centrifuged at 2000 rpm for 20 min; the plasma, the leukocytes and the platelets were separated. The packed red blood cells were mixed with 2 ml of a fraction (7 μg/ml) in physiological saline, pH 7.2-7.4. Each series consisted of two pairs of samples (experimental and control).

The test tubes were stoppered with rubber caps and the red blood cells were cautiously suspended by repeated inversion of the tubes. Five minutes later, 2 ml of 2% gelatin was added and the content thoroughly mixed. The degree of aggregation was measured from the rate of sedimentation of the red blood cells. The 30-min sedimentation of the control sample was taken as 100%.

Macrophage-Migration Inhibition. The experimental animals were albino guinea pigs of both sexes weighing from 300 to 350 g. Peritoneal exudate formation was provoked by the method of Chase (1945). The peritoneal cavity was washed with Hank's medium 72 hr following the injection of liquid paraffin. The migration of the macrophages and its inhibition were determined by the method of David *et al.* (1964). The zones of migration were calculated after photographing, copying on transparent paper, and weighing on an analytical balance. The macrophages were suspended in Hank's solution containing 10 μg/kg (10 mU/kg of protease inhibitor) of a bee venom fraction and incubated for 22 hr.

RESULTS

Adjuvant Arthritis. Figure 4 illustrates the development of adjuvant edema. Melittin and apamin caused negligible inhibition of adjuvant edema during the first 10-15 days. Edema became more marked thereafter. Bee venom protease inhibitor fraction and fractions Oa and Op caused 15%-40% inhibition. Phospholipase showed no inhibition but stimulated edema. The values of serum haptoglobins and serum beta glucorinidase are shown in Figs. 5 and 6. It is noteworthy that the inflammatory elevation of haptoglobin and glucoronidase was inhibited 30%-40% by all fractions but phospholipase. The development of adjuvant arthritis, occurring 18-20 days after treatment, is indicated in Fig. 7. The components of the venom caused 15%-25% inhibition, in contradistinction to phospholipase which stimulated arthritis by about 30%.

Granuloma Pouch of Selye. This test as well as that with turpentine oil were carried out only with apamin. Apamin, administered in a daily dose of 30 μg/kg inhibited by 34% of the exudative inflammation. The level of seromucoid and serum haptoglobin (Fig. 8) as well as that of haptoglobin in turpentine-stimulated inflammation (Fig. 9) was considerably more retarded as compared to the controls.

Inflammation of the rat hind paw. Figures 10 and 11 present the results of evaluation of the efficiency of bee venom components in the treatment of inflammation induced by various flogistic agents and mediators of inflammation. The figures include only data from animals injected once before the injection of

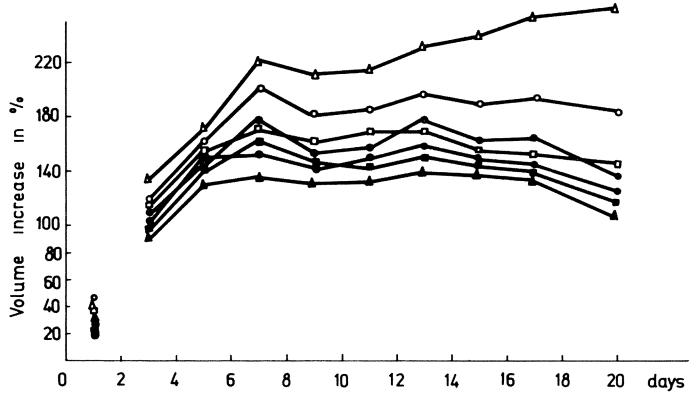

Fig. 4. Adjuvant hind paw edema. Groups consisting of 10 albino Wistar rats were injected with Freund's complete adjuvant (6-mg *Mycobacterium butyricum* in 1.0-ml paraffin oil) below the aponeurosis of the hind paw. The experimental animals each received 100 μg (200 mU protease inhibitor) subcutaneously every day. Control (white circles); phospholipase A (white triangles); fraction Oa (black circles); fraction Op (black squares); protease inhibitor (black triangles); melittin (white squares); apamin (white circles with lines). The differences between the control and the experimental group were statistically significant ($p < 0.05$) on the dates shown below: for protease inhibitor on days 7, 13, 17, and 20; for fraction Oa on days 17 and 20; for fraction Op on days 17 and 20; and for phospholipase A on days 19 and 20.

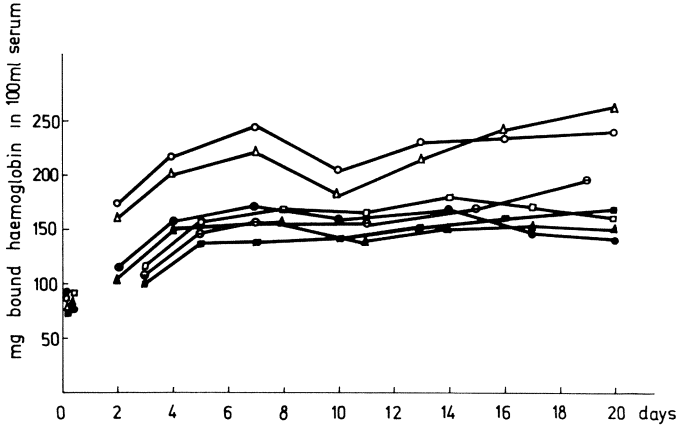

Fig. 5. Serum haptoglobin level in adjuvant treated rats. Blood was taken by cardiac puncture. The differences between control and experimental groups were statistically significant ($p < 0.05$) for the protease inhibitor on days 2, 8, 14, and 20; fraction Op on days 5 and 13; fraction Oa on days 14, 17, and 20; melittin on days 17 and 20; and apamin on day 15. In case the control and the experimental animals were not tested on the same day, the data were interpolated. The designations are the same as in Fig. 4.

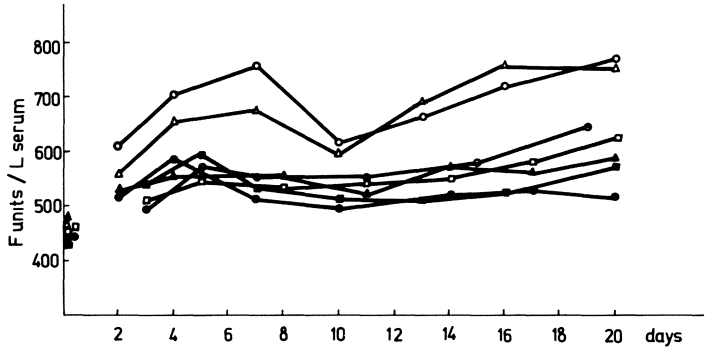

Fig. 6. Serum β-glucuronidase activity in adjuvant-treated rats. The differences between the mean values of control and experimental groups were statistically significant for fraction Oa on days 7, 14, and 17; fraction Op on days 7 and 16, and 20; the protease inhibitor on days 8, 11, 17, and 20; and melittin on days 8, 14, and 17. The evaluation and the designations are the same as in Fig. 4.

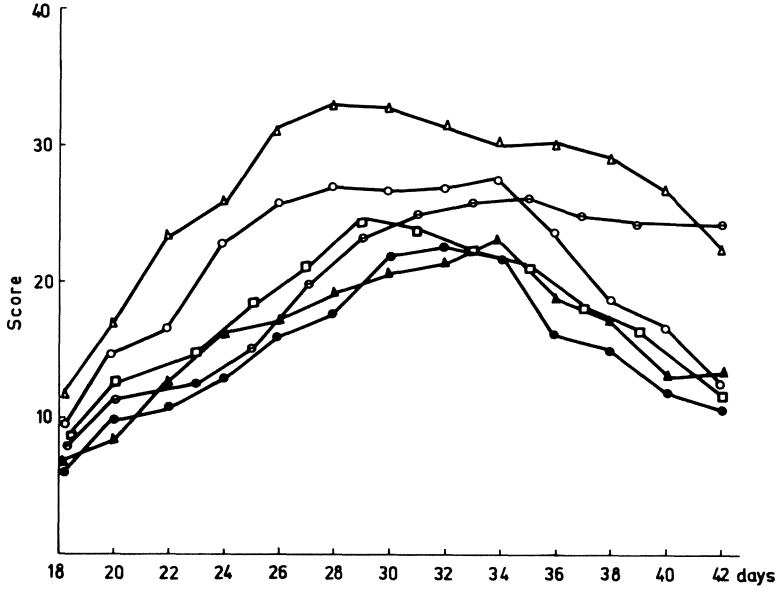

Fig. 7. Adjuvant arthritis evoked in the same rats, which had been treated as described in Fig. 4. The bees venom components were administered daily until the completion of the experiment. The severity of swelling and the redness of joints were recorded on the days shown. Each radiocarpal, tibiodorsal, and proximal interphalangeal joint of the hind and forepaw were graded separately and all metacarpophalangeal and metatorsophalangeal joints of each extremity were graded as a single joint. The grading of arthritis was based on a $0\text{-}4^+$ scale (1^+ representing the least swelling and 4^+ the largest swelling). When the animals were not examined on the same day, comparison was made by interpolation. The designations are the same as those in Fig. 4. Statistical significance was found between the controls and the experimental animals for phospholipase A on days 28 and 30; fraction Oa on days 23, 26, and 28; the protease inhibitor on days 26 and 28; and melittin and apamin on day 25.

the flogistic agent. Repeated administration of any bee venom component did not yield substantially different results. The only exception was the group with histamine-provoked inflammation, where the repeated injections of fraction Op caused 38% higher inhibition than did a single injection and 16% higher in the case of melittin.

Except phospholipase, which intensified histamine-determined inflammation, most of the components caused inhibition of various models of inflammation. Serotonin inflamation appeared to be least inhibited, apamin being the only component affecting it. The carrageenin-induced inhibition turned out to be strongly inhibited by fractions Oa and Op and the proteinase inhibitor, the prostaglandin-stimulated inflammation by fractions Oa and Op, and the bradykinin inflammation was influenced by melittin and the protease inhibitor. Fraction Oa was chromatographed further because of its favorable effect on carrageenin and prostaglandin inflammations. The data in Fig. 12 indicate that the

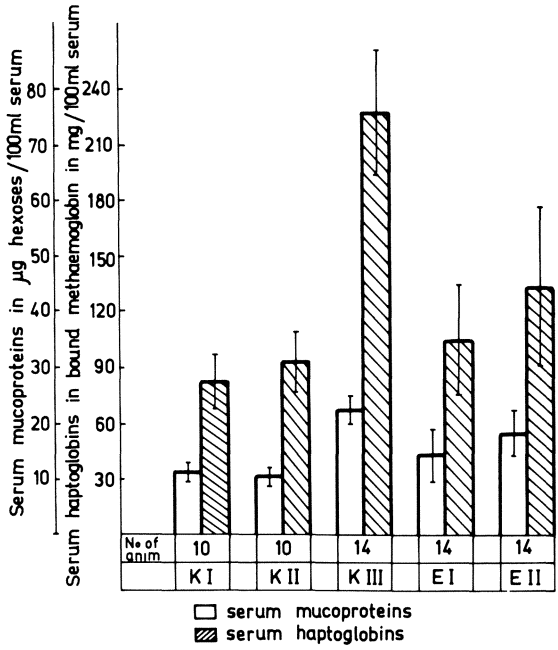

Fig. 8. Levels of the serum mucoproteins and the haptoglobins of the rats with granuloma pouches treated with apamin. K_1, serum mucoproteins and haptoglobins in the control group; K_2, serum mucoprotein and haptoglobin contents of the rats injected subcutaneously with apamin (30 µg/kg for 10 days); K_{III}, mucoproteins and haptoglobins in the rats with granuloma pouch (Selye); E_1, mucoproteins and haptoglobins in the rats with granuloma pouches given 30 µg/kg apamin subcutaneously for 10 days; E_{II}, serum mucoproteins and haptoglobins in rats with granuloma pouches given 2.5 µg/kg hydrocortisone subcutaneously for 10 days.

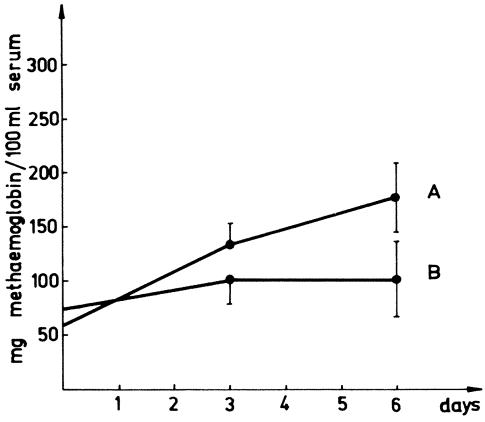

Fig. 9. Levels of the serum haptoglobin of the rabbits with turpentine-induced inflammation (1 ml subcutaneously in the back). The animals (groups of five) were injected with 30 μg/kg apamin for 6 days, starting on the day of turpentine administration. The mean error of the arithmetic means was calculated at $p = 0.05$. A, control group, B, experimental group.

Oa_1, Oa_6, and Oa_8 components of fractions Oa were most efficient with respect to prostaglandin and carrageenin inflammation. Electrophoresis in polyacrylamide gel (Fig. 3) showed that the peaks were contaminated with some components and were still heterogeneous.

The protease inhibitor recently isolated from bee venom (Shkenderov, 1973) may be regarded as an anti-inflammatory component of the venom. Its effects

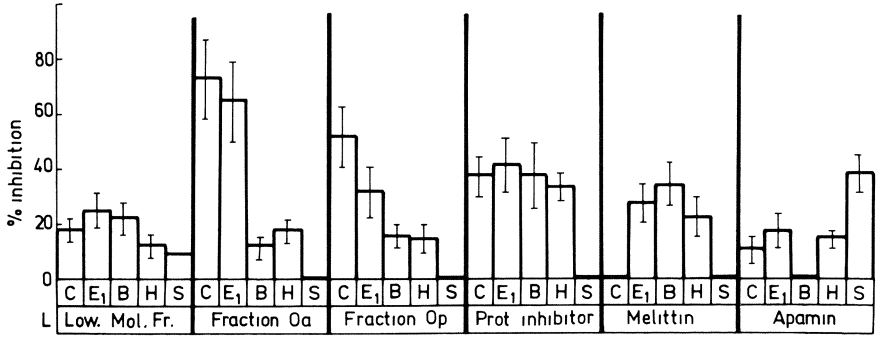

Fig. 10. Hind paw edema test. Inhibition of carrageenin (C), prostaglandin (E_1), bradykinin (B), histamine (H), and serotonin (S) hind paw inflammation induced with bee venom fractions (10 μg; 20 mU protease inhibitor) administered half an hour before the application of the inflammatory agents. Carrageenin inflammation was measured in 3 hr; prostaglandin and bradykinin in 0.5 hr; histamine and serotonin in 1.5 hr. The mean errors of the arithmetic means were calculated at $p = 0.05$. Groups of six animals were included.

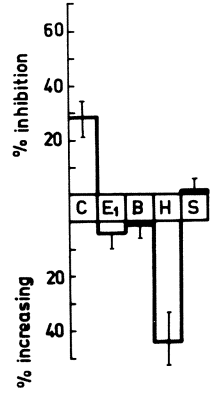

Fig. 11. Changes in hind paw edemas (the same as in Fig. 10) in the animals treated with bee venom phospholipase A (10 μg/kg).

on the proteolytic activity as well as on the trypsin-inhibitory activity of rat serum were examined. It was found that the whole venom, the low-molecular dialyzable and the partially purified protease inhibitor, in doses of 100 μg/kg or 200 mU/kg for 8 days, reduced the proteolytic activity of the serum and enhanced its inhibitory activity (Fig. 13).

Immunosuppressive Effect. Figure 14 reveals the effect of 100 μg/kg of each venom component on the number of hemolysine-forming spleen cells during the primary immune response. Melittin, apamin, and the protease inhibitor (200 mU/kg) caused about 4-fold, 3-fold, and 2-fold inhibition, respectively.

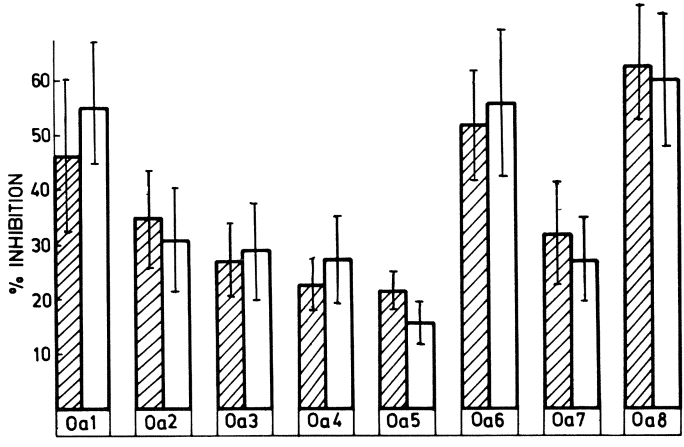

Fig. 12. Anti-inflammatory effect of Oa_1-Oa_8 fractions. The hatched columns show inhibition of carrageenin hind paw edema. Blank columns indicate the inhibition of prostaglandin E_1 edema. Oa fractions (10 μg/kg) were injected half an hour prior to the administration of the anti-inflammatory agent. Groups of six animals were included. The mean error of the arithmetic means was calculated at $p = 0.05$.

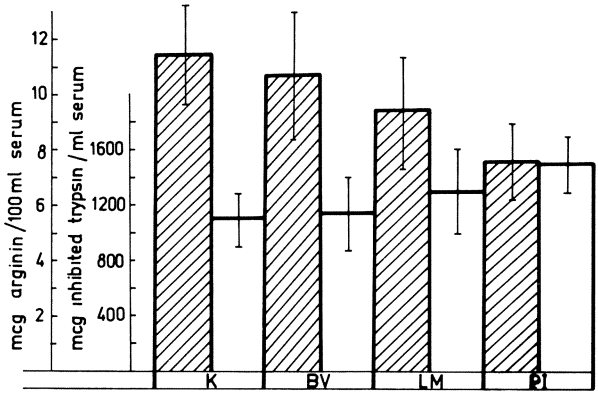

Fig. 13. Serum proteolytic activity (hatched columns) and serum trypsin inhibitory activity (blank columns) of the rats (groups of 8) injected for 8 days with 100 μg/kg whole bee venom (BV); 100 μg/kg low molecular weight fraction (LM); and 200mU bee venom protease inhibitor (PI). The control group received physiological saline.

The first fraction (Fr. I) exerted a stimulatory effect, whereas phospholipase, hyaluronidase, and fraction Op had practically no effect. Adrenalectomized animals, immunized and treated with melittin, showed a double number of the immunocompetent cells.

Inhibition of Denaturation of the Serum Proteins. The experimental data are summarized in Fig. 15. The values each are means of 3-5 determinations. The concentrations of melittin and apamin were optimal. As may be seen, both of them afforded the best protection against heat denaturation. They exerted

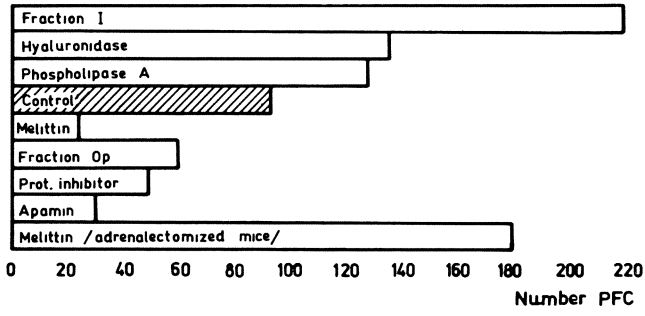

Fig. 14. Immunosuppressive effect of bee venom fractions. Number of hemolysine plaque-forming cells/10^6 spleen cells taken from the mice treated with 100 μg/kg of each venom component (200 mU/kg of protease inhibitor) on the day before, on the day of immunization, and on the following day.

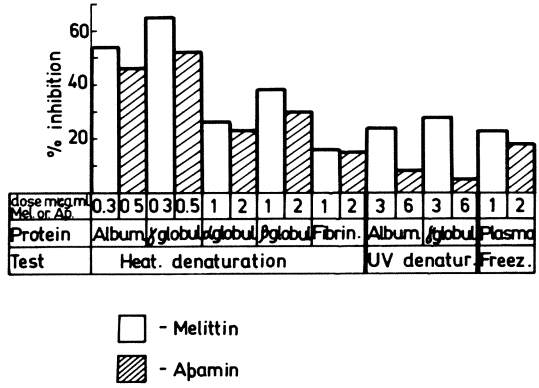

Fig. 15. Protein denaturation test. Inhibition with melittin and apamin of denaturation of serum proteins caused by heat, UV light, and freezing and thawing.

a stronger protective effect on albumin and gamma globulin in the case of heat denaturation and UV irradiation. The test for protection from denaturation of plasma on freezing and thawing is in fact related to the stability of plasma lipoproteins which are subject to denaturation on freezing and thawing.

Apamin appeared to be less inhibitory and the optimal effect was achieved at higher concentrations than those of melittin.

Melittin, in doses surpassing 50 μg/ml, brought about protein denaturation.

Inhibition of Gelatin-Produced Red Cell Aggregation. Figure 16 shows that fraction Oa caused complete inhibition contrary to melittin, apamin and the protease inhibitor which lacked activity.

Inhibition of Macrophage Migration. Preliminary results (Fig. 17) point to the strongest inhibitory effect of melittin and the protease inhibitor on the macrophage migration.

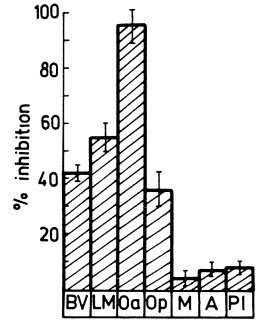

Fig. 16. Inhibition of gelatin-induced aggregation by whole bee venom (BV), low molecular weight fraction (LM), fraction Oa (Oa), fraction Op (Op), melittin (M), apamin (A), and protease inhibitor (Pi); the mean error of the arithmetic means (mean of 6 experiments) was calculated at $p = 0.05$.

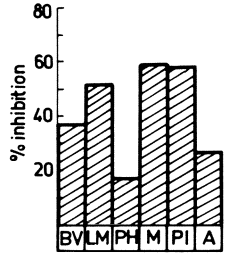

Fig. 17. Inhibition of macrophage migration. The symbols are the same as those in Fig. 16. Phospholipase A (PH).

DISCUSSION

All known peptide components of bee venom and the enzyme phospholipase A, which is presumed to be involved in the anti-inflammatory action of the venom, were included in this study. As the studies are still in progress, some of the components were not analyzed by all tests. The *in vivo* and *in vitro* tests employed are well known. They are more or less related to various aspects of the mechanism of action of nonsteroid anti-inflammatory drugs (Paulus and Whitehouse, 1973; Ferreira and Vane, 1974).

Adjuvant inflammation and polyarthritis were only slightly affected by melittin, Oa, Op, and apamin, and mildly by the protease inhibitor in the doses used. These results are in agreement with the findings on the role of protease activation in the inflammatory process (Bertelli *et al.*, 1969). It has been shown that an animal protease inhibitor, Trasylol, impairs the tissue reaction caused by Freund's complete adjuvant (Laurentaci, 1969) and inhibits various hind paw edemas (Förster, 1969; Kaller *et al.*, 1966). The parallel inhibition of the inflammatory elevation of serum haptoglobin and β glucuronidase suggests a favorable effect on the pathogenetic mechanisms of inflammation: the alteration of mucoprotein metabolism and stability of lysosomes. In the case of the granuloma pouch of Selye and turpentine inflammation, the lowered levels of seromucoid and haptoglobin following apamin treatment provided another line of evidence (Ovcharov *et al.*, 1976). The tests with edema of rat paws provide information about the direct effect of small doses of the venom components on the inflammatory activity of flogistic agents and the mediator of inflammation. Paulus and Whitehouse (1973) claimed that carrageenin- and prostaglandin-stimulated edema offer a more reliable test than adjuvant arthritis in the proper choice of drugs against chronic inflammation. The marked anti-inflammatory effect of fractions Oa and Op on carrageenin and prostaglandin inflammation, and the complete inhibition of red cell aggregation deserve special attention. As a matter of fact, the findings of Hanson *et al.* (1974) have already demonstrated that MCD-peptide, the major component of Op, manifests a strong anti-inflammatory effect and acts directly on the inflammation-induced changes. Our data on fraction Oa, which has been thought so far to be a by-

product, open prospects for its detailed investigation and eventual utilization. Preliminary data characterize it as weakly toxic (LD_{50} –20 mg/kg, i.v., for mice). Its constituents, Oa_1, Oa_6, and Oa_8, which carry most of its anti-inflammatory activity, seem especially intriguing. Oa_1 proved to have been contaminated by phospholipase A.

The protease inhibitor exhibited strong activity against carrageenin, prostaglandin, bradykinin, and adjuvant inflammation. Its capacity to lower the proteolytic activity of the serum, with protamine sulfate as a substrate, is a manifestation of its *in vitro* plasmin-inhibitory activity. Plasmin is known as a trigger factor for inflammation mechanisms (Seidel *et al.*, 1971). The chymotrypsin- (acetyl-tyrosin-ethylester as substrate) and leucine-aminopeptidase-(leucine-p-nitro-anilide as substrate) inhibitory activities of the inhibitor (Shkenderov, 1975) contribute to its anti-inflammatory activity (Förster, 1969).

It has previously been shown (Kousturkov and Shkenderov, 1974) that the low-molecular components of the venom possess an immunosuppressive effect, whereas the high-molecular components stimulate the immune response. The above data (Fig. 14) indicate that the immunosuppressive effect of the venom is a property of melittin and apamin and partially of the protease inhibitor. The immunosuppressive effect of melittin and presumably of apamin is due to the stimulation of the function of the hypophysocorticorenal system, whereas the enhanced immune response in adrenalectomized animals treated with melittin is most probably associated with the direct effect of ACTH on immunogenesis. The immunosuppressive action of bee venom protease inhibitor may be due to impairment of the cellular recognition of antigen of the associated immune response (Laurentaci, 1969). From the viewpoint of modern concepts on the mechanism of action of anti-inflammatory drugs, their immunosuppressive activity is an inherent property (Paulus and Whitehouse, 1973). The doses employed in these *in vivo* investigations were of the order of 0.0025-0.025 LD_{50}, i.v., for mice.

The inhibition of denaturation of the serum proteins by melittin and to a lower degree by apamin is of particular interest not only as a common property of most anti-inflammatory substances (Mizushima and Suzuki, 1965; Mizushima and Kobayashi, 1968), but is in fact an interesting phenomenon of macromolecular interaction. This is probably related not only to the detergent properties but to its primary structure.

Macrophage inhibition manifested by melittin and the protease inhibitor decreased the initiated part of leukocytes in inflammation and immunogenesis. According to Di Rosa *et al.* (1971), nonsteroid anti-inflammatory agents exert their action primarily on leukocyte emigration.

Our studies indicate that all investigated peptides in bee venom exhibit some of the properties of nonsteroid anti-inflammatory drugs. The venom represents a source of diverse natural anti-inflammatory substances.

Phospholipase A increases some model inflammations, but has no effect on others, and only to a certain extent inhibits carrageenin inflammation. The results do not give us sufficient evidence to assume it as an inflammatory substance. The supposition made by Zurier and Weissmann (1972), that phospholipase A is an anti-inflammatory component of bee venom, stands no chance of being confirmed. The reverse effect, increasing inflammation, is worth our notice. However, it potentiates histamine inflammation and deteriorates the development of adjuvant arthritis. Whether the former is due to the potent histamine-liberating effect (melittin and fraction Op are histamine liberators but display the reverse inhibitory effect) and the latter to its ability to degrade the lipoprotein membranes (including lysosomal) (Weissmann *et al.*, 1964) and the slight immunostimulating effect remain to be seen.

SUMMARY

Chromatographic fractions (Oa, Op, and the protease inhibitor) and pure proteins and peptides from bee venom (melittin, apamin, and phospholipase A) were tested in accordance with some of the tests for nonsteroid anti-inflammatory drugs. Adjuvant arthritis under the effect of the employed doses of 100 μg/kg is weakly affected by melittin and apamin and moderately affected by the protease inhibitor and the fractions Oa and Op. Phospholipase A aggravates the development of adjuvant inflammation. The inflammatory rise of the levels of haptoglobin and β-glucuronidase with adjuvant arthritis is considerably inhibited under the effect of the bee venom components examined, with the exception of phospholipase, which does not manifest any inhibitory effect. Seromucoid and haptoglobin also decrease in the case of granuloma pouch and turpentine inflammation under the effect of apamin. In the hind paw edema tests, the inhibition of the carrageenin and prostaglandin E_1 inflammation under the effects of Oa and Op fractions and the protease inhibitor are strongest. Melittin, apamin, and the protease inhibitor show immunosuppressive actions. The test for protection of denaturation of the serum proteins is positive with melittin and apamin. The Oa fraction strongly inhibits gelatin-induced aggregation of erythrocytes. Melittin and the protease inhibitor inhibit macrophage migration.

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Some Chemical and Pharmacological Studies on Two Venomous Jellyfish

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INTRODUCTION

Venomous jellyfish are widely distributed throughout the world. Two of these animals found in American waters are the sea nettle (*Chrysaora quinquecirrha*) and the Portuguese man-o-war (*Physalia physalis*). Both these coelenterates are capable of injuring humans with their tentacles. Painful urticarial cutaneous lesions, local muscle cramps, corneal ulcerations, and occasionally respiratory distress are symptoms which can follow contact with their tentacles (Cleland and Southcott, 1965). *P. physalis* is generally regarded as the more toxic animal. The presence of these organisms can be economically detrimental, as well as a nuisance and a health hazard. It has been estimated that recreational use of the Chesapeake Bay is reduced 30% by the sea nettle, thereby decreasing property value and tax income. Recently, fisherman off the Virginia coast reported catching sea nettle masses so great that net damage may run as high as a million dollars. The man-o-war interferes with ocean bathing and fishing along the seafront of many Florida and Gulf coast cities. The recent investigations reported below were conducted to further delineate the chemical and pharmacological properties of the venoms obtained from the nematocysts of these animals.

MATERIALS AND METHODS

Tentacles were removed from live *C. quinquecirrha* and *P. physalis* and prepared for use by grinding and centrifugation according to prior techniques

(Burnett and Goldner, 1970b; Burnett and Gould, 1971a; Burnett *et al.*, 1968b). The density of the nematocysts was determined by density-gradient centrifugation (Burnett and Calton, 1973; Calton and Burnett, 1973b). The best method of preparation of the *Physalia* nematocyst venom was by density-gradient centrifugation followed by extrusion pressure (Calton and Burnett, 1973b). Previous preparative techniques employing autolysis (Lane and Dodge, 1958) did not free the organelles from microscopic debris, and the total yield of lethal toxin was thereby reduced (Burnett and Calton, 1973). In order to prevent loss of toxin by premature nematocyst destruction, *Chrysaora* nematocyst venom was prepared by subjecting nematocyst suspensions to zonal centrifugation and destruction either by sonication or extrusion pressure (Burnett and Calton, 1973).

Molecular-weight determinations of nematocyst venoms were made by ultracentrifugation (Burnett and Calton, 1974a). Purification of the nematocyst venoms was undertaken with gel filtration, ammonium sulfate precipitation, and isoelectric focusing (Burnett and Calton, 1974a; Burnett and Goldner, 1970b; Burnett and Gould, 1971a; Calton and Burnett, 1973b; and Burnett and Calton, 1973). Analysis of the chemical contents and enzymatic constituents of the venoms was performed as previously described (Goldner *et al.*, 1969; Burnett and Calton, 1974b).

The physiological tests employed include mouse lethality, hemolysis, and dermonecrosis (Burnett *et al.*, 1968b; Burnett and Goldner, 1971). The cardiac response to nettle toxins was measured on anesthetized rats or isolated canine Purkinje fiber preparations (Burnett and Goldner, 1969; Klinehaus *et al.*, 1973). The effect of nettle toxins on peripheral nerve and muscle was recorded as described earlier (Burnett and Goldner, 1970a).

The effect of nettle and man-o-war toxins on sodium transport across frog skin and toad or turtle bladder preparations was measured (Gould and Burnett, 1971; Saladino, Calton and Burnett, unpublished).

Immunological assays including neutralization tests, immunodiffusion, complement fixation tests, and immunoelectrophoresis were also performed (Burnett and Goldner, 1970b; Burnett and Gould, 1971a,b). Intact fishing tentacles from nettle adults and polyps were examined under the electron microscope (Burnett and Sutton, 1969; Sutton and Burnett, 1969; Burnett, 1971).

The content of histamine, serotonin, kininlike compounds, prostaglandins, and histamine releasing agents in these two animal venoms was also measured (Goldner *et al.*, 1969; Burnett and Calton, 1974a; Burnett *et al.*, 1975).

Several series of experiments exposing the epilated dorsal skin of five guinea pigs to live sea nettle tentacles were conducted (Burnett *et al.*, 1968a).

RESULTS

Nematocysts. Several types of nematocysts were present on the tentacles of both the sea nettle and the man-o-war (Burnett and Sutton, 1969; Cleland

and Southcott, 1965; Calder, 1971). It was extremely difficult to differentiate nascent from adult nettle nematocysts and to state positively the function of each organelle (Sutton and Burnett, 1969; Burnett, 1971). Although numerous morphological types of sea nettle nematocysts have been described, only one band of nematocysts appeared after density-gradient centrifugation (specific gravity, 1.22). On the other hand, *Physalia*, which appeared to contain fewer morphological nematocyst types, yielded two distinct bands. Smaller nematocysts appeared at a specific gravity of 1.28, whereas the larger structures had a specific gravity of 1.32 (Burnett and Calton,.1973; Calton and Burnett, 1973b).

The isolated nematocysts of these two species differed in the threshold of mechanical trauma necessary for rupture. Most sea nettle nematocysts were ruptured by sonic treatment and some by density-gradient centrifugation, whereas *Physalia* nematocysts could withstand these procedures. Even though *Chrysaora* nematocysts were less stable they were refractory to many physical and chemical treatments (Burnett *et al.*, 1968b). It appeared that no one chemical or physical treatment could rupture all forms of the nematocysts with equal efficiency. When released, the nematocyst threads extend 200-400 μ (Rice and Powell, 1970). This is a significant depth in human skin.

Studies on the lethal factor of these toxins demonstrated that the mouse was superior to the fiddler crab as an assay animal because a larger inoculum could be accepted (Burnett and Calton, 1973; Calton and Burnett, 1973b). This was an essential feature since concentration techniques sometimes compromise toxicity. No diagnostic pathological findings were detected in mice who had received lethal doses of the toxin (Burnett *et al.*, 1968b; Lane and Dodge, 1958). The nematocysts were found to contain the important coelenterate venoms (Burnett *et al.*, 1968b; Burnett and Goldner, 1970b; Lane and Dodge, 1958).

Partial Purification of Nematocyst Venoms. The nematocyst venoms of both animals were viscous fluids which were stable to lyophilization, three cycles of freeze-thaw and storage at -90° C for over two years (Burnett *et al.*, 1968b; Burnett and Goldner, 1970b; Calton and Burnett, 1973b), but sensitive to trypsin (Lane and Dodge, 1958; Burnett and Goldner, 1970b). It appeared that the lethal factors of *Chrysaora* were less stable at 4°C than were those of *Physalia* (Burnett and Goldner, 1970b; Calton and Burnett, 1973b). Both venoms lost activity in organic solvents (Lane and Dodge, 1958; Burnett and Goldner, 1970b). Sea nettle venom was also resistant to x ray (Burnett *et al.*, 1974) and was stable over a pH range of 6-9.5 (Burnett and Goldner, 1970b).

The nematocyst venoms of both jellyfish possessed a lethal polypeptide having a molecular weight of approximately 150,000 (Burnett and Calton, 1974a). However, most of the protein in the nettle venom had a molecular weight of approximately 19,000. It is possible that the larger component is an 8-unit polymer of the smaller protein.

Only *Chrysaora* venom could be efficiently purified by ammonium sulfate

precipitation. Almost all of the pharmacologically active preparation remained in the supernate after ammonium sulfate was added to a concentration of 20% (Burnett and Calton, 1974a).

Each of these animal venoms contained two lethal fractions with similar isoelectric points. Most of the nettle lethal protein had a pI near 6.3. A smaller fraction was detected at a pI of approximately 4.0. Isoelectric focusing of nettle venom treated with 20% $(NH_4)_2SO_4$ yielded similar results. *Physalia's* lethality was primarily at a pI of 6.5 with a smaller fraction at pI 4.5 (Burnett and Gould, 1971a; Burnett and Calton, 1974a). None of the fractions obtained from isoelectric focusing of nematocyst venoms were sufficiently pure that they contained single bands on disk gel electrophoresis.

Gel filtration with Sephadex was performed at pH values ranging from 6.8 to 8.6 and with eluate solutions ranging in ionic strength from 0.001 to 0.05. In earlier experiments two lethal fractions were isolated by this method (Burnett and Goldner, 1970b; Blanquet, 1972). The largest of these two fractions was found to contain multiple bands on analytical disk gel electrophoresis (Burnett and Goldner, 1970b) and was divided into two additional subfractions by isoelectric focusing (Burnett and Gould, 1971a). Neither of these two subfractions yielded single bands on disk gel electrophoresis. When the elution process from Sephadex gel columns was continued for several bed volumes, more lethal fractions were detected, suggesting the fact that the *Chrysaora* venom adhered to the Sephadex (Burnett and Calton, 1973). A similar phenomenon occurred after inoculating *Physalia* venom onto Sephadex gels (Calton and Burnett, 1973b). In both instances lethal activity was detected in fractions which had no spectroscopically detectable protein, suggesting that some toxins are extremely potent. It was also observed that some of these purified lethal fractions were not stable after being frozen and stored at −90°C.

At least nine lethal fractions could be isolated by preparative gel electrophoresis from the nematocyst venom of each animal. A similar number of lethal fractions was also obtained from *Chrysaora* nematocyst venom partially purified by ammonium sulfate (Burnett and Calton, 1974a). These fractions contained only single bands after analysis by disk gel electrophoresis. Our results correlate closely with an earlier report of eight lethal polypeptides isolated from *Physalia* toxin by paper chromatography (Lane *et al.*, 1961). Repeated attempts to purify *Chrysaora* venoms by several anionic and cationic exchange resins failed because the lethal venoms were not eluted from the column.

Chemical Content. Since none of these toxins have been isolated, no definitive data on their chemical contents can be made. Nevertheless, some useful information is available. The nematocyst contents were primarily proteinaceous. Little fat or carbohydrate was present (Goldner *et al.*, 1969; Lane, 1960; Rice and Powell, 1970). It was possible that some of the fat was free fatty acid or cholesterol (Goldner *et al.*, 1969; Middlebrook and Lane, 1968; Stillway, 1974).

Chrysaora and *Physalia* nematocyst venoms possessed at least six or seven enzymatic actions (Burnett and Calton, 1974b). ATPase, nonspecific aminopeptidase, RNase, and DNase were present in the venoms of both animals. The ATPase and RNase in these two venoms were not identical since they had different pH optima. In the case of RNase the pH optima was 7 and 8 for the nettle and man-o-war, respectively. *Physalia* alone possessed an AMPase and a fibrinolysin. Contrariwise *Chrysaora* alone contained an acid protease, an alkaline protease, and a hyaluronidase. These enzymes were present in physiological doses of the venom. If high doses of the venoms were used, *Chrysaora* toxins contained esterases whereas *Physalia* possessed a phospholipase (Burnett and Calton, 1974b; Stillway and Lane, 1971).

No information was available to determine whether the toxins or enzymes within the unruptured nematocysts were present in active or inactive form. The nematocyst wall of the sea nettle was thick and was composed of a collagenlike material which provided the fluid venom with a stable covering (Stone *et al.*, 1970; Sutton and Burnett, 1969).

Although an amino acid analysis had been performed on the nematocyst fluids of both animals (Blanquet, 1972; Lane and Dodge, 1958), the nematocysts used in these studies were not purified by density-gradient centrifugation. Thus the toxin analyzed was not pure and contained considerable nonlethal protein (Burnett and Goldner, 1970b; Burnett and Gould, 1971a; Lane and Dodge, 1958). It was reported that high levels of glutamic acid and low levels of aromatic amino acids were present in both venoms. Aspartic acid was present in considerable amounts in *Chrysaora* venom but was not detected in that of *Physalia*. Certain procedural pitfalls might affect the results of the analysis of nettle venom: (a) Sephadex adsorbs the aromatic and basic amino acids and would repell the acidic ones allowing them to appear in abundance early in the eluate (Burnett and Calton, 1973) and (b) tyrosine can be lost during hydrolysis of fluids containing liberal amounts of aspartic acid (Blanquet, 1972). Nonetheless, we can deduce that there is considerable glutamic acid in or near the nematocysts of these animals.

Pharmacology. The toxins of both jellyfish were lethal to mice and the fiddler crab, *Uca pugilator*, and produced dermonecrosis in several vertebrates. A comparison of *Physalia* and *Chrysaora* nematocyst venoms containing equal amounts of protein showed that they were equipotent in lethal activity, yet the nettle produced more dermonecrosis (Calton and Burnett, 1973b).

Both the nettle and the man-o-war were cardiotoxic (Burnett and Goldner, 1970a; Lane, 1967). The depression of the ST wave and the production of atrioventricular block by sea nettle venom was thought to be similar to the cardiac changes induced by hypocalcemia (Klinehaus *et al.*, 1973). Indeed, these investigators demonstrated that the nettle venoms acted on the Purkinje fibers rather than the papillary muscle of the isolated dog heart. A loss of resting

potential, a diminution of upstroke velocity, a reduced conduction velocity, and a partial and later complete block between cells of the same bundle occurred after contact with nettle nematocyst venom. Finally, a reduction of spontaneous activity and an eventual loss of excitability ensued. These changes appeared to mimic the effect of decreased availability of calcium to the Purkinje fiber and were not due to chelation of ionic calcium in the surrounding medium. The electrocardiographic abnormalities were accompanied by a transient rise in the blood pressure. The blood pressure later fell when a significant A-V block appeared or when evidence of peripheral vasodilation occurred. In some instances the pulse decreased slightly, immediately after injection of the toxin.

Sea nettle and man-o-war venoms affected peripheral nerves (Burnett and Goldner, 1970a; Burnett *et al.*, 1974; Larsen and Lane, 1970a). The nettle's venom decreased the action potential of peripheral nerve without significantly altering the conduction time. These experiments were initially performed in a nerve chamber and later repeated by us using sleeve electrodes. No abnormal changes were seen in voltage clamp experiments involving the giant axon of the marine worm or the squid. This apparent conflict could be due to a difference in either the conditions of the preparation or the subject animal. Sea nettle venom in physiologic doses was capable of reducing the miniature end plate potentials (MEPP) of the rat phrenic nerve-diaphragm preparation which indicated an effect on the acetylcholine receptors. No change in MEPP frequency was found, indicating that the toxin did not depolarize nerve terminals nor change muscle membrane potentials.

The musculotoxic action of both nettle and man-o-war venoms was similar. The action potential of skeletal muscle was decreased by the injection of nettle toxins into the muscle body between the electrodes (Burnett and Goldner, 1970a). This myotoxic action may be explained by the observation that the venom of either jellyfish affected the sarcoplasmic reticulum (Calton and Burnett, 1973a).

The venom of both jellyfish was destructive to smooth muscle (Burnett and Goldner, 1970a; Burnett *et al.*, 1975). Both toxins produced ileal contraction before ablating the muscle's electrical impulses. It is possible that the gut contraction was produced by a kininlike substance before another polypeptide or enzyme destroyed the smooth muscle (Garriott and Lane, 1969; Kaplan *et al.*, 1976).

Sea nettle and man-o-war venoms can directly affect membranes. Venom from the former animal was hemolytic and ruptured mitochondria, lysosomes, and the sarcoplasmic reticulum (Burnett and Goldner, 1971; Calton *et al.*, 1973; Burnett and Gould, 1971a; Calton and Burnett, 1973a). However, nettle venom did not affect either the permeability of human stratum corneum to tritiated water or the glucose-6-phosphatase content of rat liver microsomal membranes (Burnett *et al.*, 1974). *Physalia* lacked a hemolysin (Burnett and Calton, 1974a;

Lane, 1960) but had factors which damage the mitochondria and sarcoplasmic reticulum. These factors were heat stable in contrast to those of the nettle.

Nettle venom did not inhibit the *in vivo* growth of P 388 mouse leukemia or lyse cultured human embryonic kidney or KB cells (Burnett *et al.*, 1974). *Physalia* toxins did not destroy intact *Paramecia* or *Tetrahymena* (Lane *et al.*, 1961).

Both venoms increased the transport of sodium across frog skin (Gould and Burnett, 1971; Larsen and Lane, 1970b). These changes were not accompanied by detectable ultramicroscopic changes (Saladino, Calton, and Burnett, unpublished). Similar doses of these venoms did not increase the transport of sodium across the toad or turtle bladder. Since the increased sodium transport across the frog skin occurred after 10–30 min, rather than instantaneously, we hypothesized that the measured increase in short-circuit current produced by these toxins was due to increased sodium transport and not to outward movement of chloride from the mucous glands of the frog skin (Koeford-Johnsen *et al.*, 1952). This sodium effect of the sea nettle venom occurred after the toxin was applied to either the dermis or epidermis and was not accompanied by detectable alterations of cyclic AMP- or Na-dependent ATPase (Gould and Burnett, 1971). *Physalia* venom, on the other hand, altered sodium transport of frog skin only when applied to the dermis.

The cutaneous pain inflicted by the venoms of these animals could well be due to jellyfish destructive enzymes or lysosomal rupture. The pain could also be produced by histamine, histamine releasers, serotonin, kininlike substances, or prostaglandins E and F, all of which are associated with the sea nettle venom (Burnett and Calton, 1974a). Sea nettle venom did not contain any slow reacting substance of anaphylaxis. The *Physalia* kininlike substances were not as potent as those in the nettle. The other above-mentioned mediators of inflammation were not detected in man-o-war venom (Burnett *et al.*, 1975; Garriott and Lane, 1969; Lane, 1960).

It appeared that the rapid death seen in mice after intravenous injection of the nettle venom was due to cardiotoxity (Burnett and Goldner, 1969; Klinehaus *et al.*, 1973). Delayed deaths up to 48 h were probably the result of renal failure with azotemia and hyperkalemia in guinea pigs (Table 1). The elevation in serum potassium seen within 8 min of intravenous administration of *Physalia* venom (Hastings *et al.*, 1967) was thought to be a result of an interruption of active sodium and potassium transport. However, rapid renal failure would produce the same reaction. Endotoxin production and complementary abnormalities did not accompany nettle venom injection (Burnett *et al.*, 1974).

Physalia and *Chrysaora* toxins were weakly antigenic (Burnett and Goldner, 1971; Burnett and Gould, 1971b; Calton and Burnett, 1973b). High-titer antibodies could not be produced in rabbits either by injecting active venom or heat or formalin-inactivated venom with or without adjuvants over a course of many

Table 1. Guinea Pig Serum Chemistries after Intravenous Injection of a Sublethal Dose of Sea Nettle Nematocyst Venom[a]

	Hours after injection			
	0	4	21	22
CO_2, meq/liter	15	21	17	14
Cl, meq/liter	107	106	108	104
Na, meq/liter	140	135	143	134
K, meq/liter	5.0	5.0	5.5	13.3
BUN, mg%	28	41	82	88

[a] Blood sugar, calcium, and bilirubin values were normal.

months. Evidence of low-titer cross-reacting protective antibodies was found. Some immunized rabbits failed to develop a cutaneous eruption after contact with live nettle tentacles (Burnett and Goldner, 1971). Other uses of these immunization programs have not been successful.

Human cutaneous pain was prevented by incubating nettle nematocyst venom with equal parts of either normal or hyperimmune rabbit sera but not by incubating *Physalia* nematocyst venom with low-titer sera (capable of neutralizing 8 mouse i.v. LD_{50}'s). Thus, cutaneous pain has not been effectively counteracted by specific antisera. It is possible that protective antisera or toxoids could be developed if toxin purification were more advanced or a better immunization schedule were developed.

Medical Aspects. Experiments attempting to develop a topical application to block the nettle's sting were performed (Burnett *et al.*, 1968). The thick application of an oil-base ointment was an effective prophylactic. The only known agents which, if incorporated into this base, increased its efficacy were alkali, mercaptoethanols, or detergents; none of which were cosmetically attractive.

Therapy of the sting was disappointing. Uncontrolled studies demonstrating the usefulness of topical papain have been published (Cargo and Schultz, 1967; Arnold, 1971). In our laboratory these results could not be corroborated in two series of controlled experiments using human volunteers who were stung with live nettle tentacles. Antihistamines and cortisone were ineffective in preventing the appearance of the eruption in guinea pigs (Burnett *et al.*, 1968a). The simultaneous or prior injection of high levels of calcium with nettle venom did not reduce its lethality in mice (Burnett and Calton, unpublished). Atropine did appear to temporarily arrest some of the cardiovascular abnormalities induced by the nettle venom in the rat (Burnett and Goldner, 1969). The same drug did not protect mice against the *Physalia* lethal factor (Garriott and Lane, 1969). Epinephrine adversely affected the spontaneous activity of the canine Purkinje fiber treated with nettle venom, yet calcium protected the fiber's activity

(Klinehaus *et al.*, 1973). Potassium appeared to counteract the cardiovascular effects of man-o-war toxin (Hastings *et al.*, 1967). Methysergide and antihistamines both reduced the vasopermeability produced by the nettle venoms (Burnett and Calton, 1974a). Corticosteroids did not stabilize the lysosomes against nettle venom nor inhibit the vasopermeability induced by this venom (Burnett and Gould, 1971a; Burnett and Calton, 1974a). Parenteral rabbit antisera had only a partial protective effect, whereas systemic antihistamines, anti-5-hydroxytryptamine agents, and corticosteroids had no effect on the dermonecrotic action of the venom of another jellyfish, the sea wasp (Keen and Crone, 1969; Baxter and Marr, 1974).

DISCUSSION

The sea nettle and man-o-war are distantly related animals. Although both are coelenterates they are not classified in the same subphyla. In spite of this fact there are many similarities and contrasts (Tables 2 and 3).

It is not known how many pharmacologically active factors are present in these venoms. At least six different factors are present in nettle venom (Table 4). The heat-labile human cutaneous pain factors were located in three different fractions after sucrose density centrifugation. Some of these fractions were identical with those which contained dermonecrotic, lethal, or mitochondrial lysis factors (Burnett and Calton, 1974a). Dermonecrosis was more heat stable than lethality and also carboxypeptidase-B resistant (Burnett and Goldner, 1971; Burnett and Calton, 1974a). The agents responsible for this action were either in the sediment or heavier fractions of the centrifuged 5%–20% sucrose gradient. The hemolysin was more prevalent in the lower titer than the dermonecrotic factor. Although they were both neutralized by the same hyperimmune sera, they had similar but not identical sensitivities to heat denaturation (Burnett and Goldner, 1971). The vasopermeability factor was heat stable but was carboxypeptidase-B labile. The nettle mitochondria and sarcoplasmic reticulum rupturing factors were heat sensitive and could be identical. The mitochondria rupturing factor and the multiple pain producers have different densities in a sucrose gradient. The gut contracting kininlike factor was carboxypeptidase-B resistant and heat labile. The lysosomal rupturing factors and frog skin permeability factors were heat labile and were neutralized by antilethal sera, thereby being similar to the lethal factor. The cardiotoxic action appeared to be the basis of lethality. The myotoxin and neurotoxin were also heat labile and difficult to classify because of insufficient data.

It must be emphasized that some differences in the chemical properties and pharmacological actions of these as well as other jellyfish venoms will be a result of the different preparative techniques employed by different investigators at different times. Also, it is possible that collection sampling errors explain some

Table 2. Known Differences Between *Physalia* and *Chrysaora* Nematocysts and Their Toxins

	Physalia	*Chrysaora*
Morphology	Fewer types	More types
Specific gravity of nematocysts	1.28–1.32	1.22
Quality of nematocysts	More rigid	More friable
Purification by 20% ammonium sulfate	Poor yields	Good yields
Hemolysin	Absent	Present
Heat lability at 4°C	Stable up to 6 days	Stable less than 1–2 days
Dermonecrosis	None detected at physiological doses	Potent
Factor rupturing mitochondria or affecting sarcoplasmic reticulum	Heat stable	Heat labile
Factor acting Na^+ transport in frog skin	Effective only on dermis	Effective on epidermis and dermis
Chemical mediators of inflammation	kininlike factors in low amounts	kininlike factors in high amounts contains prostaglandin, histamine, histamine releasing agents, serotonin
Duration of human pain	Longer hr	Shorter min
Enzyme differences	More ATPase and DNase, RNase and ATPase optimal at pH 8.0, has AMPase and fibrinlysin	More RNase, RNase and ATPase optimal at pH 7.0 and 6.5, respectively, has hyaluronidase and acid and alkaline proteases
Effects neuromuscular junction	Apparently none	Possible effect on acetylcholine receptor
Cardiotoxin	Initial depression of blood pressure	Initial rise of blood pressure

Table 3. Similarities between *Physalia* and *Chrysaora* Toxins

Poor antigenicity
Lethal to mice and fiddler crabs
Cardiotoxicity–ST wave changes, conduction abnormalities
Have lethal fractions which adsorb to Sephadex, have similar molecular weights, and isoelectric points
Possess kininlike factors
Possess similar nonspecific aminoesterases having similar characteristics

Table 4. Known Different Pharmacological Factors of Sea Nettle Venom

Cutaneous pain factors
Dermonecrosis factor
Hemolysin
Vasopermeability factor
Mitochondrial rupturing factor
Sarcoplasmic reticulum damaging factor
Lethal factor
Cardiotoxin[a]
Lysosomal rupturing factor[a]
Frog skin permeability factor[a]
Myotoxin[a]
Neurotoxin[a]
Erythema factor[a]
Kininlike gut contracting factor[a]

[a]Not certain of classification.

variability in the toxicity of preparations taken from different specimens of the same species.

In general, it appears that the major toxicological actions of these venoms occur at the membrane level. Alterations of calcium transport are key factors. The actions which enable the jellyfish to feed, i.e., lethality, may be produced by different factors than those which induce pain. The kininlike factor, at present, is the only common mediator of inflammation detectable in these two venoms. It appears to localize at the same level in a sucrose-density gradient as one of the human cutaneous pain factor rather than with lethality (Burnett and Calton, 1974a; Burnett *et al.*, 1975).

In order to further correlate the symptomatology of venomous jellyfish we obtained some sea wasp (*Chironex fleckeri*) venom from Dr. E. H. Baxter of the Australian Commonwealth Serum Laboratories. He did not find cross-reacting neutralizing antibodies in sea nettle, man-o-war, and sea wasp venoms (Baxter and Marr, 1974). This sea wasp nematocyst venom had been prepared by the "milking" technique (Barnes, 1967). We found that the lethal factor of this venom had a molecular weight of 20,000–80,000 as determined by sucrose density-gradient centrifugation. The lethal, dermonecrotic, and hemolytic factors of this venom were inactivated by our techniques of preparative gel electrophoresis. Sea wasp nematocyst venom lost all its lethality when treated by our technique of isoelectric focusing; however, two dermonecrotic fractions (approximate pI of 3.0 and 7.8) and one hemolysin (approximate pI of 3.0) were recovered. The toxicological factors present in *Chironex* venom were not easily separated by ammonium sulfate precipitation. More than half the lethality was present in the 20% $(NH_4)_2SO_4$ supernatant although activity was also present in

decreasing amounts in the 20% $(NH_4)_2SO_4$ sediment and both the supernatants and sediments of 60% and 100% $(NH_4)_2SO_4$ solutions. Hemolysis was present in all of the above fractions except the 100% $(NH_4)_2SO_4$ supernatant. The 20% $(NH_4)_2SO_4$ supernatant and the 60% and 100% $(NH_4)_2SO_4$ sediments were the most potent fractions. Dermonecrosis was present in all fractions but predominantly in the 20% and 60% $(NH_4)_2SO_4$ supernatants and sediments.

Chironex venom had no effect on sodium transport across either frog skin or the toad bladder. It did not rupture rat liver mitochondria, destroy the ATP, or uncouple the oxidative phosphorylation of this *in vitro* system. Sea wasp venom was more lethal and hemolytic than that of either the nettle or man-o-war. The nettle had equal or greater dermonecrotic potency with respect to lethality and protein. Sea wasp venom had a cardiotoxic action similar to that of the nettle and man-o-war (Freeman and Turner, 1969; Burnett and Calton, unpublished). We confirmed the presence of histamine releasers (Freeman and Turner, 1969). Kininlike substances were present in lower amounts than in either the nettle or man-of-war venoms (Burnett *et al.*, 1975). Histamine and serotonin were also found in this venom but detectable prostaglandins were lacking.

The medical therapy of jellyfish stings has been frustrating. An experimental sea wasp antivenom is now being evaluated (Baxter *et al.*, 1971). As mentioned above, some therapeutic agents are effective on isolated actions of these toxins in lower animals. Currently counterirritants, analgesics supportive care, and organic solvents for tentacle debridement are the only commonly employed remedies (Keen, 1970). Further effective therapeutic programs await the results of additional investigations designed to purify these venoms. The major hurdle to these experiments is the lability of the venoms and the lack of a rapid screening test accurately reflecting their major actions.

The complexity of coelenterate venoms has been well established and it is reasonable to anticipate that these multiple toxins contribute to the animal's defense and digestion. These materials are membrane coated (within nematocysts) but act promptly and effectively when released. It should be expected that the chemical and pharmacological relationship of these agents to other venoms should reflect the phylogenetic relationship of these jellyfish to other venomous animals. Assembling a defense for humans against these agents will necessitate an orchestration of accurate chemical, pharmacological and medical knowledge.

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Glycine, Theophylline, and Antitoxin Effects on Rabbit Sphincter Pupillae Muscle Paralyzed by Tetanus Toxin

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INTRODUCTION

The concept that tetanus toxin interferes with inhibitory neurotransmission in the central nervous system (CNS) has been supported by extensive neurophysiological, neuropharmacological, and neurochemical studies (Curtis and DeGroat, 1968; Fedinec, 1972; Habermann, 1970; Kryzhanousky, 1973; Laurence and Webster, 1963; Mellanby and Heyningen, 1965). Most of the symptomatology of tetanus appears to be related to this CNS inhibitory effect of the toxin. In addition, Ambache *et al.* (1948a,b) demonstrated that tetanus toxin also has a peripheral effect in that it blocks the release of acetylcholine from the cholinergic nerve terminals of the rabbit iris. More recently, Mellanby (1972) observed that in goldfish tetanus toxin produced a local paralysis of fin musculature after

injecting tetanus toxin directly into the pectoral fin muscles. She concluded that it is likely that the toxin blocked presynaptic neuromuscular transmission. However, Leonardi (1973) suggests that tetanus toxin may also inhibit acetylcholinesterase.

Considerable evidence now exists indicating that glycine is a putative inhibitory neurotransmitter in the spinal cord (Aprison and Werman, 1968; Curtis, 1969) whose release from the inhibitory spinal interneurons may be blocked by tetanus toxin (Johnston *et al.*, 1969; Fedinec and Shank, 1971). This block produces a loss of postsynaptic inhibition with a resultant hyperactivity of the motor neurons manifested by spasticity of the innervated muscles. The hyperactivity of motor neurons can be overcome by iontophoresis of glycine in the vicinity of the neurons (Curtis and DeGroat, 1968; Kryzhanousky, 1972) or by systemic administration of glycine (Fedinec, 1972). Since glycine appeared to reverse the hyperactivity induced by tetanus toxin in the CNS, it was of interest to determine whether glycine would also reverse a peripheral activity of tetanus toxin, specifically, paralysis of the sphincter pupillae muscle in the iris.

A variety of recent evidence suggests that cyclic nucleotides are involved in both neural and muscular function by controlling the release of neurotransmitters. (Singer and Goldberg, 1970). One of the effects of tetanus toxin may be to disrupt these regulatory control mechanisms. Therefore, another objective of our study was to prove the interrelationship of tetanus toxin, cyclic nucleotides, and neurotransmitters actions on the rabbit's iris. We chose to use theophylline, a drug which inhibits the breakdown of cyclic nucleotides to affect the course of tetanus toxin induced paralysis of sphincter pupillae muscle. Tetanus antitoxin was used to neutralize the activity of toxin.

MATERIALS AND METHODS

Purified tetanus toxin (Lot no. CPTxN pooled 104, 105, 502) was supplied by W. C. Latham. The toxin, from concentrated and freeze dried fraction II, contained 1500 LF or 20×10^6 mouse MLD's and 0.64-mg protein nitrogen per ml (Latham *et al.*, 1965). Antitoxin, refined equine serum, Lot. no. 964864 B (Park Davis), was used at a concentration of 2500 I.U./ml. Glycine, 99.5% ammonia free (Matheson, Coleman, and Bell) was used at a concentration of 2.5 M, adjusted to pH 7.0 with NaOH. Theophylline (Sigma) was injected at a concentration of 50 mM, adjusted to pH 7.0 with NaOH. All test substances were made up with sterile 0.15 M NaCl, kept at 4°C, and used within two hours. Optimum concentrations of glycine, 0.5-2.5 M, theophylline 50 mM, and tetanus antitoxin 100 I.U. were determined by preliminary studies (Fedinec, 1972, 1973; Fedinec and King, 1969a,b). Male New Zealand white rabbits (2.5-3 kg)

were immobilized in a restraining box. The eye was proptosed (Bennett *et al.*, 1961) and without anesthesia the test substances were injected through the cornea into the anterior chamber (intraocular, i.o.). Oblique insertion of a 30-gauge needle and injection of a standard volume of 0.05 ml prevented leakage and was well tolerated.

The toxin, diluted with saline, was tested for toxicity by bioassay in mice (Fedinec and King, 1973). In some experiments one rabbit eye was injected with 1000 mouse MLD's of tetanus toxin followed at scheduled intervals by injection of various test substances. The other rabbit eye, serving as a control, was injected with sterile isotonic saline followed by the same test substances at the same scheduled intervals.

Pupillary response to dim (40 c.p.) and bright (4000 c.p.) light, held 10 cm from the eye, was measured with calipers and a millimeter ruler. One measurement was made after a period of adaptation to dim light; the second measurement after exposure to bright light. Responses were measured at 15, 30, 60, and 120 min after injection of the test substance and daily thereafter until recovery. Owing to the known specific blocking effect of tetanus toxin on the cholinergic innervation of the sphincter pupillae muscle (Ambache *et al.*, 1948a,b) only the significant changes in pupillary constriction due to bright light are reported in this study.

Initially, separate control groups (four eyes) were intraocularly injected with the following substances: (I) sham injection; (II) 0.15 M NaCl; (III) 100 I.U. of tetanus antitoxin; (IV) 1000 mouse MLD's of tetanus toxin; (V) 1000 MLD's of tetanus toxin and 100 I.U. of antitoxin; (VI) 2.5 M glycine, pH 7.0; (VII) 50 mM theophylline, pH 7.0. All experimental eyes were injected with 1000 MLD's of tetanus toxin followed by i.o. injection of the test substances at intervals of 6, 12, 24, 48, or 72 hr after the toxin injection. Four eyes were treated in the same manner at each of the stated intervals. The test substances used in the separate groups were: (VIII) 100 I.U. of tetanus antitoxin; (IX) 2.5 M glycine, pH 7.0; (X) 100 I.U. of tetanus antitoxin and 2.5 M glycine, pH 7.0; (XI) 50 mM theophylline, pH 7.0; (XII) 100 I.U. of tetanus antitoxin and 50 mM theophylline, pH 7.0.

RESULTS

In the control group, the pupillary responses to dim and bright light were not altered by sham injections (group I), saline injection (group II), tetanus antitoxin (group III), combined tetanus toxin and antitoxin (group V), glycine (group VI), or theophylline (group VII).

The normal pupillary light response to dim light was 6.70 ± 1.32 mm ($N = 21$, mean ± S.D.) and to bright light, 2.06 ± 0.20 mm. The pupillary response to

bright light of the four eyes for each control and experimental group were measured at specific times. The ratio of the means and the standard error of the means (S.E.M.) of normal eyes to treated eyes was determined. The ratio of the means was plotted against time in Figs. 1 through 7. The S.E.M. was too small to be included in the figures. Therefore, the range (minimum and maximum S.E.M.) was indicated for each group.

Specific alterations in pupillary response occurred in group IV, the control group injected with tetanus toxin alone. Those animals developed a progressive loss of the sphincter pupillae muscle function with complete paralysis ensuing with 72 hr after injection. This loss of function persisted for 10 days after which the pupillary response to bright light stimulation gradually returned and was restored to normal within 22 days after injection.

In contrast to the sphincter pupillae paralysis, there was no significant change in the dilator pupillae muscle function in response to dim light. Indeed, the pupillary diameter increased above normal control values when the opposing contractile ability of the sphincter pupillae muscle was lost. Maximum midriasis observed was 8.7 ± 0.8 mm (mean ± standard error of mean). The pupillary diameters progressively returned to normal during the recovery period. When the contractile ability of the sphincter was sufficiently restored to enable the pupil to return to a size below that of the pupillary response to dim light (6.7 mm), the function of the dilator pupillae muscle could be tested again.

Data concerning effects of tetanus toxin followed at specific times by antitoxin on the pupillary response to bright light confirmed our former observations (Fedinec, 1973).

Figure 1 shows the ratio of the means and the range of the standard error of the means (S.E.M.) of four pupillary diameters of normal eyes in bright light to eyes treated with tetanus toxin alone (group IV) or toxin followed by 100 I.U. of antitoxin (group VIII). The time intervals between the toxin and antitoxin injections was varied from 6 to 72 hr. The results indicate that injection of antitoxin just prior to development of complete pupillary paralysis (48-72 hr) did not prevent the development of symptoms. However, the degree of paralysis was markedly diminished when the antitoxin injection was administered after only 6 hr. Complete recovery from the paralysis (8, 13, and 19 days) was directly related to the time of antitoxin injection (6, 12, and 24 hr). After complete pupillary paralysis had developed, antitoxin injection did not reduce the recovery period from the paralysis.

The effects of glycine alone (group IX) and a mixture of glycine and antitoxin (group X) on the toxin paralyzed iris are illustrated in Figs 2, 3, and 4. Figure 2 shows the short-term effect of glycine on the iris as measured at 15-120 min after injection in eyes previously treated with toxin (6-48 hr). Glycine produced a constriction of the pupil in tetanus-treated eyes. This effect was more pronounced in eyes with increased pupillary diameters and paralysis. Injection

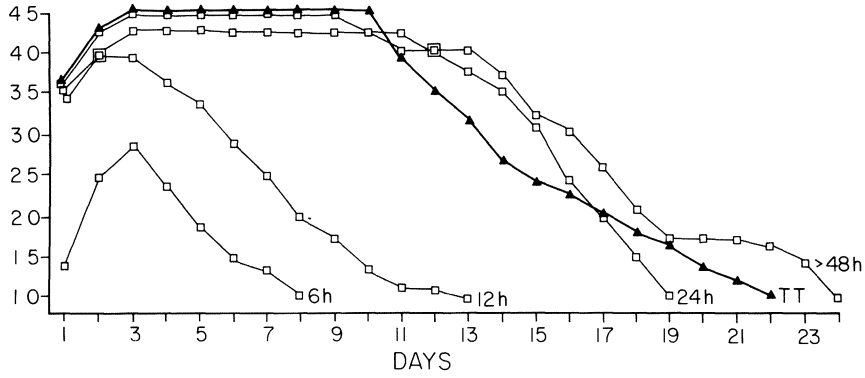

Fig. 1. Antitoxin effect on tetanus toxin paralyzed iris. Each point represents the ratio of the means of four pupillary diameters in bright light; (white squares) rabbits given i.o. injection of 1000 MLD's of tetanus toxin, followed by 100 I.U. antitoxin at varied intervals, to normal animals (group VIII); TT (black triangles) same as above but not followed by antitoxin to normal animals (group IV). h, hours after toxin injection. The range of S.E.M. for group IV was (N = 4), ± 0.00 to ± 0.26, and, for group VII was (N = 20), ± 0.00–± 0.20.

of glycine 24 hr after toxin treatment resulted in the greatest diminution of paralysis. The pupillary constriction persisted 2-5 hr before returning to the previous dilated and paralyzed state.

Daily measurements of the pupillary response to bright light, stimulation of toxin, and glycine injected eyes are plotted in Fig. 3. This graph shows the long-term effect of glycine on tetanus toxin induced paralysis of the sphincter pupillae muscle (group IX) contrasted with those injected with toxin alone (group IV). Glycine injected 6 hr after tetanus toxin did not prevent the development of pupillary paralysis; but (1) ameliorated the severity of paralysis, (2) delayed the peak of paralysis by 3 days, and (3) shortened the recovery by 4 days as compared with eyes injected with the toxin alone. Delaying the glycine injection for 12 hr or longer did not shorten the recovery period although peak pupillary paralysis was delayed 5 days.

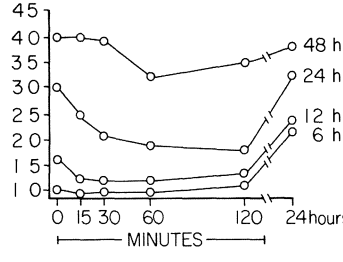

Fig. 2. Glycine effect on tetanus toxin paralyzed iris, short-term results. Each point represents the ratio of the means of four pupillary diameters in bright light; (white circles) rabbits injected with 1000 MLD's of tetanus toxin (i.o.) followed by glycine 2.5 M, pH 7.0, (i.o.) at varied intervals, to normal animals (group IX). h, hours after toxin injection. The range of S.E.M. for (N = 20) was ± 0.00–± 0.09.

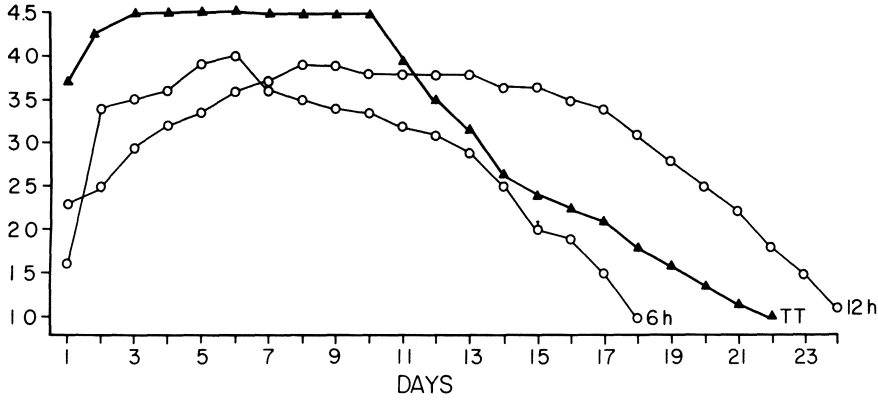

Fig. 3. Glycine effect on tetanus toxin paralyzed iris, long-term observation. Each point represents the ratio of the means of four pupillary diameters in bright light; (white circles) rabbits injected with 1000 MLD's of tetanus toxin (i.o.) followed by glycine 2.5 M, pH. 7.0 (i.o.) at varied intervals, to normal animals (group IX); TT (black triangles) same as above but not followed by glycine, to normal animals. h, hours after toxin injection. The range of the S.E.M. for group IX ($N = 20$) was ± 0.00–± 0.17.

The effect of glycine and antitoxin mixtures (group X) was compared with the antitoxin's effect alone (group VIII) on tetanus toxin paralyzed iris. Figure 4 shows that the combined effects of glycine and antitoxin are less effective in altering the progress of tetanus induced paralysis of the sphincter pupillae muscle than antitoxin alone. In eyes treated with glycine and antitoxin the degree of paralysis was greater and the time of complete recovery was prolonged. Pupillary response in eyes treated with antitoxin 6 hr following the toxin injection returned to normal on the 8th day. In contrast, when the eyes were treated with glycine and antitoxin mixtures, recovery occurred on the 17th day. A 12-hr delay in injecting glycine and the antitoxin mixture did not alter the progress of tetanus toxin induced pupillary paralysis, but injection of antitoxin alone shortened the recovery period by 9 days.

A comparison of animals injected with glycine alone and those injected with combined glycine and antitoxin shows that the time of complete recovery was approximately the same. The pupillary response of eyes injected with glycine 6 hr after toxin treatment (group IX, Fig. 3) returned to normal in 18 days; those treated with the glycine-antitoxin mixture at the same time delay (group X, Fig. 4) recovered in 17 days. A 12-hr delay in glycine injection produced a functional recovery of the pupil on the 24th day, as compared with eyes injected with the glycine and antitoxin mixture, which returned to normal on the 23rd day.

Theophylline's short-term effect (Fig. 5) and long-term effect (Fig. 6) on tetanus toxin induced sphincter pupillae paralysis (group XI) was compared

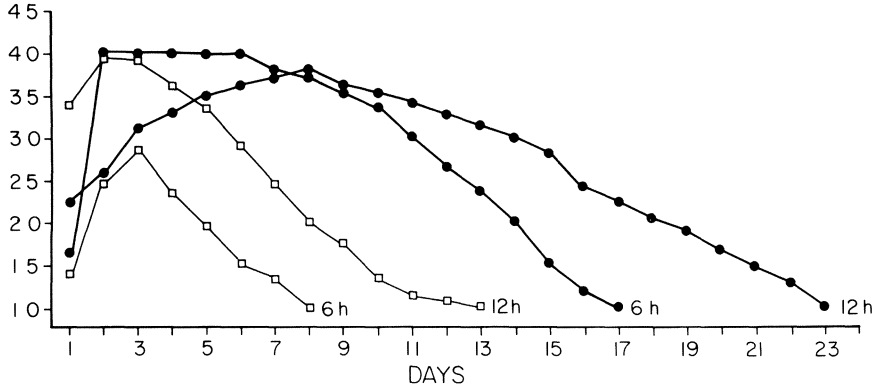

Fig. 4. Glycine and antitoxin effects on tetanus toxin paralyzed iris. Each point represents the ratio of the means of four pupillary diameters in bright light; (black circles) rabbits injected with 1000 MLD's of tetanus toxin (i.o.) followed by glycine, 2.5 M, pH 7.0 and 100 I.U. of antitoxin (i.o.) at varied intervals, to normal animals (group X); (white squares), same but not followed by glycine, to normal animals. h, hours after toxin injection. The range of S.E.M. for group X (N = 20) was ± 0.00–± 0.14.

with the ability of a theophylline and antitoxin mixture (group XII) to alter the progress of paralysis (Fig. 7).

Measurements of pupillary response to bright light stimulation at 15-120 min after i.o. injection of theophylline in toxin-treated eyes show that theophylline produced a constriction of the paralyzed and dilated pupil with a maximal effect when injected 24 hr after toxin injection. Theophylline's effect on the iris persisted for 2-5 hr after which the pupil returned to its previously dilated and paralyzed state.

When the progress of the pupillary paralysis in theophylline-injected eyes is followed until recovery (Fig. 6) and compared with eyes injected with tetanus toxin only, it is apparent that i.o. theophylline injection 6 hr following tetanus toxin has reduced the severity of paralysis and shortened the complete recovery period by 4 days. A 12-hr delay in theophylline injection was ineffective in altering the course of tetanus paralysis.

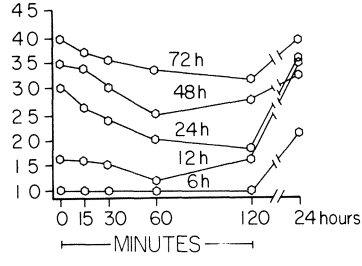

Fig. 5. Theophylline effect on the tetanus toxin paralyzed iris, short-term results. Each point represents the ratio of the means of four pupillary diameters in bright light; (white hexagons) rabbits injected with 1000 MLD's of tetanus toxin (i.o.) followed by theophylline, 50 mM, pH 7.0 (i.o.) at varied intervals, to normal animals (group XI). h, hours after toxin injection. The range of S.E.M. for group XI (N = 20) was ± 0.00–± 0.37.

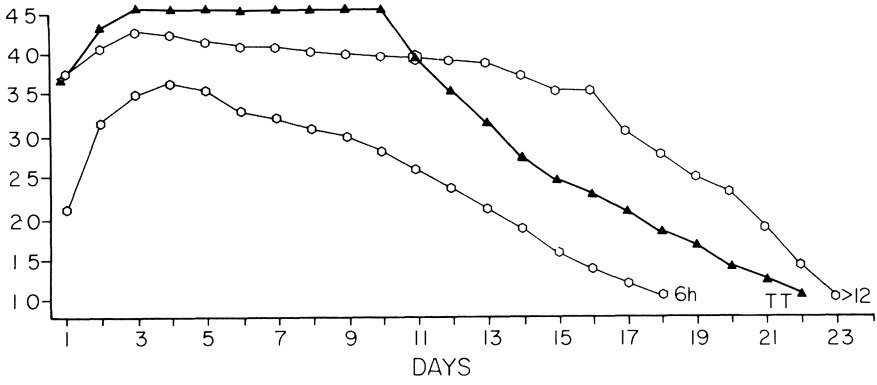

Fig. 6. Theophylline effect on tetanus toxin paralyzed iris, long-term observation. Each point represents the ratio of the means of four pupillary diameters in bright light; (white hexagons) rabbits injected with 1000 MLD's of tetanus toxin (i.o.) followed by theophylline, 50 mM, pH 7.0 (i.o.) at varied intervals, to normal animals (group XI); TT (black triangles) same as above but not followed by theophylline, to normal animals. h, hours after toxin injection. The range of the S.E.M. for group XI ($N = 20$) was ± 0.00–± 0.17.

Addition of antitoxin to the theophylline-saline mixture (group XII) was less effective than antitoxin alone (group VIII) in altering the progress of the paralysis (Fig. 7). While the severity of the paralysis was in general lowered by the theophylline and antitoxin mixture, the period of complete recovery was considerably prolonged as compared with eyes injected with antitoxin alone. Thus, a

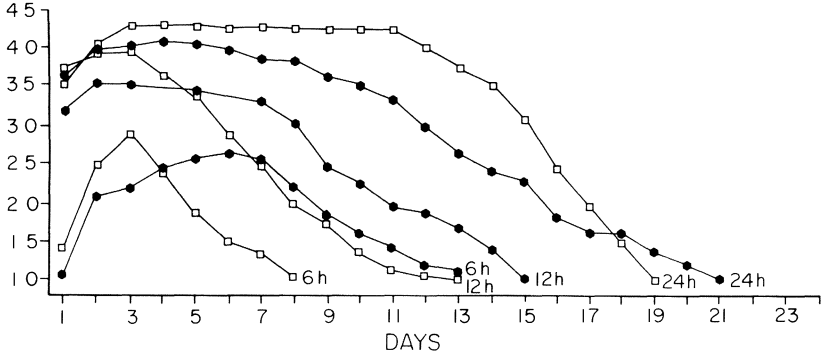

Fig. 7. Theophylline and antitoxin effect on tetanus toxin paralyzed iris. Each point represents the ratio of the means of four pupillary diameters in bright light; (black hexagons) rabbits injected with 1000 MLD's of tetanus toxin (i.o.) followed by theophylline, to mM, pH 7.0, and 100 I.U. of antitoxin (i.o.) at varied intervals, to normal animals (group XII); (white squares) same, but not followed by theophylline, to normal animals. h, hours after toxin injection. The range of S.E.M. for group XII ($N = 20$) was ± 0.00–± 0.53.

6-hr delay in i.o. injection of theophylline and the antitoxin mixture prolonged the recovery by 5 days; a delay of 12 hr prolonged the recovery by 2 days; and a delay of 24 hr prolonged the recovery by 2 days.

DISCUSSION

The rabbit eye model system (Ambache *et al.*, 1948a,b) has many advantages in screening potential therapeutic approaches to tetanus toxin induced neuromuscular paralysis. The *induction* or latent phase can be studied particularly well by experimentally altering the different steps leading to complete paralysis. Also, the *recovery* phase can be accurately followed to compare the efficacy of different therapeutic regimens. Many agents would be expected to affect one or the other of these phases and it is important to clarify the timing, sensitivity, and interactions of the system with the various agents.

Since it is assumed that in the spinal cord tetanus toxin interferes with the release of glycine, a putative inhibitory neurotransmitter substance, (Johnston *et al.*, 1969; Fedinec and Shank, 1971) and exogenous glycine produces a hyperpolarization of neurons, thus relieving the tetanus toxin induced hyperactivity of motor neurons (Curtis and DeGroat, 1968), it was not a total surprise to find that glycine was also effective in temporarily reversing the tetanus toxin induced sphincter pupillae muscle paralysis. This short-term effect of glycine appeared within 15 min and lasted 2-5 hr after i.o. injection in tetanus treated eyes. A single i.o. injection of glycine was also effective in shortening the recovery period from the paralysis but only when given in the prodromal stage before the appearance of tetanal symptoms. In this respect, theophylline was found to be almost equally as effective as glycine in that theophylline also produced a short lasting constriction of the tetanus paralyzed pupil (2-5 hr), and shortened the recovery period when injected during the prodromal stage. Both substances markedly lessened the degree of paralysis.

We have reported that tetanus antitoxin alone is effective in preventing or ameliorating and shortening the course of pupillary muscle paralysis induced by tetanus toxin when injected (i.o.) prior to development of full paralysis (Fedinec, 1973). The finding that injections of glycine and antitoxin mixtures or theophylline and antitoxin mixtures into tetanus toxin treated eyes was less effective than antitoxin alone was unexpected. When the results of glycine injections alone or theophylline injections alone are compared to the combined injections with antitoxin, it becomes apparent that the effects of glycine or theophylline predominate when combined with antitoxin. Therefore, these studies show that not only the time of administering these therapeutic agents, but also their interactions are important in determining the efficacy of treatment.

Since it is not known whether glycine is an inhibitory neurotransmitter in the iris, perhaps its effects may be related to changes in cyclic nucleotide metabolism essential for neurotransmission. Ferrendelli *et al.* (1974) demonstrated that the inhibitory and excitatory amino acids, glycine, GABA, and glutamate, are capable of altering the levels of cyclic nucleotides in slices of mouse cerebellum. In this respect, we may speculate that the effect of theophylline on tetanus toxin paralyzed iris is due to its effects on cyclic nucleotide metabolism. However, theophylline may affect not only cyclic AMP and/or cyclic GMP phosphodiesterase, but also calcium efflux and ATP depletion (Goldberg *et al.*, 1973). Therefore, additional work must be done to separate clearly the various possible actions of theophylline and the role cyclic nucleotides play in this model system before a valid hypothesis can be made.

SUMMARY

The effectiveness of glycine and theophylline in altering the course of tetanus toxin induced sphincter pupillae muscle paralysis was tested in rabbits. Purified tetanus toxin was injected into the anterior chamber of rabbit eyes followed at varied intervals of time by i.o. injection of glycine or theophylline alone or in combination with tetanus antitoxin.

The results indicate that both glycine and theophylline temporarily reversed the toxin-induced paralysis for two to five hours. The remissions were more pronounced when paralysis was severe. Both glycine and theophylline were also effective in ameliorating the symptoms of pupillary paralysis and shortening the recovery period when injected (i.o.) during the prodromal stage (six hours after toxin injection and before the development of symptoms). The combination of glycine or theophylline with tetanus antitoxin was found to be less effective than antitoxin alone in altering the tetanus toxin induced pupillary paralysis.

These studies show that the time of administration of these agents as well as their interactions determine the efficacy of the treatment.

ACKNOWLEDGMENTS

We are grateful to Mrs. M. Patterson for skillful assistance and Mrs. G. Reed for critical comments. This investigation was supported in part by U.S.P.H.S. Research Grant No. AI-08610 from the National Institute of Allergy and Infectious Diseases, General Research Support Grant No. RR-05432 from the National Institute of Health, and Research and Education Associateship Award (Dermatology) to L. E. King Jr., from the Veterans Administration.

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The Effects of Tetanus Toxin on the Extensor and Flexor Muscles of the Hind Leg of the Cat

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INTRODUCTION

Tetanus intoxication was thought to be primarily a disease of the central nervous system. On the other hand, there are a number of papers concerning the effect of tetanus toxin on the endplate and muscle.

The similarity in effect of strychnine and tetanus toxin was described by Sherrington as early as 1905. He explained that the toxin converted inhibition to excitation, which is difficult to recognize with the knowledge of the mechanisms of inhibition and excitation of modern neurophysiology, showing the excitatory and inhibitory postsynaptic potentials. However, his comments are even now taken as correct, since as we observe the reflex to be an integrated action.

Brooks *et al.* (1957) showed that the tetanus toxin blocks the spinal inhibition of alpha-motoneurons in the spine of the cat and imagined that the toxin could affect the presynaptic side of the postsynaptic inhibition.

Liljestrand and Magnus (1919) demonstrated that procaine injection into the muscle diminished the symptom of local tetanus and argued that the rigidity (better termed as a spasticity in spinal type) of local tetanus might be caused by sensory excitation originating mainly in the intoxicated muscle. If the sensory inflow as reflex input increases, it causes the abnormally strong rigidity of the muscle. This old paper suggests the hypersensitivity of the proprioceptors, namely of the muscle spindles.

The muscle spindle is a receptor measuring the change of length of the muscle in which it lies. Its sensitivity and threshold are controlled by gamma-motor fibers, which innervate the intrafusal muscle fibers in the muscle spindle. The effect of the toxin on the gamma-motor system was suggested (Schaefer, 1944; Rushworth, 1960) and demonstrated by Erzina (1961), but others have questioned it (Andrew and Barr, 1958; Laurence and Webster, 1963).

In previous publications (Takano and Kano, 1968; Kano and Takano, 1969) we presented evidence that the gamma system plays a major role in the spasticity of the muscle in the local tetanus of the cat. Finally we could measure the change of gamma bias (Eldred, *et al.*, 1953), showing the greater enhancement of gamma activity far beyond the physiological range, which brings the motor system to the spasticity (Takano and Kano, 1973).

Takano and Henatsch (1973) have observed the extensor spasticity in the cat in which toxin was injected into the flexor muscle. This suggests on the one hand, that the local tetanus is dominant in the extensor muscle, because of its more highly developed gamma influences, than in the flexor. On the other hand, it suggests a stronger peripheral effect, especially on the endplate of the flexor muscles than on the extensor. The latter cannot, however, be taken to confirm the results of Duchen and Tonge (1973), i.e., that the toxin has a stronger blocking effect on the endplate of the extensor than that of the flexor (muscle) in the mouse.

This paper presents some tentative results to clarify the differential effects of tetanus toxin on the flexor and extensor systems. Detailed reports on the special points of every motor system will appear elsewhere (Takano, 1976; Takano and Wienecke, 1976).

MATERIALS AND METHODS

Twenty-seven adult cats of both sexes weighing 1.4–3.5 kg were used. Preceding the acute experiment, tetanus toxin, noncrystalized toxin from *Clostridium tetani* (Harvard), T_9 (Behringwerke), was injected into the muscles of the left hind leg (*M. gastrocnemius* and/or *M. tibialis anterior*) in the morning, Levels of the toxin doses were between 10^3 mouse MLD/kg and 10^4 mouse MLD/kg. In 4 cats the dose of toxin 10^4 mouse MLD/kg was injected into the gastrocnemius muscle, in 12 cats the same dose into the tibialis anterior muscle and in 3 cats 5×10^3 mouse MLD/kg was injected into the gastrocnemius and the same dose into the tibialis anterior muscles. In 2 cases, $4/5 \times 10^4$ mouse MLD/kg were injected into the gastrocnemius and $1/5 \times 10^4$ mouse MLD/kg into the tibialis anterior. The ratio of the weight of the gastrocnemius to that of the tibialis anterior is approximately 4 to 1. In 2 cats 10^3 mouse MLD/kg was injected into the tibialis anterior and in 4 cats the same dose into the gastrocnemius.

On the second day (the next day after the day of injection, see Results) the first symptom of local tetanus appeared. Some hours later the animal was anaesthetized through the intraperitoneal injection of a mixture of 280–300 mg/kg urethane and 28–30 mg/kg chloralose. Ether was also used during the operation, when necessary. One of the cats for the experiment of long latency reflex was decerebrated precollicularly under anaesthesia with ether, which was removed after the decerebration.

Hind limbs of both sides were denervated except for the muscles under the experiment (*M. triceps surae*, i.e., *M.gastrocnemius*, *M. soleus*, and *M. tibialis anterior*). For the experiments measuring the tension of the muscles, the muscles were freed from the surrounding tissue. For the experiments recording only the action potential and tension of the muscle, this surgical procedure was omitted.

The cat was firmly fixed at the hip, knee, and ankle of both sides. The tendon was cut and connected to the myograph which was connected to a pneumatic muscle stretcher. The muscle was stretched 10, 12, or 14 mm at a constant rate of 1 mm/sec to measure the passive tension curve, or at variable rates to measure the active tension. The length change of the muscle was recorded with a rod potentiometer. The muscle tension was picked up by the strain-gauge carry amplifier. The action potential of the muscle was recorded with fine insulated wires exposed for 2–3 mm at the tips, which were inserted into the muscle.

The oscilloscope picture was recorded mainly on running paper film or on the film in a Polaroid camera. The passive tension curve was directly registered with an X-Y recorder. For further details of the methods see Takano and Henatsch (1971, 1973).

RESULTS

Incubation Time. As demonstrated in the preceding paper (Takano and Henatsch, 1973) there is a good linear relation between the incubation time and the cube root of body weight of animals. The incubation time is the time between the injection of toxin and first symptom, namely slight hobbling. This incubation time can be determined fairly precisely, because the symptom developed rapidly and was observed clearly in the quarter hour after determination of the incubation time.

The curve (1) in Fig. 1 shows the relation between incubation time and body weight, when the toxin was injected into the gastrocnemius muscle at a dose of 10^4 mouse MLD/kg. When the same dose of toxin was injected into the tibialis anterior of the hind limb, the leg did not show the flexion but extension. Curve (2) shows the relation between the incubation time and body weight in the cases of injection into the tibialis anterior. Deviation of the measured incubation time after injection into the tibialis anterior was larger than that for the gastrocnemius. The difference between the incubation time in both curves will be supposed

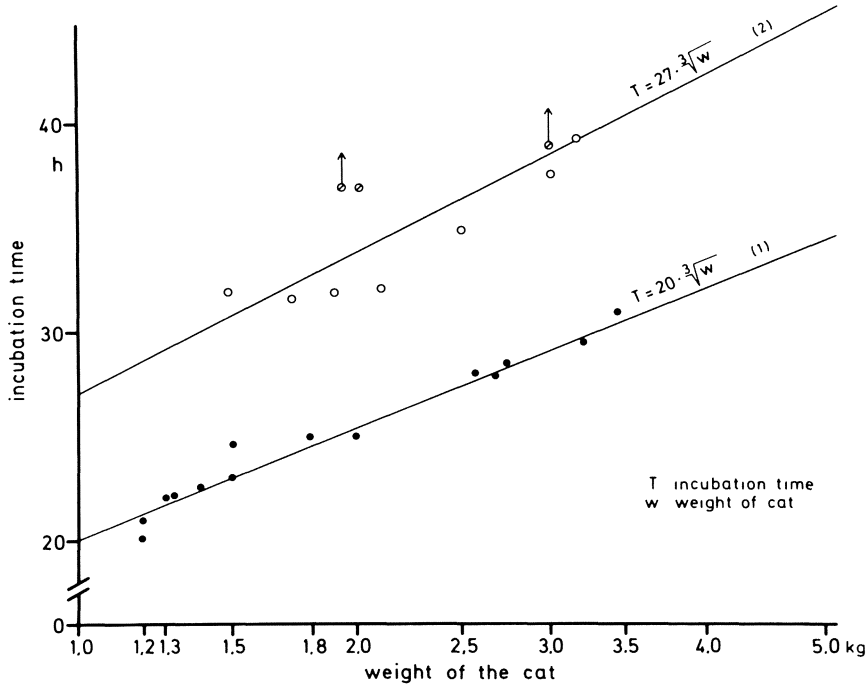

Fig. 1. Relation between incubation time and weight of the cat. The incubation time (ordinate) is the time interval between injection of toxin and the first signs of intoxication (slight hobbling). The cube root of the weight of the cat was taken for the abscissa scale. The straight line in the graph shows the relation $T = 20\sqrt[3]{w}$ (curve 1) and $T = 27\sqrt[3]{w}$ (curve 2), where T is the incubation time in hr and w is the body weight of cats. Solid circles show the incubation time in the animals in which the toxin (10^4 mouse MLD/kg) was injected into the gastrocnemius. Open circles show result for the cases of injection into the tibialis anterior muscle at same doses. Circles with a hatch mark show the results of the extensor injection with 10^3 mouse MLD/kg. Some data in curve (1) were taken from the previous paper (Takano and Henatsch, 1973, Fig. 1).

to be the time of transport of the toxin in the spinal cord and the time for the accumulation of the toxin near the extensor motoneurons.

When the toxin dose injected into the gastrocnemius is as low as 10^3 mouse MLD/kg the incubation time became longer than that of curve (1) in which the dose of toxin was 10^4 mouse MLD/kg. This difference of time might be the time in which enough toxin had accumulated in the spinal cord to activate the motor system.

Clinicals. As already stated we have only observed the extensor rigidity of the hind limb independent of whether the toxin was injected into the extensor or into the flexor muscle. This rigid extension under both types of injection at the early period could be removed by the injection of diazepam (e.g., 2 mg/kg,

i.m.) for a long time, and then the leg could be flexed easily. The marked extension in the contralateral side during flexion of the intoxicated side is noteworthy.

We mentioned in the Introduction that we have found that the gamma-motor system is activated and plays a major role at least in the early period of the local tetanus. On the other hand we have divided the action of diazepam on the stretch reflex into two phases, "dip" and "plateau." The former comes from the general action of diazepam including alpha- and gamma-motor system. The latter depends on the special action of this drug on the gamma-motor system (Brausch *et al.*, 1973; Student and Takano, 1974). The application of diazepam depressed the activity of the gamma system which was elevated by tetanus toxin. That is, pathologically activated reflex activity was normalized by diazepam.

The active tension–extension diagram shows a pure parallel shift to the left during the early period of intoxication in the triceps surae muscle (Takano and Henatsch, 1973). After the application of diazepam to the decerebrated cat (Brausch *et al.*, 1973) or during the selective blockade of gamma filters with procaine in the intoxicated preparation (Takano and Henatsch, 1973) this diagram shifts parallel to the right.

Four or five days after the application of toxin the extension rigidity of the injected leg was not removed by the application of diazepam. This symptom of the extended leg might be better called contracture rather than spasticity or rigidity. Since diazepam shows no further effect on the extension rigidity in the time corresponding to the dip phase, it would seem to follow that the peripheral system was affected by tetanus toxin and not the spinal cord at the later period of intoxication. The change of the passive tension of the curve in the extensor muscle, namely the augmentation of stiffness and characteristic in this approximately exponential curve (Takano and Henatsch, 1973), is obviously one of the peripheral effects of the toxin, directly or indirectly.

Change of Passive Tension. The toxin was injected into the tibialis anterior muscle at a dose of 10^4 mouse MLD/kg and the passive tension curve of this muscle and that of the contralateral side 56 hr after the toxin injection is shown in Fig. 2a. In general the passive tension was not greater in the tetanus intoxicated muscle than in the control one in all four cases. The passive tension–extension diagram is almost exponential. Therefore, it can be represented by the following formula (Takano and Henatsch, 1973):

$$t = k \cdot a^l$$

(t, tension; k, constant; a, characteristic; l, length of stretching). The passive tension–extension curves in Fig. 2a were replotted on semilogarithmic scaling (Fig. 2b). The two curves showed almost the same slope, that is, the same characteristic (a in the exponential formula). In the extensor muscle the passive tension curve always showed greater tension in the intoxicated muscle than in the contralateral one and the value of a also became greater during intoxication

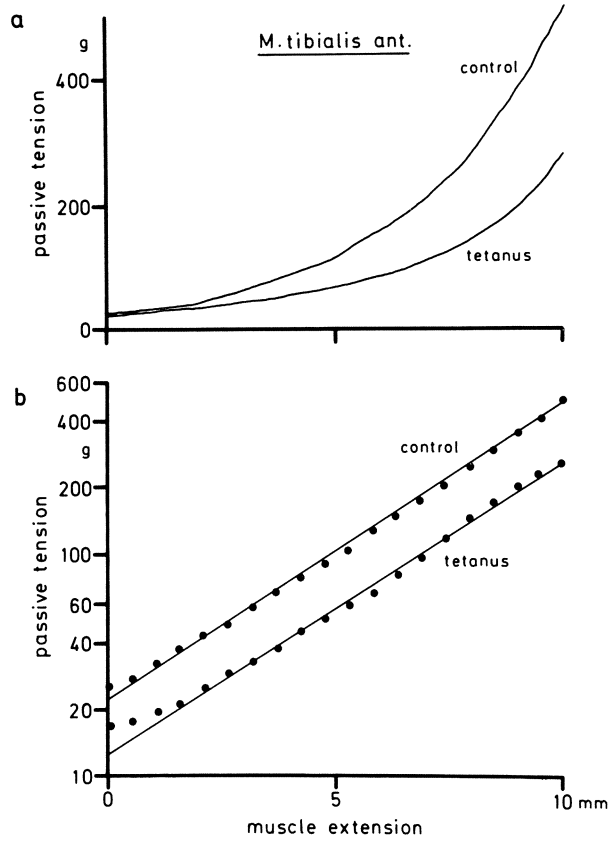

Fig. 2. Passive tension–extension curves of the tibialis anterior muscle (a) and the same relation in semilogarithmic plotting (b) of the intoxicated (tetanus) and of the contralateral (control) sides. Slope of the two curves in (b) are identical, which indicates the same characteristics in these exponential curves (56 hr after the injection of the tetanus toxin of 10^4 mouse MLD/kg into the tibialis anterior muscle).

with the exception of one cat, which was spinalized immediately after the toxin injection (Takano and Henatsch, 1973).

This was not the case in the tibialis anterior. The characteristic (*a*) of the curve of intoxicated muscle was not greater than that of the muscle in the contralateral side.

Change of Amplitude of the Muscle Action Potentials and Twitch Tension. The tension developed by the triceps surae and by the tibialis anterior muscles in response to indirect muscle stimulation is shown in Fig. 3a. In this cat 5×10^3 mouse MLD/kg toxin was injected into each muscle. The twitch tension of the triceps surae muscle became smaller but preserved fairly good contraction at the

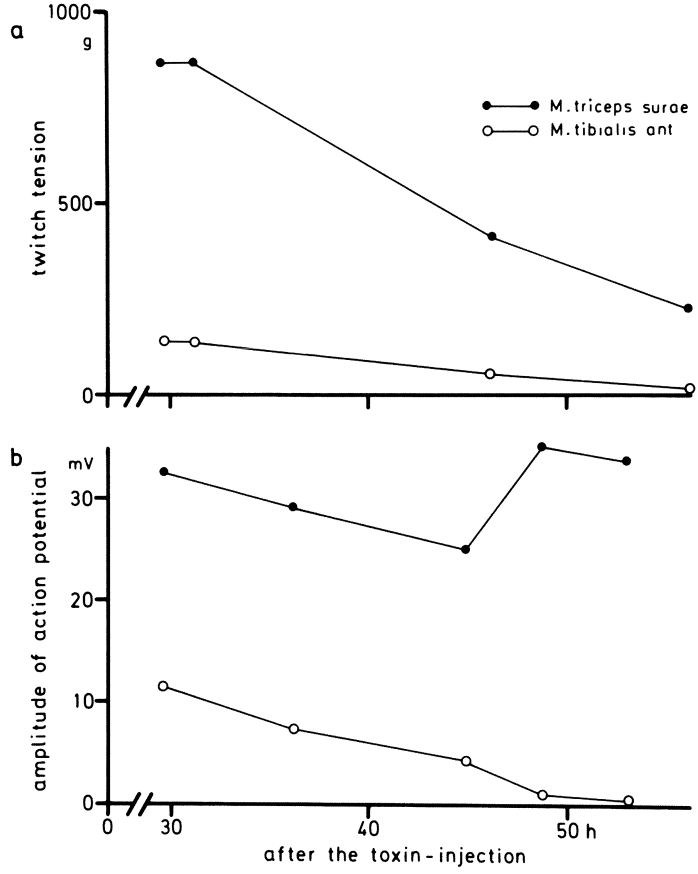

Fig. 3. Change of twitch tension (a) and amplitude of action potential (b) of the triceps surae muscle (open circles) and tibialis anterior muscle (solid circles) responding to the indirect stimulation (5×10^3 mouse MLD/kg was injected into both triceps surae and tibialis anterior muscles).

end of the experiment (50 hr after the toxin application). On the other hand the tension of the tibialis anterior almost faded away at the end of the experiment. The direct stimulation of the muscle could not develop the tension in the tibialis anterior at this time. Fig. 3b shows the change of the amplitude (peak to peak) of the action potential of the triceps surae and the tibialis anterior in response to the indirect stimulation of these muscles. The same tendencies were observed in this figure as in Fig. 3a. We ascribe no particular significance to the enhanced change of amplitude of the action potential of the triceps surae 49 hr after injection. It might depend on the state of the electrode which was inserted for a long time in the muscle.

Change of Duration of Action Potentials of the Muscles. The action potentials of the tibialis anterior in response to the indirect stimulus in both right (not injected, *a*) and left (injected, b) sides 30 hr after toxin injection are shown in Fig. 4. The latencies from stimulation to the beginning of the action potentials in both muscles are the same, but duration of the action potentials in the intoxicated muscle was markedly lengthened. Note the ten times greater amplification factor of the record of the intoxicated muscle.

Action Potentials in the Denervated Muscles. In two cats, 5×10^3 mouse MLD/kg each of tetanus toxin were injected both in triceps surae and tibialis anterior muscles and 7.5 and 8 hr after that the sciatic nerve was severed at the height of hip. Figure 5 shows the action potential of the triceps surae and of the tibialis anterior muscles 34(a) and 58 hr (b) after injection of the toxin in one of these cases. In the other cat the same test was performed between 52 and 75 hr after the toxin injection and we found well-preserved action potentials during this period. The amplitude of the action potential of both muscles did not change appreciably, but had diminished strongly in the tibialis anterior of the cat with the intact reflex arc. The durations of the action potentials were lengthened both in triceps surae and tibialis anterior muscles.

In the third cat used for this problem the sciatic nerve was severed at the height of the hip immediately after toxin injection. 55 hr after the injection of toxin the amplitude of action potential of the triceps surae was almost the same as shown in the foregoing experiments, but that of the tibialis anterior had almost faded away. The action of the tetanus toxin on the flexor was stronger

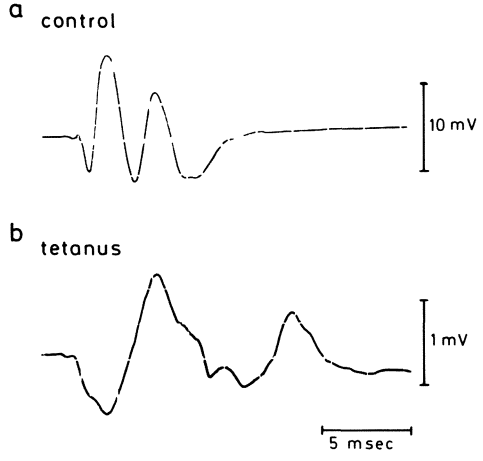

Fig. 4. The action potential of the tibialis anterior muscle of the intoxicated (tetanus) and of the contralateral (control) sides in response to the nerve stimulation. (30 hr after toxin injection 5×10^3 mouse MLD/kg each into the gastrocnemius and tibialis anterior muscles).

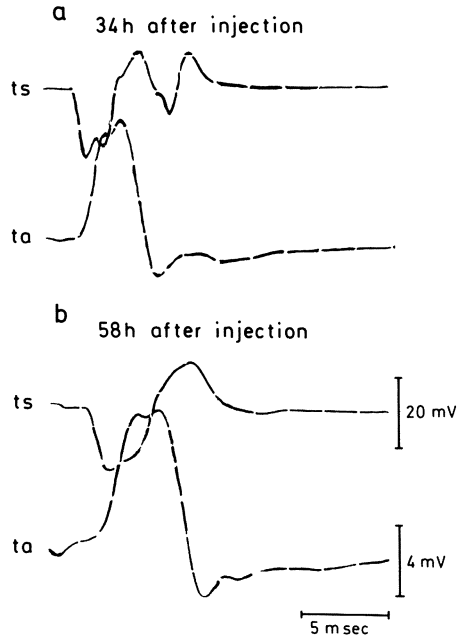

Fig. 5. The action potential of the triceps surae muscle and the tibialis anterior muscle 34 and 58 hr after injection of toxin in response to the supramaximal nerve stimulation. Sciatic nerve was cut 7 h after the injection of the toxin (10^4 mouse MLD/kg) into the gastrocnemius muscle. The amplitudes of action potentials in both muscles did not change practically, but the durations are longer in (b) than in (a). Oscilloscope sweep was triggered at the beginning of stimulus pulse (duration 0.1 msec). The latencies to the beginning of the action potential are the same in four recordings, which shows no change of conduction velocity in the nerve trunk and synaptic delay.

in this earlier denervated preparation than in the muscle which was denervated 7–8 hr after injection of tetanus toxin.

Stretch Reflex. As described above, the tibialis anterior muscle shows only small residual activity within one day after the toxin injection into this muscle. Thus, there is of course no stretch reflex in this muscle. But it shows also a higher activity if the toxin is injected into its antagonist, namely into the gastrocnemius muscle. The tibialis anterior muscle under such intoxication shows some spontaneous activity, while the triceps surae muscle shows greater activity (Fig. 6a). The foot is now slowly extended (the tibialis anterior is stretched) and both muscles show high activity (Fig. 6b). When the foot is slowly flexed (the triceps surae is stretched) the triceps surae show much stronger activity, but the tibialis anterior shows only a little stronger activity than in the relaxed state in which no force is applied.

In the physiological condition, when one muscle is stretched, the antagonist is inhibited by reciprocal innervation. This inhibition is not shown in our records, while we can see the facilitation during the antagonist stretching. This is particularly great in the triceps surae. As already described in the introduction, Sherrington (1905) stated "strychnine and tetanus toxin transform certain inhibitions into excitation." This "transformation" is observed in this Fig. 6. We have already demonstrated this transformation in the active tension-extension diagram (Takano and Henatsch, 1973).

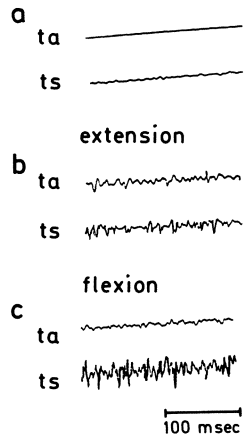

Fig. 6. Electrical activity in the tibialis anterior muscle (upper traces, ta) and in the triceps surae muscle (lower traces, ts). (a) control; (b) the foot was extended; (c) the foot was flexed. Note that there is no reciprocal inhibition (78 hr after toxin injection 10^3 mouse MLD/kg into the gastrocnemius muscle).

The muscle was stretched 10 mm at variable velocities. The maximum active tension was measured at each stretching. It is known that a flexor muscle like the tibialis anterior has only weak stretch reflex. Further, it is known (Takano, 1966) that this muscle responds only when it is stretched at a rate greater than about 20 mm/sec while the extensor, viz. soleus, is very sensitive for lower rates. The relation between stretch rate and the maximum active tension of tibialis anterior is shown in Fig. 7a.

Crosses show the relation for the decerebrated cat as a reference. The responses of the tibialis anterior of the injected side are shown by solid circles and that of the contralateral side are shown by the open circles. The tibialis anterior muscle under tetanus intoxication is very sensitive to the lower rate and shows great tension like the triceps surae muscle in the decerebrated cat (Takano, 1966).

The relation between maximal active tension and stretch rate in the triceps surae was not different from that of a decerebrate cat (Fig. 7b). We cannot expect such a curve in the nonintoxicated anaesthetized animal, which shows only small or no reflex tension.

Crossed and Uncrossed Long Way Reflex. Because of the weak and non-synchronized nature of the electrical reflex response, which has a long latency such as the crossed extensor reflex, it is difficult to record its electrical response to nerve stimulation in the nonintoxicated anaesthetized animal (e.g., Perl, 1957; Holmquist, 1961; Bosemark, 1966). This reflex can be recorded without any difficulty from the ipsi- and contralateral side of the intoxicated animal.

In Fig. 8a the right sural nerve was stimulated and the action potential was recorded from the intoxicated triceps surae. In Fig. 6b the response of the intoxicated muscle to the ipsilateral sural nerve stimulation is shown. The response of the tibialis anterior to the contralateral sural nerve stimulation is shown in Fig. 6c and d respectively. The reflex responses shown in this figure

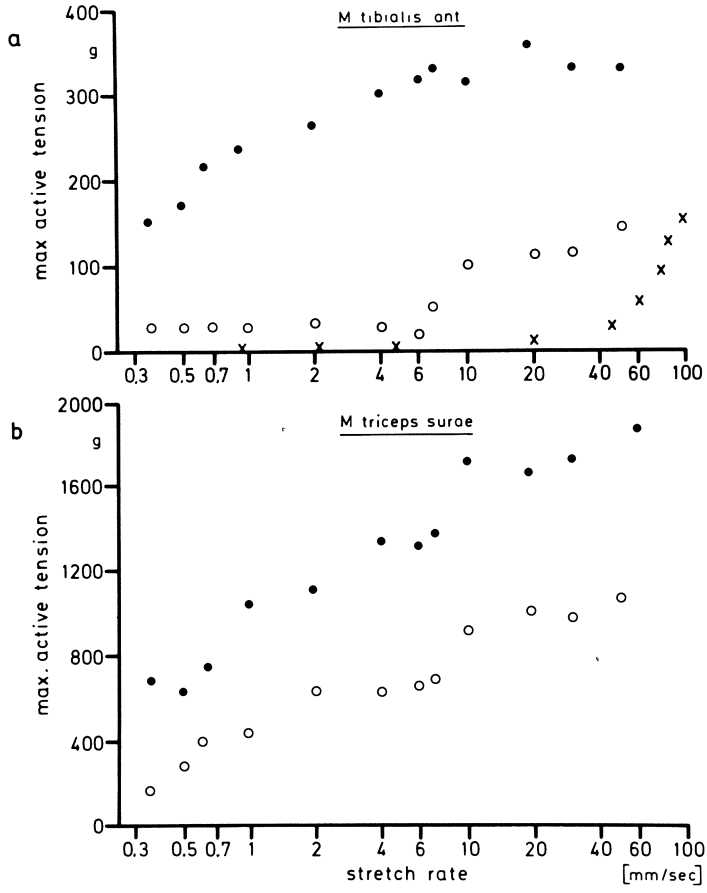

Fig. 7. The relation between stretch rate and maximal active tension in tibialis anterior muscle (a) and triceps surae muscle (b). Crosses in (a) show the case of the nonanaesthetized but decerebrate cat. Solid circles are that of the intoxicated side and open circles of the contralateral side. The muscle was stretched 10 mm in the tibialis anterior muscle and 12 mm in the triceps surae muscle (54 hr after the toxin injection at the dose of 10^4 mouse MLD/kg into the gastrocnemius muscle).

have great (a) or relatively great (b, c, and d) amplitude. The crossed and uncrossed reflex response will be reported in detail elsewhere.

DISCUSSION

Extensor Rigidity. There are several probable explanations of the genesis of extensor rigidity in the hind limb under local tetanus. When the motoneurons both of the flexor and the extensor muscles are equally activated, the leg will be

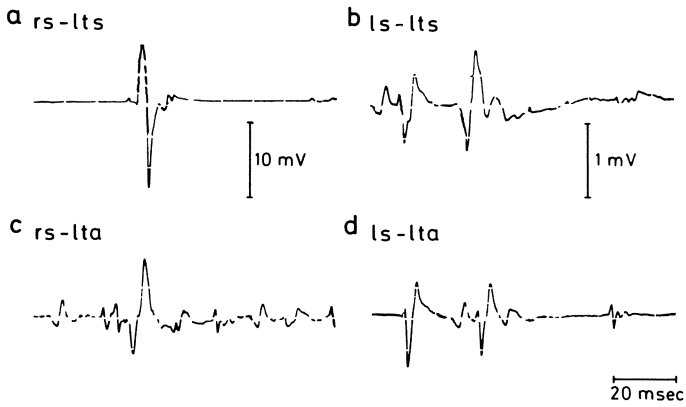

Fig. 8. Crossed (a, c) and uncrossed (b, d) reflex with long latency. rs-lts means: stimulation on the right sural nerve and recording from left triceps surae muscle; r, right; ta, tibialis anterior muscle. 10 mV calibration for record (a), 1 mV for records (b), (c), and (d).

extended only because the extensors are bigger and stronger and suited for tonic activity. This is a simple discussion but we should not forget it. Comparing the spontaneous action potentials in the triceps surae muscle and the tibialis anterior muscle in the case that the toxin is injected into any muscle, the action potential of the triceps surae muscle is always far bigger than that of the tibialis anterior muscle. This is also the case for the response to the electrical stimulation on the nerve. In the case of injection into the tibialis anterior, there is little blocking of the endplate in the tibialis anterior as well. Then we can seek the genesis of extension in the connection and function in the spinal cord under intoxication. On the development of extensor rigidity in the animal in which toxin was injected into the flexor we have hypothesized that the gamma system is activated and brings the muscle to the hyperreflex. Since the gamma system is less effective in the stretch reflex in the flexor than in the extensor, the stretch reflex is also stronger in the extensor under tetanus intoxication. This statement would be true in the weak intoxication which yields no or little amount of blocking of endplate, corresponding to most cases of tetanus intoxication in man. Interpreting our results we must find the genesis of extension now in the stronger blocking effect on the endplate or on the muscle excitation of the flexor muscle than that on the extensor muscle. In our intoxicated animals, by flexor-injection, the tibialis anterior muscle was flaccid on the third day for all doses in the experiments, while the extensor triceps surae muscle was rather intact.

Peripheral Action of Toxin

Effect on the Endplate. In spite of the fact that no binding of the toxin has been demonstrated in the motor endplate, there are many authors who found a

change of electrical activity, endplate potential, and muscle action potential (e.g., Harvey, 1939; Parsons *et al.*, 1966; Kryzhanovsky, 1967; Mellanby and Thompson, 1972; Duchen and Tonge, 1973).

Kryzhanovsky (1967) observed that the change of electrical activity occurred after the time when the toxin reached the CNS. It is important to know whether the effect on the endplate is the primary and direct action of the toxin or a more indirect and secondary one through the abnormal efferent inflow from the motoneurons with hyperactivity. Our results show that there is a direct action at least at the dose of 5×10^3 mouse MLD/kg. We have observed that the action potential of the injected tibialis anterior muscle shows a smaller amplitude than that of an uninjected muscle if it was denervated 7–8 hr after the injection. But this action potential was well preserved when we compared it with that of the intoxicated muscle with the intact reflex arc. We have chosen the time of denervation on the following consideration: in this time, toxin will be transported through the nerve trunk and the doses in the muscle may become smaller, but the toxin dose does not reach the CNS or the spinal cord (Kryzhanovsky, 1967, 1973; see also Fig. 1). In the preparation in which the nerve was cut directly after the injection, the action potential of tibialis anterior muscle was far smaller than that with later denervation. Owing to the early denervation the toxin might not be pumped out (Ponomarew, 1928; Wellhöner *et al.*, 1973) of the muscle and remains in the muscle, therefore having some greater effects on the muscle. The primary (direct) action of toxin might be dose dependent. Then in the case of lower doses change of amplitude was smaller and the tibialis anterior muscle under early denervation showed less activity (see above). The secondary change might not be directly dose dependent but dependent on the time of intoxication, because the symptom of local tetanus was so strong in every dose, suggesting the saturation of both in gamma- and alpha-motor systems.

Effect on the Muscle. In agreement with Ranson (1928) and others (Schottelius and Schottelius, 1959) we also found a change of muscle dynamics. The change of the characteristic (a) of the exponential passive tension-extension curve and that of stiffness was greater in the triceps surae muscle than in the tibialis anterior muscle. This suggests more secondary action of toxin because of the greater change in the triceps surae muscle, which is more strongly activated than the tibialis anterior muscle. The denervated and intoxicated tibialis anterior and the triceps surae of the spinalized preparation under intoxication (see Table 1, Takano and Henatsch, 1973) have no change of a value. The passive tension-extension curve was also unchanged in the intoxicated muscle whose tendon was cut before the toxin had arrived at the spinal cord (Takano, unpublished). The a value can be augmented by a prolonged period of tetanic stimulation in the muscle without intoxication (Takano, unpublished).

There may be some direct action on the muscle. The flaccid state of tibialis anterior in the case of early denervation which showed response neither to the

indirect stimulation nor to the direct stimulation, suggests the direct action of the toxin. Zacks and Sheff (1968) showed that the toxin is localized in the tubulus system and sarcoplasmatic reticulum.

The change of the amplitude and duration of action potentials could be interpreted as a part of the direct action of the toxin on the muscle membrane. The increased duration of action potential suggests the slowing of the electrical events in the muscle. One of them might be the change of conduction velocity of the muscle fibers. The membrane of the muscle fiber could be changed *directly* because it was also observed in the denervated as in the nondenervated intoxicated muscle.

Role of Gamma System. The change of active tension-extension diagram and diazepam effect on the gamma system have been discussed. An effective stimulation for the extensions of the contralateral leg is the muscle stretch (flexion) of the intoxicated side, which shows the strong participation of spindle afferents. In the cat with totally or partially failed function of the gamma-motor system, but with almost normal cutaneous sensation (Takano and Student, 1974), there were very weak local tetanus symptoms in spite of the fact that these animals showed tetanus symptom in the forelegs in the later period and died (Takano, 1973). Change of active tension-stretch rate relation also suggests a change of afferent inflow in the spinal cord from the muscle spindles.

Reflex with Long Latency. In the intoxicated animal the postsynaptic inhibitions on the motoneurons were depressed or blocked (Brooks *et al.*, 1957). We can activate the facilitatory route very easily without disturbance of inhibitory action: this was demonstrated in Fig. 8. We had a chance to survey the facilitatory system in the intoxicated animal which would otherwise not have been detectable.

SUMMARY

Tetanus toxin was injected into either the triceps surae muscle or into the tibialis anterior muscle, or into both. The contralateral muscle was compared with the muscle under intoxication.

The incubation time was measured. In both cases, injection into the tibialis anterior muscle and the triceps surae muscle, the symptom was almost always the same (namely extensor rigidity), but the incubation time was longer in the case of injection into the tibialis anterior muscle.

The early symptom of local tetanus faded away during diazepam application. Diazepam had little effect on the rigidity of the muscle at the later period.

The tension-extension curve of the tibialis anterior muscle did not show the augmentation of stiffness and the characteristic in this exponential curve, which was always observed and increased in triceps surae muscle.

The amplitude of the action potential and twitch tension of both triceps

surae muscle and tibialis anterior muscle by nerve stimulation was depressed. Fast muscle is more sensitive to the effect of toxin than slow muscle in the cat.

In the preparation which was denervated 7-8 hr after toxin injection, the action potential of the tibialis anterior muscle was well preserved. It was discussed that there might be two actions of tetanus toxin, one directly on the endplate and on the muscles; the other, indirect, caused by the hyperactivity of CNS. The intoxicated tibialis anterior muscle showed this type of extensor in the stretch reflex at variable stretch rates. The well synchronized reflex responses with long latencies by sural nerve stimulation were shown in the intoxicated muscle.

ACKNOWLEDGMENTS

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Some Pharmacological Properties of Palythoatoxin Isolated from the Zoanthid, *Palythoa tuberculosa*

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INTRODUCTION

During the course of the survey of causes of ciguatera poisoning, a pharmacologically very active substance was obtained from the zoanthid (*Coelenterata*), *Palythoa tuberculosa* Esper (Kimura *et al.*, 1972; Kimura and Hashimoto, 1973), found in the digestive tract of a filefish, *Alutera scripta*, one most widely reputed as ciguatoxic in the tropical and subtropical regions (Hashimoto *et al.*, 1969). This substance has been found to be selectively distributed in female polyps of the zoanthid, especially concentrated in their matured stage (Kimura *et al.*, 1972). The lethal dose of this substance has been estimated to be 0.6 μg/kg in mice when intraperitoneally injected (Kimura and Hashimoto, 1973). This toxicity far exceeds that of tetrodotoxin or saxitoxin, and may even exceed that of batrachotoxin, the most potent of natural toxins of low molecular weight ever known (Daly *et al.*, 1965). This substance, here designated as palythoatoxin, has been reported to be very similar, if not identical, to palytoxin previously isolated by Moore and Scheuer (1971) from closely related *Palythoa* species (*P. vestitus* and *P. toxica*) (Kimura and Hashimoto, 1973). The

latter reportedly has an estimated molecular weight of about 3300, is not a polypeptide or a protein nor does it contain sugar, and has lethal doses of 0.4 μg/kg (i.p.) and 0.15 μg/kg (i.v.) in mice (Moore and Scheuer, 1971; see also Sheikh, 1970).

Detailed pharmacological investigation of palythoatoxin has not been done yet except for the estimation of the toxicity in mice, and hence we attempted, as the first step, to obtain a general pharmacological profile of the toxin. Results to be presented will demonstrate an extremely potent membrane depolarizing activity of the toxin, which is tentatively ascribed to an increase in Na permeability of the membrane, and explains some of the various effects of the toxin in electrically excitable tissues. A part of this work has already appeared (Deguchi *et al.*, 1974).

MATERIALS AND METHODS

Palythoatoxin. Purified toxin (palythoatoxin, PTX henceforth) isolated from *Palythoa tuberculosa*, having a toxicity of 0.6 μg/kg (i.p.) in mice (MLD) (Kimura and Hashimoto, 1973) was generously donated by Professor Hashimoto of the University of Tokyo. The LD_{50} in mice by the intravenous route of this purified toxin was 0.53 μg/kg (95% confidence limit, 0.38–0.73 μg/kg).

The toxin was dissolved in distilled water at a concentration of 10^{-5} g/ml and stored in frozen state. This stock solution was appropriately diluted for daily experiments. As long as it was kept frozen, the potency of the stock solution was fairly stable, and did not change at least for a few weeks.

***In Vivo* Experiment in Anesthetized Cats.** Animals were anesthetized with pentobarbitone sodium (Nembutal) of 35 mg/kg, intraperitoneally applied. A polyethylene cannula, inserted in the femoral artery, was connected with a pressure transducer for recording arterial blood pressure. Another cannula inserted in the femoral vein served for intravenous injections. When artificial respiration was applied, tracheotomy was made and a Y tube was attached to the trachea and connected to a respirator. Respiration was picked up with a strain gauge attached to the thoracic wall. The electrocardiogram was registered with lead A–B for small domestic animals (Takahashi, 1969).

In Vitro Experiments in Excised Tissue Preparations

The Guinea Pig Heart Muscle Strip Preparations. Male guinea pigs of the Hartley strain weighing more than 500 g were used. The animal was killed by a blow on the head and subsequent decapitation. The heart was quickly removed and transferred to a Tyrode solution bath for dissectioning at room temperature. The right atrium, left atrium, or right ventricular papillary muscle was carefully dissected out and suspended in an organ bath of 15 or 40 ml in volume filled

with Tyrode's solution, oxygenated with 95% O_2 + 5% CO_2. Bath temperature was kept at 28-30°C or at 35-37°C. The left atrium and papillary muscle were electrically driven at a rate of 1 Hz with pulses of 1-3 msec duration applied through a pair of platinum wire electrodes placed in close contact with the tissue. The contractile activities were picked up with a strain gauge transducer, and registered with a chart recorder.

The Guinea Pig Ileum and Taenia Coli Strip Preparations. These tissues were similarly excised and suspended in an organ bath of 15 ml in volume. Bath temperature was set at 36°C. Tyrode's solution was aerated with the same gas mixture as above and served as bathing medium. The contractile activity of the ileum strip was isotonically registered with a frontal writing lever on a kymograph. That of the taenia coli was isometrically recorded by way of a strain gauge transducer.

The Phrenic Nerve-Diaphragm Preparation of the Mouse. Male albino mice of dd/Y strain weighing 30-35 g were used. The animals were killed by dislocation of the cervical vertebrae. The hemidiaphragm was quickly dissected out with the phrenic nerve and the costae attached, and transferred to a Tyrode solution bath for dissection. The tissue was carefully cleaned of adherings under a dissecting microscope, and mounted in an organ bath filled with 40 ml of Tyrode's solution, which was kept at 30°C and aerated with the same gas mixture as above. The bath was equipped with a small side chamber for nerve stimulation. The phrenic nerve was passed through a small hole to the side compartment, and the stimulation was effected through the hole by passing current between a pair of Pt wire electrodes put separately in the main and the side baths. The stimulation pulses of 0.01-0.05 msec, 0.1 Hz were applied to the nerve, and in addition, train pulses of 100 Hz, 10 sec, and of the same intensity were applied at 5-min intervals. With this stimulation schedule, it was possible to examine the drug for the effects on twitch tension, tetanic contraction, and posttetanic potentiation simultaneously. The tension was isometrically registered through a strain gauge transducer on a chart recorder.

The Frog Sciatic Nerve-Sartorius Muscle Preparation. Frogs used were *Rana brevipoda* of both sexes weighing 10-30 g. The frog was killed by decapitation, and the sartorius muscle carefully dissected out with a branch of the sciatic nerve innervating the muscle attached. The preparation was suspended in the same bath as used for the mouse diaphragm preparation, which was in this case filled with frog Ringer solution aerated with air and kept at room temperature (20-23°C). Stimulation was alternately applied to the nerve and muscle through two separate pairs of Pt wire electrodes. A pair of pulses 1 sec apart, one for nerve and the other for muscle stimulation, was applied once every 10 sec. Twitch tensions were isometrically registered through a strain gauge transducer.

The Frog Rectus Abdominus Muscle Preparation. The excised muscle was suspended in a 15-ml organ bath filled with frog Ringer solution, aerated with

air, and kept at room temperature. The contracture tension was isometrically recorded through a strain gauge transducer.

Measurement of the Membrane Potential in the Frog Sartorius Muscle. Conventional glass microelectrodes of less than 0.5 μ at the tip filled with 3 M KCl, and having a resistance of 10-20 MΩ were used. The electrode was connected with a thin silver wire to the head of the cathode follower preamplifier and the membrane potential was registered on the chart recorder. The electrode was driven by a micromanipulator. The frog sartorius muscle was mounted with its inner surface up in a perfusion bath of about 2 ml in volume, and the microelectrode was impaled under direct observation with a dissecting microscope. The bath was continuously perfused with bathing solution from the reservoir, overflowing solution being continuously sucked away. The perfusion rate was usually 0.5-1 ml/min. When the solution was changed the bath was flushed with new solution. Solution change was effected by turning a switch cock placed close to the inlet to the organ bath.

Solutions. Tyrode's solution was used for excised tissues from mammals. Ringer solution was used in experiments with frog muscle preparations. Compositions of these solutions were as follows. Tyrode's solution: 136.8 mM NaCl; 5.4 mM KCl; 2.5 mM $CaCl_2$; 1.0 mM $MgCl_2$; 0.4 mM NaH_2PO_4 and 11.9 mM $NaHCO_3$, pH 7.2-7.4. Frog Ringer solution: 115 mM NaCl; 2.5 mM KCl; 1.8 mM $CaCl_2$; 0.85 mM NaH_2PO_4 and 2.15 mM Na_2HPO_4, pH 7.0-7.2. In the membrane potential studies, a low-Na solution was used, where 99% or 99.9% of Na in frog Ringer solution was substituted with choline and the pH was adjusted with tris-(hydroxymethyl) aminomethane.

Drugs and Reagents. Atropine sulfate (E. Merck AG), *d*-tubocurarine chloride (Yoshitomi Pharmaceutical Co.), chlorpheniramine maleate (Sankyo Co.), tetrodotoxin (Sankyo Co.), gallamine triethiodide (Teikoku Kagaku Sangyo Co.), acetylcholine chloride (E. Merck AG), choline chloride (E. Merck AG), tris-(hydroxymethyl) aminomethane (E. Merck AG), and pentobarbitone sodium (Abbott, Nembutal®) were used. Other inorganic reagents used for physiological solutions were all reagent grade from Wako Pure Chemical Industries. The concentrations of the drugs were referred to the salts.

RESULTS

Cardiovascular and Respiratory Effects in Anesthetized Cats. In cats anesthetized with Nembutal, PTX intravenously applied at a dose of 0.2 μg/kg (which was lethal in 8 of 15 cases) caused a rise in the arterial blood pressure with a concomitant increase in pulse wave amplitude shortly after injection. The blood pressure rise attained the peak in 5-20 min, then gradually subsided in 30-60 min, and was followed thereafter by a decrease (Fig. 1, middle traces). Profound changes were observed in the electrocardiogram during the intoxication: brady-

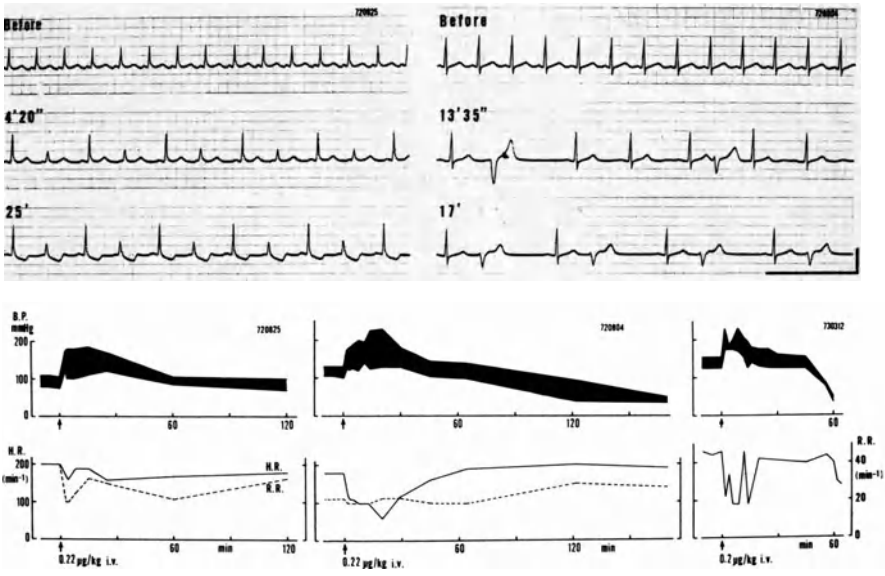

Fig. 1. Cardiovascular and respiratory alterations in anesthetized cats induced by PTX administration. Top traces are two sample records of ECG changes produced by 0.22 μg/kg (i.v.) of PTX. Time after administration is shown to the left of each trace. Calibration: 1 mV, 1 sec. Time courses of alterations in blood pressure (B.P., shaded stripe), heart rate (H.R., solid line) and respiratory rate (R.R., dotted line) in the corresponding experiments are shown below the ECG recordings. Blood pressure and heart rate are calibrated at the left and respiratory rate at the right. Alterations in blood pressure and heart rate illustrated on the right at the bottom are from a case under artificial respiration. Animal died of heart failure 60 min after 0.2 μg/kg i.v. PTX injection.

cardia, ectopic beats, bigeminity, ventricular arrhythmias, and fall in ST segment with ensuing T inversion. Some of these are clearly seen in sample records on the top in Fig. 1. These alterations were finally followed by cardiac arrest. Arrhythmias were reflected on the blood pressure as discontinuous changes in the instantaneous pressure amplitude. The respiration was gradually depressed and became abdominal type, shallow and rapid; the animal became anoxic, cyanotic, and stiff toward the end. Defecation and micturition were often observed during the intoxication.

At lower doses of 0.02–0.05 μg/kg (i.v.), PTX caused a biphasic rise in the arterial blood pressure of a slight to moderate degree (20–40 mmHg). The initial rise appeared shortly after injection, attained its peak in about 10 min and declined thereafter. The second phase of hypertension started some 20 min after administration and reached the peak in 60 min or longer after application, and continued for another hour or so. During these phases of hypertension, the pressure pulse amplitude also increased, suggesting a cardiotonic action of PTX. The

heart rate tended to decrease during the initial phase of hypertension, but no clear trend was seen during the second phase.

In several instances, PTX was administered during artificial respiration to test whether respiratory failure was the cause of the death. As exemplified in Fig. 1, at the bottom, the fatal effect of 0.2 μg/kg of PTX could not be prevented in 3 of 4 cases in spite of artificial respiration. Death occurred at an average of 48 min (range, 32-64 min) after administration under forced ventilation as opposed to the mean death time of 67 min (range, 13-180 min) in 5 of 11 cases under natural respiration at this dose level. Accordingly, the death was not attributable to respiratory collapse, but to some other causes, most probably heart failure.

Effects on the Excised Cardiac Muscle Strip Preparations. Prominent cardiac action of PTX could also be reproduced in *in vitro* experiments using excised heart muscle preparations of the guinea pig, as shown in Fig. 2. Traces A, B, and C-C′ are records from spontaneously beating right atria, C and C′ being consecutive ones, trace D, that of an electrically driven papillary muscle, and E and F, from electrically driven left atria of the guinea pig. As seen in A, spontaneously beating right atrium suspended in an organ bath responded to 1×10^{-8} g/ml PTX with gradual increases in the contractile force and rate. In B and C, a concentration of PTX twice as high was applied. In addition to the positive ino- and chronotropic effect, there appeared an initial transient phase of depression for 1-2 min, and further, arrhythmia and secondary depression ensued to the positive ino- and chronotropism. These changes eventually proceeded to arrest. It is noticed in traces B and C′ that the resting tension has risen considerably after cessation of spontaneous contractions, showing that the muscle was in the state of sustained contracture. In C′, the preparation was repeatedly washed in PTX-free Tyrode's solution for more than 1 hr after the blockage. In spite of this no complete recovery was attained; only rather weak, arrhythmic contraction reappeared on the raised baseline. Trace D shows that positive inotropic and subsequent arrhythmic effects were also produced in the electrically driven papillary muscle by PTX of a similar concentration. The papillary muscle also showed contracture after prolonged immersion in PTX solution of this strength. Records in E and F demonstrate a slowly developing positive inotropic effect of PTX attaining to its peak in 5-10 min in as low a concentration range as 10^{-10} g/ml in the left atria.

Thus, it can be stated that PTX has a cardiotonic effect at an extremely low concentration of 10^{-10} g/ml or up, and biphasic effect of initially stimulative, subsequently arrhythmic, depressive, and eventually contracture-inducing, at high concentrations of 10^{-8} g/ml.

Effects on the Excised Intestinal Strip Preparations. PTX also exerted stimulant effects on smooth muscle tissues. An isolated ileum strip of the guinea pig suspended in an organ bath exhibited a transient contraction in response to PTX.

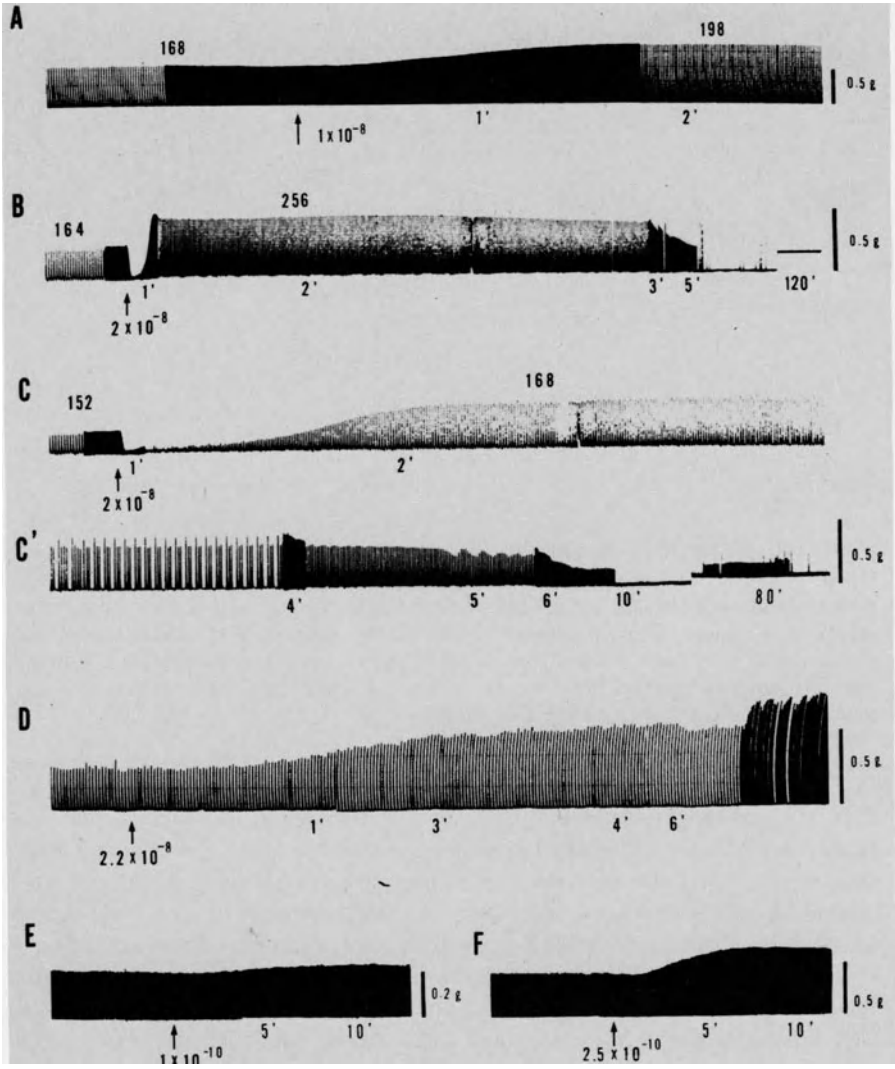

Fig. 2. Effects of PTX on various heart muscle preparations. A–C′ are from the spontaneously beating right atria of the guinea pig. C and C′ are consecutive records from the same preparation. D, from an electrically driven papillary muscle, and E and F, from electrically driven left atria of the guinea pig. Numbers on the traces indicate the spontaneously beating atrial rate in beats/min. Time in min after drug application are shown underneath. Upward arrows indicate time of PTX application. Tension calibration is shown to the right of each trace.

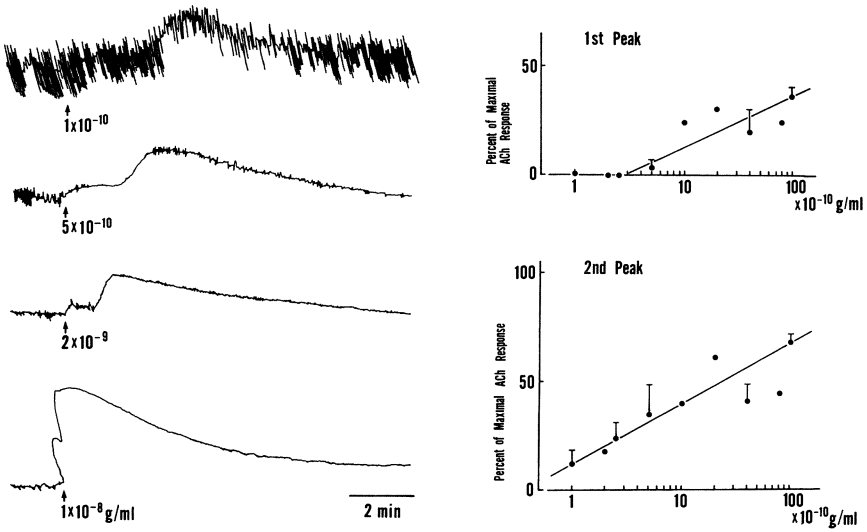

Fig. 3. Effects of PTX on the isolated ileum strips of the guinea pig. On the left are shown sample recordings of the isotonic contractions induced by PTX at various concentrations. Dose-response relationships are illustrated on the right. Vertical bars on the points in the graphs show standard error of means. Points bearing bars show the means of at least several measurements, and those without bars, means of only a few measurements. As the magnitude of contraction varied between preparations, it was normalized in reference to the maximal response induced by 2×10^{-5} g/ml ACh.

In Fig. 3, traces on the left are isotonically registered contractions induced by PTX of doses ranging from 1×10^{-10} to 1×10^{-8} g/ml. The response had two peaks; one was an initial small contraction, and the other, a subsequent large contraction. After the peak the contraction slowly subsided in 10-15 min and attained a level somewhat raised above the initial baseline. The amplitude of and the time to peaks varied depending on PTX concentration. The higher the concentration the shorter the time to peaks. The positive correlation of the magnitude of contractions and the dose of the toxin is shown as the dose-response curves drawn separately for the two peaks on the right. Magnitude of the responses were expressed as percent of maximal ACh contracture for each preparation to cancel out variation in actual size of contraction between preparations, and plotted on the ordinates. Abscissas are PTX concentrations in log scale. It can be seen in the graphs that the inclination is almost the same for the two curves, and the minimal concentration of PTX to produce contraction is somewhere around 10^{-10} g/ml. Preliminary observations showed that both of these responses were not influenced by either atropine (1×10^{-5} g/ml) or chlorpheniramine (1×10^{-7} g/ml), indicating direct action, if not all, of PTX on the smooth muscle. It was also shown that isolated strips of the taenia coli of the

guinea pig, exhibiting spontaneous contractile activities, responded to a PTX level of 10^{-10} g/ml or higher with a sustained contracture when isometrically registered.

Effects on the Excised Frog Rectus Abdominis Muscle Preparations. PTX caused slowly developing contracture in the frog rectus muscle. More than 30 min elapsed before the contracture reached the plateau in response to 1×10^{-8} g/ml PTX, as shown in the sample record on the top in Fig. 4.

The dose-response curve in Fig. 4 was constructed with the maximal ACh contraction as the reference. The curve tailed in the low concentration range toward the minimal effective concentration of 10^{-10} g/ml, and rose at around $2\text{-}4 \times 10^{-9}$ g/ml on the abscissa toward high concentrations. The curve thus seemed to be composed of two parts: one in the lower concentration range with a rather small inclination, and the other in the higher concentration range

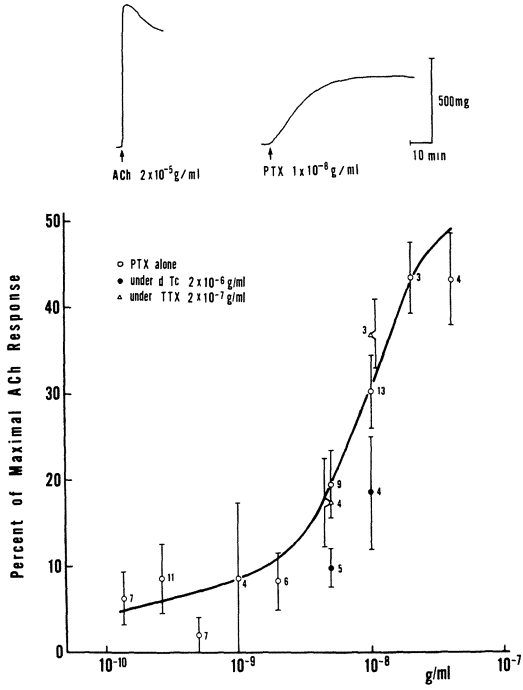

Fig. 4. Dose-response relationship for PTX contraction in the frog rectus abdominis muscle. Traces on the top depict time courses of contractions induced by ACh and PTX. Contractions induced by PTX were normalized with reference to maximal ACh contraction and plotted on the ordinate. Abscissa, concentration of PTX in log scale. Vertical bars indicate standard error of means. Numbers of observations are indicated close to each point. Hollow circles are responses to PTX in normal Ringer solution; triangles, in the presence of 2×10^{-7} g/ml tetrodotoxin; and black dots, in the presence of 2×10^{-6} g/ml *d*-tubocurarine.

with a moderately steep slope. This might be a reflection of two separable processes connected with the generation of PTX contracture. Preliminary analysis showed that *d*-tubocurarine (2×10^{-6} g/ml) only slightly depressed, and tetrodotoxin (2×10^{-7} g/ml) did not affect at all, the contracture induced by 0.5–1×10^{-8} g/ml of PTX. This observation indicated that PTX acted directly, if not solely, on the muscle to produce contracture.

Effects on the Excised Neuromuscular Preparations. In the mouse phrenic nerve-diaphragm preparations, PTX of 1×10^{-8} g/ml or higher concentrations increased the twitch tension temporarily for 20–30 min, and then gradually decreased it. The decrement of 50% in the twitch height by nerve stimulation occurred in less than one hour in this dose range. Reduction in the magnitude of posttetanic potentiation (as expressed by the product of the maximal height and the half-decay time of the posttetanic potentiation) usually preceded that of twitch tension. The dose-effect relation, as obtained after more than 40 min of PTX application, showed that twitch tension was depressed by 50% of control height at a concentration of 2.5×10^{-8} g/ml, and posttetanic potentiation was likewise depressed at a concentration of 2×10^{-8} g/ml. Figure 5A shows that at a high concentration of 3×10^{-8} g/ml, the twitch depression occurred in 30 min and in addition contracture appeared after the depression, which can be seen as a rise in the baseline. These effects of PTX were again difficult to remove even by repeated washing with the toxin-free bathing medium.

Effects of PTX on the frog sciatic nerve-sartorius muscle preparations are shown in Fig. 5B. The twitch contractions induced through nerve stimulation and direct stimulation of the muscle were depressed almost simultaneously in the presence of 2×10^{-8} g/ml PTX.

Effects on the Membrane Potential of the Excised Frog Sartorius Muscle. The membrane potential of the excised frog sartorius muscles was measured during treatment with PTX in the organ bath. PTX exerted a strong depolarizing action. The time course of the change in membrane potential after application of the toxin at several different concentrations is shown in Fig. 6. Resting membrane potential gradually decreased at 1×10^{-8} g/ml of PTX to a level below −50 mV after one hour of treatment. The curve looks as if the depolarization has not leveled off yet. The eventual level of depolarization would have further exceeded this level. As the concentration of PTX increased, the rate and the extent of depolarization increased. At a concentration of 3.2×10^{-8} g/ml or higher, the membrane was depolarized to less than −50 mV in 10 min. When PTX at concentrations higher than 1×10^{-7} g/ml was introduced into the organ bath fibrillation occurred and membrane potential diminished quickly and reached a level of less than −10 mV within 1 hr.

The data of the same experiment were replotted in terms of the dose-response relation in Fig. 7, where the ordinate was the magnitude of depolarization and the abscissa the concentration in log scale. Although data at concentra-

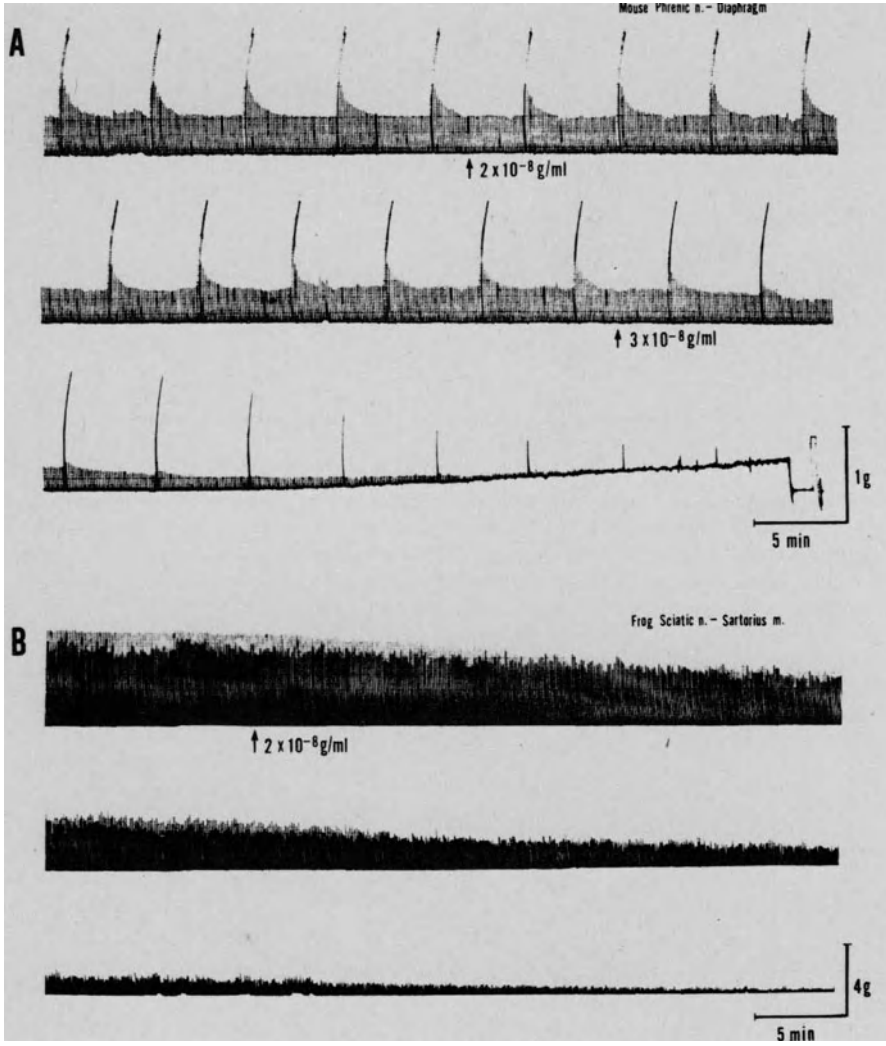

Fig. 5. Effects of PTX on the neuromuscular preparations. Traces in A show PTX effects on the phrenic nerve-diaphragm preparation of the mouse and those in B, that on the frog sciatic nerve-sartorius muscle preparation. Note the rise of baseline after blockage of transmission in A, and the parallel depression of twitch contractions evoked by nerve and muscle stimulations in B.

tions lower than 1×10^{-8} g/ml are lacking, it may be possible to estimate the minimal depolarizing concentration by extrapolating the curve for 60-min values toward lower concentrations. Thus PTX will exert a minimal depolarizing effect at a concentration well below 10^{-8} g/ml, probably at around 10^{-9} g/ml. If more

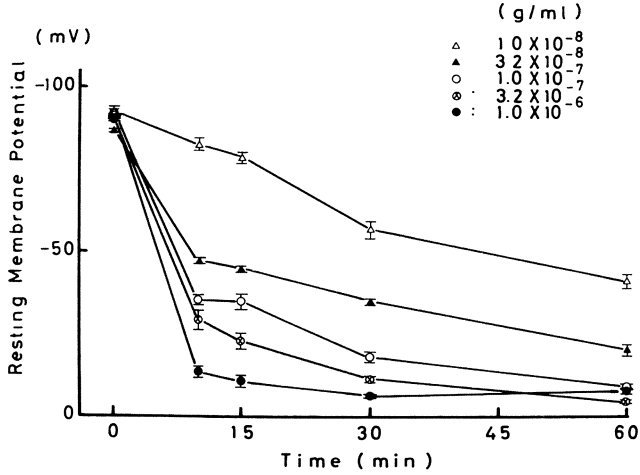

Fig. 6. Time course of membrane potential changes in the frog sartorius muscle preparations during treatment with PTX of various concentrations. Ordinate shows membrane potential in –mV, abscissa, time in min after PTX application. Vertical bars indicate standard error of means. Open triangles, 1×10^{-8}; solid triangles, 3.2×10^{-8}; open circles, 1×10^{-7}; circles with crosses, 3.2×10^{-7}; solid circles, 1×10^{-6} g/ml.

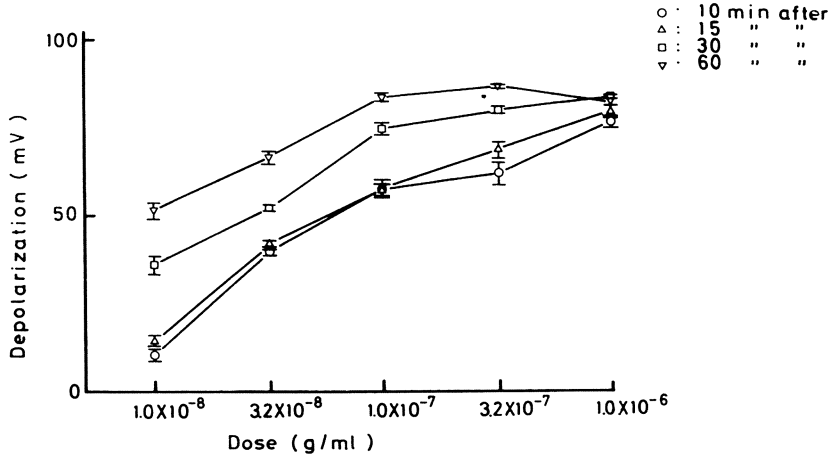

Fig. 7. Dose-response relationship for the depolarization in the frog sartorius muscle preparations at various time intervals after PTX application. Ordinate shows magnitude of depolarization in mV, abscissa, concentration of PTX in log scale. Circles, 10 min; triangles, 15 min; squares, 30 min; and inverted triangles, 60 min after PTX application.

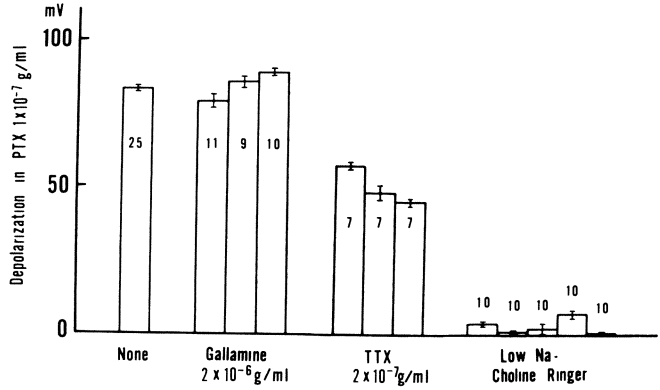

Fig. 8. Effects of gallamine, tetrodotoxin, and Na deficiency on the magnitude of depolarization in the frog sartorii after 60 min treatment with 1×10^{-7} g/ml PTX. The muscles were equilibrated for at least 30 min with drug-containing or low-Na Ringer solution before PTX was applied. See text for explanations.

time is allowed for PTX to act, the range of depolarizing concentration may be extended further to a lower level.

Figure 8 compares the extent of depolarization after 1 hr at 1×10^{-7} g/ml PTX in normal Ringer solution with that in the presence of gallamine or tetrodotoxin or in low-Na choline Ringer solution. The depolarization was not affected by gallamine triethiodide, a curare-like neuromuscular blocker. This shows that PTX exerted depolarizing action directly, if not solely, on the muscular membrane. The depolarization was diminished by 2×10^{-7} g/ml tetrodotoxin, and almost totally abolished in low-Na Ringer. These results indicate that PTX depolarizes muscle membrane by increasing Na permeability.

DISCUSSION

PTX with a toxicity of 0.6 μg/kg (i.p., MLD) and 0.53 μg/kg (i.v., LD_{50}) in mice exerted a fatal effect also in anesthetized cats at a dose of 0.2 μg/kg (i.v., lethal in 8 of 15 cases). The toxicity may exceed, or is at least as potent as, that of batrachotoxin, the most potent of natural toxins of low molecular weight ever known, having an LD_{50} of 2 μg/kg (s.c.) in mice (Daly *et al.*, 1965). There seem to be apparent similarities in pharmacological properties between the two toxins. Both toxins have a strong membrane depolarizing effect attributable to an increase in Na permeability of the membrane, potent cardiotoxic and skeletal muscle depressant effects, and a contracture-inducing effect in mammalian skeletal muscles (Albuquerque *et al.*, 1971; and also Albuquerque, 1972). How

much these two toxins have in common in their mechanism of action awaits further analysis.

The close similarities of PTX and palytoxin in chemical properties have suggested the identity of the two toxins (Kimura and Hashimoto, 1973). The toxicological symptomatology of palytoxin in mice has led to the conclusion that death was from respiratory collapse (Moore and Scheuer, 1971). In the present study, however, it was demonstrated that death from intravenously applied PTX was most probably ascribable to cardiac failure, rather than to respiratory collapse in anesthetized cats. Recently toxicological evaluation of palytoxin in several animal species has been made (Wiles *et al.*, 1974), and its LD_{50} in the dog, rabbit, monkey, guinea pig, rat, and mouse estimated to be in a range of 0.025-0.45 μg/kg, when applied intravenously. The toxin has also been demonstrated to have irritant and tissue-damaging effects when topically applied to the skin or eyes. Direct pharmacological comparison of PTX and palytoxin may help clarify the problem of possible identity of the two toxins. In any case elucidation of the chemical structures of the two toxins is awaited.

PTX was shown to stimulate the cardiac muscle, the intestinal smooth muscle, and frog rectus muscle at such an extremely low concentration as 10^{-10} g/ml, and depolarize the skeletal muscle membrane at a concentration of 10^{-8} g/ml or less. The depolarizing action of the membrane was attributable to an increase in Na permeability since it was totally abolished in low-Na Ringer solution. This depolarization was diminished by 2×10^{-7} g/ml of tetrodotoxin. This may also support the notion that the depolarization is due to the Na permeability increase. Thorough investigation, however, into the interrelation between tetrodotoxin and PTX actions remains to be accomplished. The potent depolarizing action may be correlated with some of PTX effects, e.g., cardiotoxic effect and depressant effects on twitch contractions in skeletal muscles. Contractures produced in the skeletal and cardiac muscles by high doses of PTX may also result from depolarization. However, cardiostimulation and intestinal contraction, as well as contracture in the frog rectus muscle at low concentrations, may not be reconciled with the membrane depolarization. A mechanism of action directly connected with contraction mechanism may be involved in these actions of PTX.

Apparently more work is needed to clarify the pharmacological properties of this powerful toxin, and to evaluate it for possible therapeutic uses, or as a tool for fundamental research.

SUMMARY

Pharmacological properties of palythoatoxin (PTX), an extremely potent toxin from the zoanthid, *Palythoa tuberculosa*, having an i.v. LD_{50} of 0.53 μg/kg in mice, were studied in *in vivo* and *in vitro* experiments.

In anesthetized cats, PTX, at an i.v. dose of 0.2 μg/kg (a lethal dose) caused a transient rise in the arterial blood pressure followed by a gradual decrease. Arrhythmias to various degree appeared, and in fatal cases, were followed by cardiac arrest. Artificial respiration did not prevent the fatal effect. The cardiotoxic effect was thus suggested as the primary cause of death. At lower doses than this, PTX induced slowly developing hypertension with concomitant cardiostimulation.

PTX showed a positive inotropic effect at a concentration of 10^{-10}-10^{-8} g/ml, and arrhythmias and contracture ensued at higher concentrations than this in the excised guinea pig heart muscle preparations. It also induced a slowly developing contracture in the isolated ileum of the guinea pig and in the frog rectus abdominis muscle at a concentration above 10^{-10} g/ml by directly, if not solely, acting on the muscular tissues. In the mouse phrenic nerve-diaphragm preparation, it depressed twitch contractions induced by either nerve or muscle stimulation at a concentration above 10^{-8} g/ml, and caused contracture after the depression. The resting membrane potential of the frog sartorius muscle gradually decreased in PTX of 10^{-8} g/ml or higher. The depolarization was prevented in the low-Na medium and diminished by tetrodotoxin. The depolarizing action, tentatively ascribed to an increase in Na permeability of the membrane, could account for some of PTX effects observed in electrically excitable tissues.

NOTE ADDED IN PROOF

Since submission of this work a preliminary report on some pharmacological properties of palytoxin has appeared (Kaul, P. N., Farmer, M. R. and Ciereszko, L. S. (1974) Pharmacology of palytoxin: the most potent marine toxin known, Proc. West. Pharmacol. Soc., *17*, 294-301).

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Pharmacological Studies on Surugatoxin, the Toxic Principle from the Japanese Ivory Mollusk, *Babylonia japonica*

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INTRODUCTION

In September 1965, food poisoning occurred from ingestion of a carnivorous gastropod, the Japanese ivory mollusk (*Babylonia japonica*), captured in the Shizuoka district of Suruga Bay. The patients complained of visual disorders, mydriasis, abdominal distention, dryness of mouth, constipation, and vomiting. The poisoning was apparently due to toxins, which were later demostrated in the mid-gut gland of the mollusk found in a very limited area of the bay (Kimura and Sugiyama, 1967; Hashimoto, 1967).

In 1972, Kosuge *et al.* (1972) isolated one of the toxic principles in crystalline form and determined the chemical structure (molecular formula: $C_{25}H_{26}N_5O_{13}Br \cdot 7H_2O$; molecular weight: 810.53) (Fig. 1). The toxin was named surugatoxin (SGTX) after Suruga Bay. SGTX causes a mydriasis with an effective minimum s.c. dose of 0.05 μg/g of body weight (in mice), approximately equivalent to that of atropine sulfate.

Only a few toxins such as saxitoxin from Pelecypod and murexine from several *Murex* species (gastropod) have so far been clarified for their pharmacological and toxicological properties. Saxitoxin is known to block action potentials in nerves and muscles by preventing, in a very specific manner, an increase in the ionic permeability which is normally associated with the inward flow of sodium (Kao and Fuhrman, 1967). On the other hand, murexine produces paralysis in

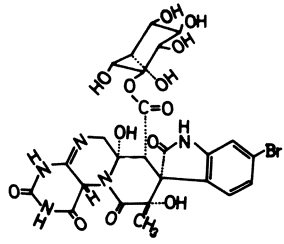

Fig. 1. The chemical structure of surugatoxin.

skeletal muscle probably through its depolarizing action on the endplate (Erspamer and Glasser, 1957).

We have studied the pharmacological properties of SGTX and have found that the toxin has a ganglion-blocking action and the toxic effect is mainly mediated through this action. Some accounts of this work were communicated to the Japanese Pharmacological Society (Yamada *et al.*, 1973).

MATERIALS AND METHODS

Measurements of Blood Pressure and Contractile Response of Nictitating Membrane in Cats. Cats of either sex weighing between 2.5 and 4.5 kg were anesthetized with an intraperitoneal injection of a mixture of urethane (500 mg/kg) and chloralose (40 mg/kg). The blood pressure was recorded from the right femoral artery with a pressure transducer, and the contractile response of the nictitating membrane was recorded with a force-displacement transducer connected to a multipurpose polygraph (Nihon Kohden RM-150). Drugs were administered intra-arterially close to the superior cervical ganglion, to the nictitating membrane through a polyethylene cannula inserted into the lingual artery (Trendelenburg, 1956), or intravenously into the femoral vein.

Autonomic Nerve Stimulation. The nerves were placed on a pair of platinum electrodes kept moist and electrically insulated from the surrounding tissues by warm liquid paraffin. Stimulation was applied with an electrical stimulator (Nihon Kohden MSE-3R) delivering rectangular pulses of submaximal or supramaximal voltage, 1 msec duration, 5–30 Hz frequencies for 10 sec at 2–3 min intervals. The responses recorded were: an increased and decreased blood pressure during splanchnic and vagal nerve stimulation, respectively, and a contraction of the nictitating membrane and the action potential during pre- and postganglionic cervical sympathetic nerve stimulation. Cats sectioned at C_1 of the spinal cord were artificially ventilated.

Construction of Dose-Response Curves for Agonists in Isolated Guinea Pig Ileum. Male guinea pigs weighing 300–500 g were killed by a blow on the head

and a segment of the ileum was dissected at least 8 cm from the ileocaecal junction. A preparation was suspended in Tyrode's solution in a thermostatically controlled organ bath (10 ml capacity) at 37°C and gassed with oxygen. Responses of the ileum to drugs were recorded on a smoked drum with a isotonic frontal writing lever. Acetylcholine and histamine were added by means of the cumulative-dose method described by van Rossum *et al.* (van Rossum, 1963; van Rossum and Ariens, 1962), and nicotine, DMPP, and 5-hydroxytryptamine by the single-dose method. SGTX and some other antagonists were added to the organ bath 3-5 min before an addition of the agonist at 3-5 dose levels. The dose-response curves for the agonists were obtained by plotting the log concentration of agonist used on the abscissa and the percent of the control maximal contraction on the ordinate.

Transmural Stimulation. Transmural stimulation was carried out by a technique essentially similar to that described by Paton (1955, 1957). The electrodes were made of platinum and the intraluminal electrode was made the anode. Rectangular pulses were used of 0.4 msec duration at a frequency of 0.1 Hz and a strength sufficient to give a maximal response. The responses were isometrically recorded.

Drugs. The drugs used were obtained from the following sources: acetylcholine chloride (Daiichi), histamine diphosphate (Wako), nicotine tartrate (Wako), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Aldrich), 5-hydroxytryptamine (Wako), adrenaline hydrochloride (Daiichi), 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) (McNeil Laboratories, Inc.), hexamethonium chloride (Wako), tetraethylammonium chloride (Wako), mecamylamine hydrochloride (Sigma), atropine sulfate (Merck), propranolol hydrochloride (Sumitomo), phenoxybenzamine hydrochloride (Tokyo Kasei), urethane (Merck), alpha-chloralose (Wako). Crystalline surugatoxin was generously provided by Professor Takuo Kosuge of this college. Drugs were dissolved in a 0.9% wt./vol. NaCl solution (saline) and the injected doses refer to the weights of the salts.

RESULTS AND DISCUSSION

Effect on Blood Pressure and Ganglionic Transmission in Anesthetized Cats. Intravenous injection of SGTX (50 nmol/kg) produced a hypotension of 1-2 hr duration in anesthetized cats (Fig. 2). The hypotension was neither blocked by pretreatment with atropine and propranolol nor by spinal cord transection. The pressor effect produced by a nicotinic ganglion stimulant, DMPP was completely blocked by SGTX (50 nmol/kg, i.v.), while the pressor effect by adrenaline and a muscarinic ganglion stimulant, McN-A-343, was not blocked. SGTX reduced the pressor or depressor response to both splanchnic and vagal nerve stimulation (Fig. 3).

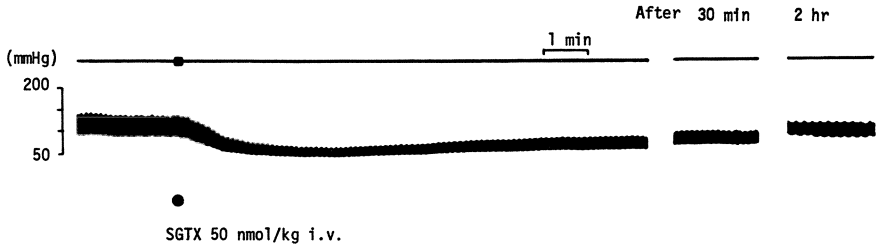

Fig. 2. The effect of SGTX on the systemic blood pressure in anesthetized cat.

As these results suggested that SGTX exerted a ganglion-blocking action, the effect of SGTX on the superior cervical ganglion was investigated using the cat's cervical sympathetic nerve-nictitating membrane preparation.

Arterial injection of SGTX close to the superior cervical ganglion blocked the contractile response of the nictitating membrane to preganglionic stimulation (Fig. 4a) and to a close-arterial injection of DMPP, but not that to McN-A-343 (Fig. 5). The response to postganglionic stimulation (Fig. 4b) or adrenaline (injected into the external carotid artery) were also unaffected by SGTX.

In addition, the postganglionic nicotinic action potential induced by preganglionic stimulation of the cervical sympathetic nerve was blocked by SGTX. These experiments clearly indicate that SGTX exerts a ganglion-blocking action through the nicotinic receptor blockade like hexamethonium.

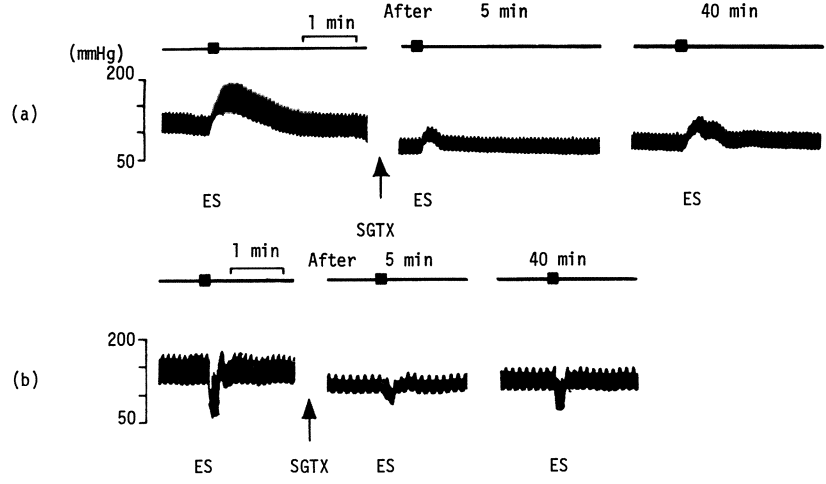

Fig. 3. The effect of SGTX (50 nmol/kg, i.v.) on the hypertensive (a) and hypotensive (b) responses to electrical stimulation of splanchnic and vagal nerves, respectively, in the anesthetized cat. Dots: supramaximal stimulation for 10 sec.

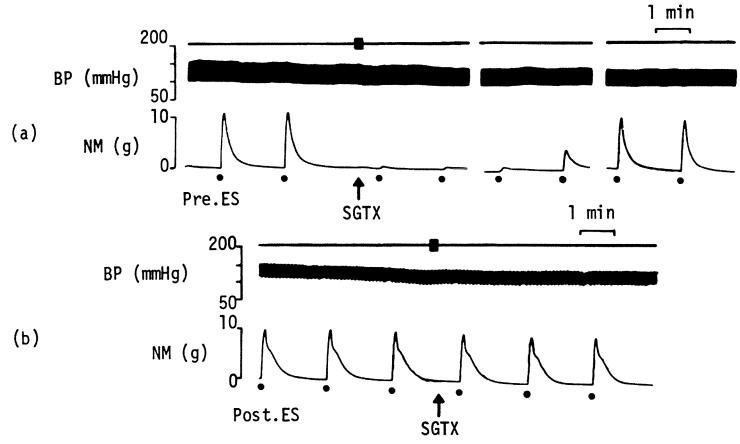

Fig. 4. The effect of SGTX (12.3 nmol/kg, i.a.) on the contractile response of the nictitating membrane (NM) to preganglionic (a) and postganglionic (b) stimulation of the cervical sympathetic nerve. Dots: supramaximal preganglionic (a) and submaximal postganglionic (b) stimulation for 10 sec at 2 min intervals. BP: blood pressure from the femoral artery.

Effect on Isolated Guinea Pig Ileum. The dose-response curves for acetylcholine and histamine were not affected by SGTX at the concentrations less than 12.3 μM. The curves for nicotine and DMPP were shifted to the right and gradually depressed as the concentration of SGTX was increased from 12.3 nM to 1.23 μM (Fig. 6). The antinicotinic action of SGTX on the isolated guinea pig ileum was then compared to the other ganglion blockers such as hexamethonium, tetraethylammonium, and mecamylamine. Hexamethonium (3.66–36.6 μM) and tetraethylammonium (6.0–60.0 μM) shifted the curves for nicotine

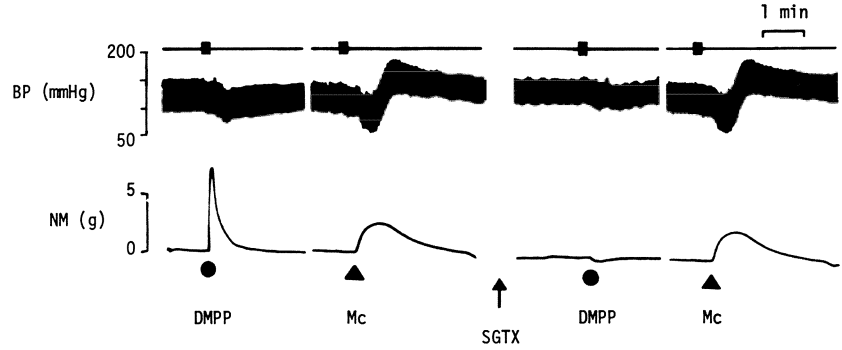

Fig. 5. The effect of SGTX (12.3 nmol/kg, i.a.) on the contractile response of the nictitating membrane (NM) to injected DMPP (6.3 nmol/kg, i.a.) and McN-A-343 (Mc) (31.6 nmol/kg, i.a.). BP: blood pressure from the femoral artery.

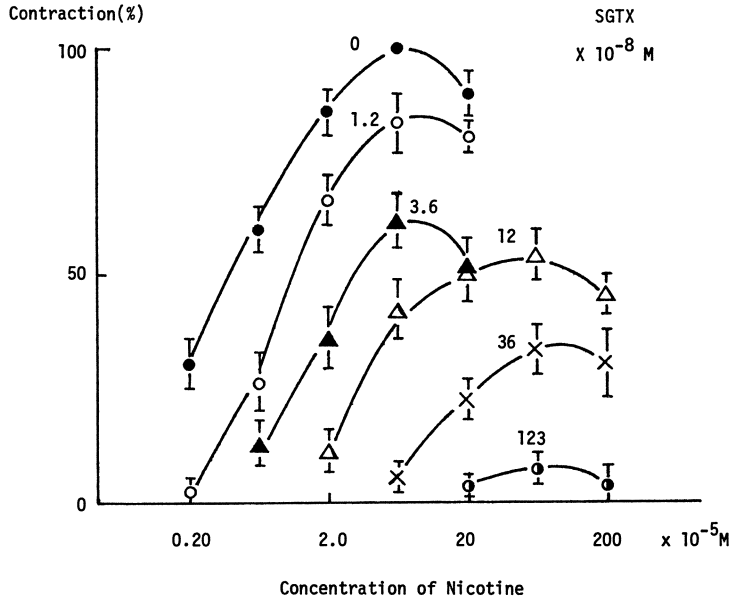

Fig. 6. The effect of SGTX on the dose-response curves for nicotine in the isolated guinea pig ileum. Each point represents the mean of at least six experiments and vertical bars show the standard errors of the mean.

to the right without the depression, whereas mecamylamine (0.49-4.9 μM) made a shift to the right with the gradual depression resembling SGTX.

SGTX appears to act preferentially upon the intrinsic nerve supply of the ileum rather than on the smooth muscle, because it antagonized the effects of nicotine and DMPP without affecting direct muscle responses to acetylcholine and histamine. Antagonism to nicotine and DMPP may largely result from ganglion blockade. However, this antagonism did not appear to be strictly simple competitive blockade of ganglionic receptors in that the maximum response was depressed, perhaps resulting from an additional component of activity upon the postganglionic nerves *per se* rather than the nicotinic receptors.

The above concept is further supported by the following experiments. SGTX inhibited the phenoxybenzamine-resistant component of the contractile response to 5-hydroxytryptamine, which has been attributed to the stimulation of intramural nerves (Gaddum and Picarelli, 1957) and in addition, partially inhibited the transmurally induced contraction (twitch response) of the ileum which is commonly assumed to be mediated by liberation of acetylcholine from the postganglionic cholinergic nerves (Paton, 1955, 1957) (Fig. 7). Mecamylamine affected both responses as well, while hexamethonium did not.

Structurally, SGTX differs appreciably from other known ganglion-blocking agents. Its potency is fifty to one hundred times greater than that of hexametho-

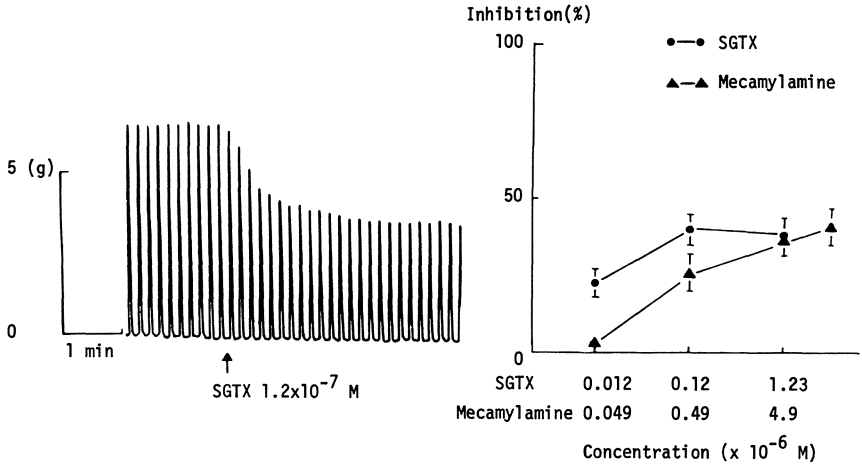

Fig. 7. The effect of SGTX and mecamylamine on the twitch response to transmural stimulation in the isolated guinea pig ileum. (a) the inhibitory action of SGTX on the twitch response, (b) the dose-inhibition curves of SGTX and mecamylamine on the twitch response. Each point represents the mean of at least five experiments and vertical bars show standard errors of the mean.

nium, and twenty to thirty times greater than that of mecamylamine. Therefore, SGTX appears to be of some pharmacological interest as a new type of ganglion-blocking agent.

Most clinical symptoms resulting from ingestion of the mollusk appear to be mediated by the specific ganglion-blocking action in the various sites. These symptoms include visual disorders and mydriasis, due to cilliary ganglion block; dryness of mouth, due to submaxillary and otic ganglion block; constipation and abdominal distention, due to block of the intrinsic nerve in the intestine.

SUMMARY

Surugatoxin (SGTX) (50 nmol/kg, i.v.), isolated from the mollusk, *B. japonica*, produced a prolonged hypotension in anesthetized cats. The toxin inhibited the pressor and depressor response to DMPP and electrical stimulation of the splanchnic and vagal nerve. Intra-arterial injection of SGTX (12.3 nmol/kg) close to the superior cervical ganglion inhibited contractile response of the nictitating membrane to preganglionic stimulation and DMPP, but not to postganglionic stimulation and McN-A-343.

These results indicate that SGTX has a ganglion-blocking action. The mode of antinicotinic action of SGTX seemed to differ from that of hexamethonium and to resemble more closely that of mecamylamine in the isolated guinea pig ileum.

ACKNOWLEDGMENTS

The authors wish to thank Dr. T. Kosuge, Shizuoka College of Pharmaceutical Science and Dr. M. Noguchi, Shizuoka Prefectural Institute of Public Health, Shizuoka, Japan for their kind supply of surugatoxin and to Dr. A. P. Roszkowski, McNeil Laboratories, Inc., Fort Washington, Pennsylvania, U.S.A., for his generous gift of McN-A-343.

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Immunological Aspects of Venom of Sea Snakes from the Indo-Pacific

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INTRODUCTION

The most abundant venomous snakes are those of the *Hydrophiidae*. Although none are found in the Atlantic Ocean, members of this family range broadly throughout the Pacific Ocean. High concentrations occur in the Indo-Pacific and near the Malay Archipelago. At least 50 species are known, all of which are venomous, a dozen of which have been implicated in human envenomation. Nevertheless, very little is known about the general biology of the majority of these snakes and much less is known regarding specifics for their venom. Therefore, an expedition was arranged under the direction of the Research Vessel Alpha Helix program to study these animals in their natural habitat. Ashmore Reef was a logical choice because of the known abundance and diversity of sea snakes there.

It is the object of this report to describe the preliminary observations of the immunology of a few of the snakes found during that expedition. These observations concern the *in vitro* neutralization with a commercial antivenin and efforts to produce antisera with unmodified venom in mice and guinea pigs.

MATERIALS AND METHODS

Crude venoms from the sea snakes *Aipysurus laevis*, *Astrotia stokesii*, *Hydrophis elegans*, *Hydrophis ornatus*, and *Hydrophis belcheri* were used in this study. The snakes were collected at Ashmore Reef and the venom was obtained with a pipette inserted directly over the fangs of the animal. Crude freshly milked venom was either used immediately or freeze dried and stored under refrigeration for later use. Refrigerated freeze dried venom was reconstituted in either isotonic saline or distilled water and used immediately in subsequent studies.

A commercial sea snake antivenin was obtained from Commonwealth Serum Laboratories, Melbourne, Australia. The single-antigen-derived antivenin, lot number 26-1, manufactured July 1972, was produced from horses hyperimmunized against *Enhydrina schistosa.*

Male Swiss Webster mice weighing 18–20 g were used for MLD, LD_{50}, and neutralization determinations. The LD_{50} values were calculated by the method of Litchfield and Wilcoxon (1949) after intramuscular injection of different doses of the venom into 5 mice per dose. Venom, freeze dried and stored at 5°C for approximately 10 months, was reconstituted in isotonic saline. For the *in vitro* neutralization test, the reconstituted venom was mixed with antivenin and allowed to stand at 25°C for 30 min prior to injection.

Male Swiss Webster mice initially weighing 18–20 g and male guinea pigs weighing 300–400 g were used for antiserum production studies. The general method was that described for small animals by Campbell *et al.* (1964). Animals were injected for various periods of time ranging from 3 weeks to 6 months. Doses injected varied from 0.1 to 1.0 LD_{50}. The diluted venoms were emulsified with equal parts of Freund's complete adjuvant (Calbiochem, San Diego, California) prior to injection. Mice were sacrificed by cervical dislocation and blood was obtained by cardiac puncture. Guinea pigs were sacrificed or, less often, anesthetized with ether prior to blood sampling by cardiac puncture. Blood sampling of anesthetized animals allowed sequential use of the same animal for longitudinal evaluation of antibody production. Serum was separated by centrifuging and further used in immunoelectrophoresis and immunodiffusion experiments.

The microgel diffusion technique was that of Ouchterlony (1949). For immunoelectrophoresis (equipment supplied by Beckman Instrument Co., Fullerton, California) the venom was separated for 60 min at 250 V. Antisera or antivenin was placed in the troughs and allowed to diffuse for 24 hr at 25°C, then the gel plate was allowed to stand with 5% amido black to be stained.

RESULTS AND DISCUSSION

The results are shown in Table 1 and Figs. 1 and 2. With fresh crude venoms from *A. stokesii*, *A. laevis*, *H. elegans*, *H. belcheri*, and *H. ornatus* in gel-diffusion

Table 1. Neutralization Capacity of a Single-Antigen-Derived Commercial Sea Snake Antivenin

Snake	MLD,[a] μg/kg	LD_{50},[b] μg/kg	Venom neutralized by 100 units of antivenin, mg
Aipysurus laevis	100	90	3.5
Astrotia stokesii	300	250	8.8
Hydrophis elegans	350	200	12.2

[a] Minimum lethal dose of crude venom injected intramuscularly into mice.
[b] Lethal dose in 50% of mice intramuscularly injected with crude venom and calculated according to Litchfield and Wilcoxon (1949).

experiments against the commercial sea snake antivenin, strong precipitin bands were obtained against *H. ornatus* and *H. belcheri* (Fig. 1). Moderate precipitin bands were formed against *H. elegans* and *A. stokesii*, whereas only a single weak band could be demonstrated against *A. laevis* (Fig. 1). Venoms of various species of sea snakes appear to contain common antigens. It would appear that the commercial sea snake antivenin might provide protection against all of the sea snake venoms tested above.

The use of *A. laevis*, *A. stokesii*, and *H. elegans* in the *in vitro* neutralization test further bore out the protection obtained with the antivenin. The amounts of antivenin required to neutralize the MLD of each of these three venoms are listed in Table 1. No *in vivo* experiments have as yet been conducted in our laboratories with these venoms. However, the *in vitro* results indicate that even though a weak precipitin band was obtained by immunodiffusion, there may be good neutralization of *A. laevis* venom.

Use of commercial antivenin has inherent problems of cross antigen reaction due to the use of horses for alternate antigen types in hyperimmunization procedures (Baxter and Gallichio, 1974). For further testing it was considered desirable to prepare antisera against these venoms in our own laboratory. Our

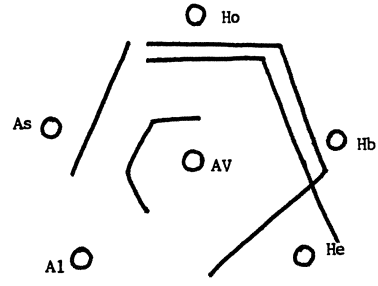

Fig. 1. Precipitin bands obtained in the Ouchterlony microgel diffusion test with commercial sea snake antivenin (AV) against fresh crude venom of *Astrotia stokesii* (As), *Aipysurus laevis* (Al), *Hydrophis elegans* (He), *Hydrophis belcheri* (Hb), and *Hydrophis ornatus* (Ho).

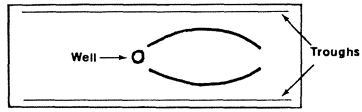

Fig. 2. Immunoelectrophoretic pattern of *Aipysurus laevis* antiserum prepared in guinea pigs against crude venom of *A. laevis*. Crude venom was placed in center well and commercial antiserum in top trough, our animal antiserum in bottom trough.

initial studies have attempted to do so without modification of the venom. Modification is often time consuming and can result in loss of the starting material; our supplies of venom were limited. We also wished to determine whether the venom was too toxic to use unaltered for small animals. Mice and guinea pigs offer several advantages for immunologic studies. They require minimal space and maintenance, toxicity is easily evaluated in these animals, and homogenous strains are available.

To date numerous bleedings have been done from both mice and guinea pigs injected with *A. stokesii*, *A. laevis*, or *H. elegans* venom. Only, *A. laevis* venom has produced the antibody detectable by the immunodiffusion method and in this case only a single weak band was visible (Fig. 2). Antiserum to animal proteins derived from guinea pigs was used as a control and indicated that we were dealing with complete serum. It appears that the unaltered venom is too toxic to use for production of antisera in small animals. Future studies will concern modification of the venom as indicated by Sato and Tamiya (1970).

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Some News and Comments about a Rational and Efficient Antivenomous Serotherapy

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INTRODUCTION

Among all the venomous animals, the snakes and the scorpions have constituted the most worrying problem in serotherapy. Up to now only crude antigens have been used in practice, either whole venoms, especially for snakes (Latifi and Manhouri, 1966; Minton, 1967; Kankonkar *et al.*, 1971) or ground telsons for scorpions (Balozet, 1955; Monroy Velasco and Monroy Nieto, 1960–1961). In some cases such empiric serotherapy would have led to excellent results; in Mexico, for example, where lethality by *Centruroïdes* genus type scorpions used to be very important, the mortality, according to Galvan Cervantes (1966), has been considerably reduced (but has not yet vanished) by the application of equine serum prepared with ground telsons.

Since about twenty years ago, several research groups have succeeded in purifying many active principles from these venoms; in our laboratory, we isolated and characterized eighteen neurotoxins that belong to six different scorpion species. The amino acid sequences of three of these neurotoxins were established (Rochat *et al.*, 1976) and the positions of disulfide bridges of toxin II of *Androctonus australis Hector* were determined (Kopeyan *et al.*, 1974). Regarding the *Elapidae* and *Hydrophidae* snakes, mostly responsible for the deadly ophidian bites, about thirty primary structures of neurotoxins and also the positions of the disulfide bridges of four of them are known (see the review by Yang,

1974). In addition to these neurotoxins, having very high specific toxicities, some less active molecules, though involved to a certain extent in the toxicity, were purified from *Elapidae* venoms, viz., cardiotoxins (Lee *et al.*, 1970; Weise *et al.*, 1973; Louw, 1974a; Carlsson and Joubert, 1974) and phospholipases (Walhström, 1971; Eaker, 1976). The amino acid sequences of seven cardiotoxins (Narita and Lee, 1970; Hayashi *et al.*, 1971; Takechi *et al.*, 1972; Fryklund and Eaker, 1973; Louw, 1974b) and the positions of disulfide bonds of one of them (Takechi and Hayashi, 1972) have been established.

The possibility to now work with pure antigens gives a new outlook to the antitoxic serotherapy. Scorpion and snake neurotoxins which share in common the property of being low molecular weight basic molecules (57–74 amino acid residues, 4 or 5 disulfide bridges) were first studied as they are entirely or to a major part responsible for the lethality due to the venoms containing neurotoxins, and they were claimed to be poor immunogens. But only a few immunological studies have been dedicated to pure neurotoxins; this is true for scorpion neurotoxins (Boquet *et al.*, 1972) as well as snake neurotoxins (Chang, 1969; Chang and Yang, 1969; Clark *et al.*, 1972; Chang and Yang, 1973; Boquet *et al.*, 1973).

We have been using pure antigens or purified fractions from these two types of venoms available in our laboratory to show how interesting and important it is to deal with a molecular concept in antivenomous serotherapy.

MATERIALS AND METHODS

Venoms. The venom of *Androctonus australis Hector* (*A. a. Hector* or A.a.H) was prepared in this laboratory from animals collected in North Africa. The venom of *Naja mossambica mossambica* (*N. m. mossambica* or N.m.m.) was obtained from D. Muller (Johannesburg, South Africa). The venom of *Naja haje* (*N. haje* or N.h.) and *Naja nigricollis* (*N. nigricollis* or N.n.) were the gifts from Dr. Boquet (Institut Pasteur, 92380, Garches, France).

Antigens. The references for the preparation of antigens are given in Table 1.

Immunizations. Each antigen was dissolved in 0.15 M NaCl (0.5 ml), then emulsified with an equal volume of Freund's complete adjuvant (Merieux, 69620, Marcy-l'Etoile, France) and injected (1 ml) into 1.5–3-kg rabbits (on an average we used five animals per program). The injections were first done intradermally at several points and, later, subcutaneously according to different immunization schedules (Delori *et al.*, 1976). All bleeding was made by cardiac puncture. The sera obtained were stored either at −30°C or in a freeze dried state; in the latter case, in order to reconstitute 1 ml of immune serum, 75 mg of freeze dried serum was dissolved in redistilled water.

Table 1. Definition of Different Antisera Obtained in Rabbits

	Antigens[a]	Total amount used per rabbit, mg	Designation of the antisera
A.a.H.	Sephadex G-50 fraction unmodified (Miranda *et al.*, 1970a)	8,100	A.a.H.–S_1
	Sephadex G-50 fraction acetylated (Delori *et al.*, 1976)	32,000	A.a.H.–S_2
	Toxins (Miranda *et al.*, 1970a) toxin I	4,300	A.a.H.–S_3
	Toxins (Miranda *et al.*, 1970a) toxin II	2,500	A.a.H.–S_4
N.m.m.	Crude venom	31,500	N.m.m.–S_1
	Unmodified toxin I (Rochat *et al.*, 1974)	10,075	N.m.m.–S_2
	Acetylated toxin I (Delori *et al.*, 1976)	10,075	N.m.m.–S_3
	Phospholipase A fraction (Rochat *et al.*, 1974)	25,050	N.m.m.–S_4
N.h.	Toxin III (Miranda *et al.*, 1970b)	4,050	N.h.–S_1

[a]References give the methods for preparation of the different antigens.

Toxicity Determinations. The toxicity of antigens was measured according to the method of Behrens and Karber (1935) with the modifications described elsewhere (Miranda, 1964; Rochat *et al.*, 1967). The unit of toxicity was the LD_{50} for a 20-g mouse.

Neutralization Assay. The neutralizing capacity of immune sera was estimated by a technique that derived from Ipsen's graphical method (Ipsen, 1938). In a set of test tubes, increasing amounts of an antigen were added to a given volume of immune serum; the mixtures were incubated at 37°C for 30 min. The volume in each tube was then made up to 1 ml with 0.15 M NaCl and the solution injected into four mice at a dose of 0.25 ml/20-g body weight (mice weighing slightly less than 20 g were chosen so that we could perform four injections with the 1-ml mixture). For that given volume of serum, we noted the amount of antigen necessary to kill two of the four mice (LD_{50}). We then plotted the LD_{50} amount of the antigen versus the volume of the serum. We got two types of curves; type I (Fig. 1) and type II (Fig. 2).

The type-I curve (Fig. 1) is just a straight line and its slope expresses the neutralizing capacity in weight of antigen (μg or mg) per volume of serum (ml). Since all these experimental points are on the straight line (a) it looks as if we were dealing with a single antigen, but one may extrapolate experimental results only when he is dealing with a pure antigen. In the case of a mixture of antigens, one has a right to express the slope of the curve in weight of antigen neutralized per ml of serum, but one must specify thereby the range of the volume of the serum injected to one mouse in which the phenomenon holds true. At higher doses in the presence of multiple antigens, the antigen that is more poorly neu-

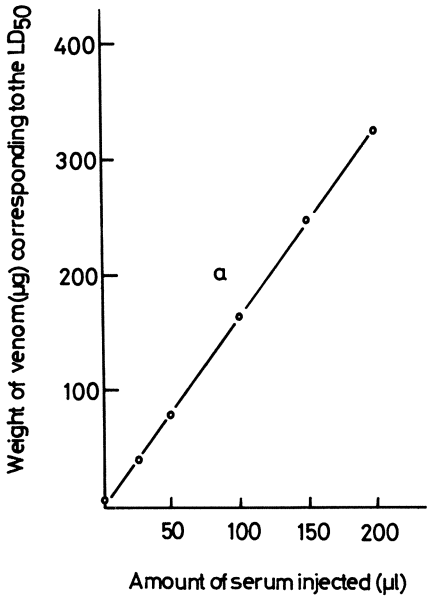

Fig. 1. Type I neutralization curve. Four mice were subcutaneously injected with *A. a. Hector* Tozeur) venom preincubated with a mixture of two monospecific sera: A.a.H.–S_3 and A.a.H.–S_4 (see Table 1). Experimental details are given in Materials and Methods; for neutralization assays, see Table 7.

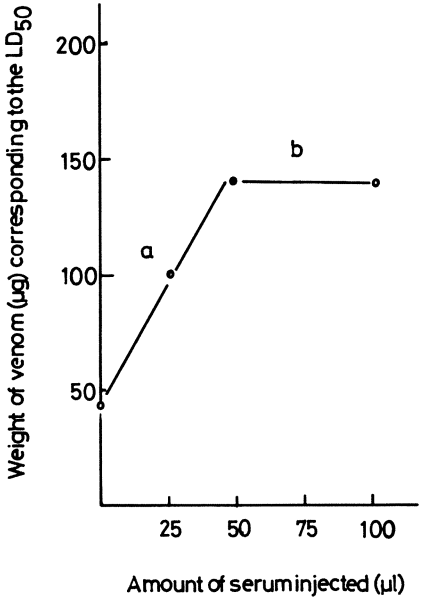

Fig. 2. Type II neutralization curve. Four mice were subcutaneously injected with *N. m. mossambica* venom preincubated with a monospecific serum: N.m.m.–S_2 (see Table 1). Experimental details are given in Materials and Methods; for neutralization assays, see Table 6.

tralized by the serum would become responsible for lethality. Additional neutralization points then form the second straight line with a gentler slope. This second type of curve is shown in Fig. 2. In that extreme case, one of the antigens has a low toxicity but it is not neutralized at all and from a certain dose onward it becomes entirely responsible for lethality; in that case the straight line (b) is parallel to the abscissa axis.

In Tables 6 and 7, we indicate which type of line (a or b) corresponded to the experimental points.

Preparation of Artificial Sera by Mixing Monospecific Sera. Artificial sera (Tables 6 and 7) are obtained by mixing different monospecific sera in proportions based upon their own neutralizing capacities and the relative concentrations of antigens which are present in different venoms. Such relative concentrations of antigens were calculated either by preliminary absorption experiments of crude venoms with monospecific antisera (Delori *et al.*, 1976) or from the purification data (Miranda *et al.*, 1970a,b; Kopeyan *et al.*, 1973; Rochat *et al.*, 1974).

RESULTS

In Table 1 are listed all the types of antisera we obtained by injecting rabbits either with a pure antigen (scorpion neurotoxins of *A. a. Hector* or snake neurotoxins of *N. m. mossambica* and *N. haje*) or a mixture of antigens (different fractions obtained by gel filtration on Sephadex G-50 of venom extracts). Some of these antisera were obtained with antigens whose toxic activities were abolished by treating with acetic anhydride: Sephadex G-50 neurotoxic fraction of the *A. a. Hector* venom (Table 2) and the toxin I of *N. m. mossambica* (Table 5).

The neutralizing capacities of these antisera were tested against pure antigens (scorpion or snake neurotoxins in Tables 2, 4, and 5) and also against different mixtures of antigens (phopholipase-active fraction from the venom of *N. m. mossambica* in Table 3; crude snake venoms in Table 6; and crude scorpion venoms in Table 7).

Table 2. Neutralizing Capacities of A.a.H.–S_1 and A.a.H.–S_2 against Pure Toxins of *Androctonus australis Hector* (Chellala)

Toxins	Antigen neutralized/ml of serum, μg	
	A.a.H.–S_1	A.a.H.–S_2
I	48	54
II	58	24
III	58	81

Table 3. Comparison of the Neutralizing Capacities of N.m.m.–S_4 from Different Rabbits against the Sephadex G-50 Phospholipase-Active Fraction of *Naja mossambica mossambica*

Rabbit number	Neutralizing capacity/ml of antiserum[a]	
	Weight of antigen, μg	Number of LD_{50}[b]
75	2,100	35
74	1,500	25
72[c]	900	15
73[c]		

[a] Between 0 and 0.075 ml of serum injected per mouse.
[b] We are allowed to express the result in number of LD_{50} as the toxicity of this phospholipase-active fraction is only due to two phospholipases that have same mode of action and same specific activity (unpublished results).
[c] Pooled.

Table 4. Comparison of the Neutralizing Capacities of Monospecific Antisera from Different Rabbits against the Corresponding Toxins

Antigens	Antisera[a]	Rabbit number	Neutralizing capacity/ml of antisera		
			Weight of antigen, μg		Number of LD_{50}
A.a.H. toxin I	A.a.H.–S_3	63	157	102 (average)	413
		60, 61, 65 pooled	120		316
		62, 64 pooled	47		124
A.a.H. toxin II	A.a.H.–S_4	54 to 59 pooled	93		423
N.m.m. toxin I	N.m.m.–S_2	27	157	101 (average)	192
		30	133		166
		32	72		90
		28	40		50
N.h. toxin III	N.h.–S_1	67	156	95 (average)	120
		66, 71 pooled	130		100
		68, 69, 70 pooled	52		40

[a] Defined in Table 1.

Table 5. Neutralizing Capacities of Monospecific Antisera (N.m.m.–S_2 and N.m.m.–S_3) against Pure Neurotoxins of *Naja mossambica mossambica*

Toxins	Antigen/ml of serum, μg	
	N.m.m.–S_2	N.m.m.–S_3
I	157	84
II	160	84
III	150	72

Table 6. Neutralization Assays of *Elapidae* Venoms by Different Sera or Mixtures of Sera

Venom	Sera[a]	Volume of serum,[b] ml	Amount of preincubated venom (μg) to reach the LD_{50}	Reference to the graphical representation[c]	Slope of the (a) curve[d]
N.h.	None	—	11	—	—
	N.m.m.-S_2	0.025	25	b	—
	N.h.-S_1	0.050	30	b	—
	N.m.m.-S_4	0.025	11	b	—
	N.m.m.-S_1	0.025	11	b	—
	Mixture: N.m.m.-S_2 + N.h.-S_1 (7:11, by vol.)	0.150	112	a	0.67[e]
N.n.	None	—	12	—	—
	N.m.m.-S_2	0.225	110	a	0.43[f]
	N.m.m.-S_4	0.025	12	b	—
	N.m.m.-S_1	0.025	12	b	—
N.m.m.	None	—	42	—	—
	N.m.m.-S_2	0.075	140	b	1.96[g]
	N.m.m.-S_4	0.025	42	b	—
	N.m.m.-S_1	0.075	90	b	—
	Mixture: N.m.m.-S_2 + N.m.m.-S_4 (1:1, by vol.)	0.150	170	a	0.85[e]

[a] Defined in Table 1.
[b] For one mouse.
[c] See the text and Figs. 1 and 2.
[d] In mg of antigen per ml of serum.
[e] Highest experimental point on curve (a): 0.150 ml of serum per mouse.
[f] Highest experimental point on curve (a): 0.225 ml of serum per mouse.
[g] Highest experimental point on curve (a): 0.050 ml of serum per mouse.

Table 7. Neutralization Assays of Scorpion Venoms by Mixtures of Monospecific Sera and by Commercial Sera

Venom	Sera[a]	Volume of serum,[b] ml	Amount of preincubated venom (μg) to reach the LD_{50}	Reference to the graphical representation[c]	Slope of the (a) curve[d]
A.a.H. (Chellala)	None	—	8	—	—
	Mixture: A.a.H.-S_3 + A.a.H.-S_4 (1.00:1.38, by vol.)	0.200	450	a	2.21[e]
	–Pasteur Institute (Alger)	0.200	110	a	0.51[e]
	–Pasteur Institute (Tunis)	0.200	165	a	0.78[e]
A.a.H. (Mecheria)	None	—	10	—	—
	Mixture: A.a.H.-S_3 + A.a.H.-S_4 (1.00:1.38, by vol.)	0.200	460	a	2.25[e]
A.a.H. (Tozeur)	None	—	5	—	—
	Mixture: A.a.H.-S_3 + A.a.H.-S_4 (1.00:1.00, by vol.)	0.200	330	a	1.62[e]
	–Pasteur Institute (Alger)	0.200	40	a	0.17[e]
	–Pasteur Institute (Tunis)	0.200	65	b	0.48[f]

[a]Defined in Table 1.
[b]For one mouse.
[c]See the text and Figs. 1 and 2.
[d]In mg of antigen per ml of serum.
[e]Highest experimental point on curve (a): 0.200 ml of serum per mouse.
[f]Highest experimental point on curve (a): 0.125 ml of serum per mouse.

In Tables 6 and 7, one can also find the neutralizing capacities of different monovalent, mixed, and commercial sera against snake and scorpion venoms.

DISCUSSION

Contrary to what generally appears in publications dedicated to antitoxic serotherapy, one must vigorously denounce the use of the LD_{50} (or even more the less accurate LD_{100}) to express the potency of the serum; the potency

should be expressed only (see Materials and Methods) as the weight of antigen neutralized by a certain volume of serum (within the experimental limits). Indeed, the expression of LD_{50} is always the function of the sensitivity of the test animal and it can vary a great deal, even within the same species (we found that, depending on the strain of animals tested, the sensitivity of mice to scorpion neurotoxins varied in a ratio of about 1 to 50); the extrapolation to man, that underlies all serotherapeutic work, is even more hazardous. Moreover, one may calculate the neutralization capacity of antigens in terms of LD_{50} only if all the antigens show not only the same mode of action on the molecular level but also the same specific activities. Otherwise, unconsciously, one may introduce an error due to the fact that, in the absence of serum, on the level of the LD_{50} for the 20-g mouse, the toxicity of the venom is due to the most active component alone. So there is only one strict way of proceeding; that is, with the different neutralization points that have been tested, to draw the neutralization curve which should be of one of the two types described in Materials and Methods. For every segment of straight line, one can calculate the neutralizing capacity in weight of antigen neutralized per ml of serum. Finally, when dealing with a complex antigen, in order to standardize the sera, it is essential to accurately define this antigen for a venom, to collect maximum information about the identification of the venomous animals that gave this venom and for a toxic fraction, as the one obtained by chromatography, to specify the experimental conditions and the exact delimitations of the fraction on the elution pattern. But it is clear that there is no more precise way to express the neutralizing capacity than to show the weight of pure antigen, from a known zoological origin and of a known chemical structure, that is neutralized per ml of serum.

When preparation of anatoxins is to be considered, very careful chemical modification studies can be made on well-defined pure proteins (Sampieri and Habersetzer-Rochat, 1976) and can be extended further to less pure preparations (a Sephadex G-50 fraction for instance). Acetylation of the amino groups could well be a general preparation method of scorpion and snake anatoxins. Anatoxins (like the Sephadex G-50 acetylated fraction, see Table 1) allow the injection of high doses of antigens without harm to the animals (this advantage is even more important when dealing with horses, used commonly in most of the serotherapeutic institutes) and can rapidly give sera with high neutralizing titer when compared to preparations containing the native toxins (Table 2). When equal amounts of toxin and anatoxin are separately injected into rabbits, the neutralizing titer of the sera obtained from the former are generally higher than those obtained from the latter (case of toxin I of *N. m. mossambica*: Tables 1 and 5); this indicates that anatoxins can loose some of the antigenic determinants which are present in the unmodified molecules. This phenomenon is in some cases so obvious that even by injecting much higher doses of anatoxin to prepare the serum, its titer remains lower (see the antitoxin II titer of A.a.H.–S_2 in Table 2).

Pure antigens not only present an irreplaceable advantage in determination of the neutralizing capacity of the sera but also constitute a remarkable tool for preparation of highly protective immune sera. Indeed, it is obvious that the higher the concentration of some antigens in the preparation that is injected into the rabbit, the higher the concentration of the corresponding antibody will be in the serum. In addition, when the antigens that are concerned are of low molecular weight molecules, another qualitative notion appears. They are, as is generally admitted, less immunogenic than high molecular weight proteins of the whole venom. The results in Tables 3 and 4 allow comparison between toxic phospholipases (M.W. $\simeq$ 14,000) and scorpion or snake neurotoxins (M.W. $\simeq$ 7000). If we presume that the amounts of both antigens that are injected into the rabbits corresponded to the optimum for the antibody response and if we relate the neutralizing activity of the sera to the molecular weights of the antigens, we can establish that the neurotoxins give rise to five times less antibodies than the phospholipases.

We can verify this phenomenom in Table 6 that expresses the reduction in toxicity of snake venoms by different antisera. The venoms of *N. nigricollis* and *N. m. mossambica* contain only type I (short) neurotoxins and the contact with antitoxin I serum considerably increases the amount of venom necessary to reach the LD_{50}; that is not the case when the venom is put in contact with the serum against crude venom (N.m.m.–S_1). This phenomenom is slightly reduced but still exists when comparing the activities of sera made with purified extracts (Table 2) and of sera made with pure antigens (Table 4).

The practical interest of all these theoretical considerations in view of an effective serotherapy leads to conceiving the idea of artificial sera that are prepared according to the neutralizing capacities of the monospecific sera and the relative proportions of the different toxic antigens in the venoms.

The strictest determinations of the nature and the concentration of each of these antigens is only possible after intensive purification of the venom that is to be considered. But the fact that venoms secreted by animals of closely related species contain antigens with closely related structures makes it possible to identify and titrate a certain type of antigen with the use of a monospecific antiserum that was prepared with an antigen purified from another venom of the same type. This is very true in the case of *Naja* genus type venoms as their neurotoxins belong only to two different groups; toxins containing approximately 60 amino acid residues (e.g., toxin I of *N. m. mossambica*) and those containing approximately 70 amino acid residues (e.g., toxin III of *N. haje*). We have already seen examples of reduction in toxicity of the venoms of *N. m. mossambica* and *N. nigricollis* after preincubation with antitoxin I of *N. m. mossambica* serum (Table 6).

Once we have at our disposal monospecific sera directed against the principal toxic antigens and determined the proportions of these antigens by preliminary

neutralization tests with these sera, we can then just mix these sera together according to their neutralizing capacities and the relative amounts of the toxic antigen types and we obtain sera with neutralizing capacities much higher than those of any natural serum (Tables 6 and 7).

The absolute limitation of the efficiency of serotherapy would come from the existence in some venoms of antigens that are, eventually, not very toxic but present at very high concentration making it impossible to neutralize them by injecting the corresponding amounts of antibodies. This is the case with some *Naja* genus type venoms, for example, the venom of *N. haje* that contains up to 50% cardiotoxin (unpublished results), whose molecular weight is about 7000. The injection of 100 mg of dry venom corresponds thus to the introduction of 50 mg of cardiotoxin. The molecular weight of immunoglobulins (IgG) is about 20 times higher than that of cardiotoxin. If we assume a reaction of 1 antibody with 1 antigen and if the concentration of the specific antibodies in the monospecific serum is 10 mg/ml, we would need 100 ml of serum to be injected just at the time and at the place of the wound in order to completely neutralize the antigen. The cardiotoxins are toxic at a level of 300 μg per 20-g mouse when subcutaneously injected but only at a level of 20 μg when intravenously injected. We have no information about their toxicity in man but they may well represent the kind of limitation we were speaking about.

We think that the preparation of artificial sera by mixing monospecific sera obtained with pure antigens or, for a reason of convenience, with purified extracts (obtained, for instance, by Sephadex G-50 gel filtration) should contribute to promising development of the antitoxic serotherapy.

SUMMARY

The use of pure antigens for antitoxic serotherapy offers many advantages; an objective measure of the neutralizing capacity of a serum, the best way of preparation of anatoxins, the production of immune sera particularly rich in specific antibodies, and finally the preparation of artificial mixtures of monospecific sera leading to efficient neutralization of crude venoms.

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Immunological Properties of Two Proteins of *Naja nigricollis* Venom

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The α toxin of the venom of *Naja nigricollis* is transformed into a toxoid composed of populations of monomers and small soluble polymers when treated with low concentrations of formaldehyde (Dumarey, 1971; Dumarey and Boquet 1972). The toxoid itself and the isolated monomers and polymers are immunogenic when injected into rabbits with Freund's adjuvant. However, the insoluble higher polymers obtained by treating the α toxin with higher concentrations of formaldehyde or with glutaraldehyde as previously described (Dumarey and Boquet, 1972), do not stimulate the synthesis of α-toxin-specific antibodies.

This communication describes the effects of (1) the nature of the adjuvant, (2) the dose of antigen injected, (3) the method of preparation of the toxoid, and (4) preimmunization with α toxin, γ toxin, or a mixture of both on the production of specific immune sera.

MATERIALS AND METHODS

The venom of *Naja nigricollis* (One LD_{100} of crude venom by i.v. route to a 20-g mouse is equal to 16 μg) came from Ethiopia. The α and γ toxins (one LD_{100} of α toxin = 2.2 μg; one LD_{100} of γ toxin = 22.5 μg) were extracted from the venom by the normal procedures (Karlsson *et al.*, 1966; Izard *et al.*, 1969; Dumarey, 1971). The α toxin electively binds on the cholinergic receptors (Tazieff-Depierre and Pierre 1966; Meunier *et al.*, 1971). The γ toxin alters the cell membranes (Boquet, 1970). Both toxins have molecular weights slightly

less than 7,000. The soluble α and γ toxoids were obtained by treating solutions of these proteins with formaldehyde as previously described (Dumarey, 1971). The α toxin was also transformed into a soluble toxoid by mixing a 25% solution of glutaraldehyde with the toxin buffered at pH 7.0 (final concentration of reagent 2.5×10^{-3} M, protein 1.47×10^{-4} M). This protein lost 90% of its toxicity when held at 20°C for 18 hr. The reaction was arrested by the addition of lysine (final concentration 0.1 M). The molecular weight of the product, as determined by filtration on Sephadex G-50, corresponded to that of a dimer of the toxin.

The insoluble higher polymers of the α protein were prepared either by means of formaldehyde, according to the procedure previously employed (Dumarey and Boquet, 1972), or by treating the same toxin with glutaraldehyde, the final concentration of the two reagents being 6.1×10^{-3} and 4.4×10^{-2} M, respectively. Under these conditions the mixture precipitated in less than one hour at 20°C. This precipitate was collected, washed, resuspended, and then injected into the animal.

Tyrosine was incorporated into the soluble toxoid by adding 2.6×10^{-3} M of the amino acid to a mixture of α toxin and formaldehyde prepared under the usual conditions (Dumarey, 1971). Chromato-electrophoresis of the hydrochloric-acid hydrolysate of the α toxoid free from acid labile formaldehyde and any excess of polymerized tyrosine demonstrated a compound "lysine-methylene-tyrosine" (Blass *et al.*, 1968) which did not appear in the chromato-electrophoregram of α toxin treated with formaldehyde alone (Dumarey, 1971) because of the hidden position of the only tyrosyl residue of the molecule. The presence of this compound demonstrated that tyrosine molecules were attached to the protein.

The adjuvants associated with a α toxin were: (a) bentonites (Clarsol F 135) in the proportion of 1% to 10% wt./wt.; (b) sodium alginate (Carlo Erba) in a 1% solution (the toxoid was incorporated into this preparation in a proportion of 10% vol./vol.); and (c) Freund's complete adjuvant (Merieux) emulsified in a solution of toxoid in a proportion of 10% vol./vol. (this adjuvant was also used in the experiments performed with the γ toxin).

Rabbits (mostly of the fauve variety) of 2.0–3.0 kg were injected at several points under the skin of the back with the toxin, toxoid, highly polymerized toxin, or venom associated with an adjuvant. The first two injections were made at a 5–7 day interval, and after a rest of 3 weeks the rabbits were reinjected once per week.

They were bled by cardiac puncture 52 (I), 86 (II), and 114 (III) days after the first injection. The "bleeding white" was done after various periods.

The activity of the serum was measured by the procedure recommended by Christensen (1966) slightly modified because of the small quantities of reagents available. The activity was expressed as the number of LD_{100} neutralized by 1 ml of the serum (for the mouse tested intravenously).

The precipitating power of the serum was demonstrated by immuno-precipitation according to Ouchterlony (1958-1962) and in certain experiments by quantitative precipitation.

RESULTS

The formylated α toxoid, adsorbed on bentonite, was administered to 5 rabbits of the first group, to 7 rabbits of the second, and to 8 rabbits of the third, in total doses of 59,934, and 5910 μg, respectively. The best sera obtained (rabbits of groups 2 and 3) neutralized at most 5 LD_{100} of α toxin per ml but did not precipitate.

The sera of 6 rabbits immunized by injections of the same toxoid associated with sodium alginate at the total dose of 1900 μg neutralized on average 12 LD_{100} of α toxin per ml. Only three sera were precipitants.

The best results were obtained by incorporating the same toxoid in Freund's complete adjuvant. While low doses of antigen (total 375 μg) either did not stimulate, or only slightly stimulated, synthesis of antibodies. Higher doses (1900 μg) allowed the recovery of strongly precipitating sera which neutralized an average 142 LD_{100} of α toxin per ml days after the first injection (Table 1).

Under the same conditions, the γ toxoid produced precipitating antibodies but no neutralizing antibodies. When the dose of antigen was increased (to 3850 μg total) 1 ml of the serum of 6 rabbits neutralized on average 8.5 LD_{100} of γ toxin (Table 2). In the absence of Freund's adjuvant injection of the same doses of γ toxoid produced neither precipitant nor neutralizing antibodies.

In 1971 we demonstrated that injection of rabbits with toxin, detoxified and highly polymerized by glutaraldehyde, did not stimulate the synthesis of α protein-specific antibodies. Recent experiments have established that this treatment does not create a state of tolerance because the formylated soluble α toxoid administered to rabbits according to the usual procedure 3 weeks after

Table 1. Immunization with α Toxoid (Formaldehyde) Plus Freund's Adjuvant[a] (Number of LD_{100} Neutralized by 1 ml of Serum)

Number of bleeding	Number of rabbit										Average
	201	203	205	207	209	221	223	225	227	229	
I	47	47	94	31	<6	31	31	125	31	31	47
	P[b]	*P*	*P*	*P*	*0*[b]						
II	Dead	125	31	Dead	25	190	190	190	190	190	142
		P	*P*		*P*						
III	–	125	125	–	48	–	–	–	–	–	99

[a]Total dose of α toxoid injected: 1900 μg.
[b]*P*, precipitating antibodies; *0*, no precipitating antibodies.

Table 2. Immunization with γ Toxoid (Formaldehyde) Plus Freund's Adjuvant[a] (Number of LD_{100} Neutralized by 1 ml of Serum)

Number of bleeding	Number of rabbit								Average
	1	3	5	7	9	11	13	15	
I	10	<10	10	<10	10	<10	<10	<10	5
	P[b]	P	P	P	P	P	P	P	
II	10	10	10	Dead	10	<10	10	10	8
	P	P	P		P	P	P	P	
III	10	20	10	–	20	10	20	10	14
	P	P	P		P	P	P	P	

[a]Total dose of γ toxoid injected: 3850 μg.
[b]P, precipitating antibodies.

the last injection of highly polymerized toxin behaved like an immunogenic protein.

At a low concentration (2.5×10^{-3} M) glutaraldehyde inactivated a 0.1% solution of α toxin (1.47×10^{-4} M) in 20 hr at 20°C without suppressing its immunogenic power when the reaction was blocked after this period by the addition of lysine (0.1 M). Eighty-six days after the first injection of this toxoid, 5 rabbits supplied serum neutralizing an average of 34 LD_{100} of α toxin per ml, but having an antitoxic ability less than that of sera obtained from animals immunized under the same conditions by injections with formylated toxoid.

The fixation of tyrosine to gelatin conferred immunogenic properties to this substance (Sela and Arnon, 1960). The question arose as to whether the fixation of tyrosine to the α protein would increase its immunogenic power. Injected by the method described above into 13 rabbits, of which 9 survived, the α toxoid with tyrosine provoked the synthesis of α toxin antibodies at lower doses (240 μg). The average titer of the sera collected was higher than that from 8 control rabbits immunized by injections of toxoid without tyrosine (Tables 3 and 4).

Table 3. Immunization with α Toxoid (Formaldehyde) Plus Freund's Adjuvant[a] (Number of LD_{100} Neutralized by 1 ml of Serum)

Number of bleeding	Number of rabbit								Average
	231	233	235	237	239	241	245	249	
I	12	<10	<10	30	<10	<10	<10	<10	5
II	<15	20	10	10	<10	<10	<10	<10	5
III	25	16	<10	30	<10	<10	<10	<10	9
last bleeding	75	10	<10	Dead	<10	<10	<10	<10	12

[a]Total dose of α toxoid injected: 240 μg.

Table 4. Immunization with α Toxoid (Formaldehyde) Plus Freund's Adjuvant, Plus Tyrosine[a] (Number of LD_{100} Neutralized by 1 ml of Immune Serum)

Number of bleeding	Number of rabbit													Average
	570	572	574	231	233	235	237	239	261	263	265	267	269	
I	20	Dead	<5	10	<10	45	Dead	<10	<10	10	20	Dead	10	10
II	>87	–	>125	45	<10	75	–	30	10	15	30	–	15	>43
III	33	–	33	60	10	75	–	45	15	Dead	30	–	15	35
Last bleeding	30	–	290	63	<10	47	–	25	Dead	–	20	–	–	68

[a]Total dose of α toxoid plus tyrosine: 240 μg.

A final series of experiments examined the effect of preimmunization with formylated α and γ toxoids administered alone or jointly, and of preimmunization with formylated venom, on the production of antivenom antibodies in response to booster injections of venom treated with formaldehyde. The results of four series of experiments are shown in Tables 5–8.

Preimmunization with α toxoid or a mixture of α and γ toxoids, followed by a second immunization with the venom of *N. nigricollis*, increased the activity of the serum with respect to the native α toxin. Preimmunization with γ toxoid had apparently no effect. However, owing to the low toxicity of γ protein, about one-tenth that the α protein, the monovalent anti-α and anti-γ toxin sera we obtained neutralized, in the same volume, equal amounts of the two proteins. In other words, the number of molecules of toxin neutralized by the anti-γ specific serum was in the same order of magnitude as that neutralized by the anti-α. This was not the case when the neutralizing power of sera from rabbits, preimmunized by injections of α or γ or a mixture of both toxoids and then immunized with formylated venom of *N. nigricollis*, was examined. In effect, whatever the

Table 5. Preimmunization[a] with α Toxoid (Formaldehyde)[b] Plus Freund's Adjuvant (Number of LD_{100} Neutralized by 1 ml of Serum)

Number of bleeding	Agent neutralized	Number of rabbit								Average
		33	35	37	39	41	43	45	47	
I	*N. nigricollis* venom	<10	<10	<10	<10	<10	<10	<10	<10	<10
		P[c]	*P*	*0*[c]	*P*	*P*	*P*	*P*	*P*	
II		15	20	<10	<10	10	20	20	20	13
		P	*P*	*0*	*P*	*P*	*P*	*P*	*P*	
III		30	20	20	20	40	30	40	30	30
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	
I	α toxin	40	75	12	125	40	100	75	120	73
		P	*P*	*0*	*P*	*P*	*P*	*P*	*P*	
II		90	75	45	75	50	85	80	120	77
		P	*0*	*0*	*P*	*P*	*P*	*P*	*P*	
III		50	37	25	87	50	62	87	62	57
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	
I	γ toxin	<10	<10	<10	<10	<10	<10	<10	<10	<10
		0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	
II		10	10	<10	<10	10	10	10	10	7
		P	*P*	*P*	*0*	*P*	*P*	*P*	*P*	
III		10	10	10	10	20	10	20	10	12
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	

[a]Booster injections: *Naja nigricollis* venom (formaldehyde). Total dose of venom: 3600 μg.
[b]Total dose of α toxoid injected: 75 μg.
[c]*P*, precipitating antibodies; *0*, no precipitating antibodies.

Table 6. Preimmunization[a] with α Toxoid (Formaldehyde)[b] Plus Freund's Adjuvant (Number of LD_{100} Neutralized by 1 ml of Serum)

Number of bleeding	Agent neutralized	Number of rabbit								Average
		17	19	21	23	25	27	29	31	
I	*N. nigricollis* venom	<10	<10	Dead	<10	<10	Dead	<10	<10	<10
		P[c]	*P*		*P*	*P*		*P*	*P*	
II		<10	<10	–	<10	<10	–	<10	20	3
		P	*P*		*P*	*P*		*P*	*P*	
III		<10	20	–	10	10	–	20	40	17
		P	*P*		*P*	*P*		*P*	*P*	
I	α toxin	<10	<10	–	<10	<10	–	<10	<10	10
		0[c]	*0*		*0*	*0*		*0*	*0*	
II		<10	<10	–	<10	<10	–	<10	<10	2
		0	*0*		*0*	*0*		*0*	*0*	
III		10	20	–	10	10	–	10	30	15
		0	*0*		*0*	*0*		*0*	*P*	
I	γ toxin	10	<10	–	10	10	–	<10	10	7
		P	*0*		*P*	*P*		*P*	*P*	
II		10	<10	–	10	10	–	<10	10	7
		P	*P*		*P*	*P*		*P*	*P*	
III		10	10	–	20	20	–	<10	20	13
		P	*P*		*P*	*P*		*P*	*P*	

[a]Booster injections: *Naja nigricollis* venom (formaldehyde); total dose of venom: 3600 μg.
[b]Total dose of γ toxoid injected: 250 μg.
[c]*P*, precipitating antibodies; *0*, no precipitating antibodies.

method of preimmunization may be, the number of molecules of α toxin neutralized by a given volume of any of these sera was less than that of γ toxin.

DISCUSSION AND CONCLUSION

Compared to the effects of bentonites or sodium alginate, the addition of Freund's complete adjuvant to the α toxin of *N. nigricollis*, transformed into toxoid with formaldehyde, conferred a relatively high immunogenic power to this low molecular weight protein. This observation suggests that the α toxin belongs to a group of antigens which stimulate *in vivo* antibody synthesis by bringing into play the complex mechanism of the T lymphocyte cells. The same adjuvant increased the immunogenic power of the γ toxoid. Substantial quantities of this antigen associated with Freund's adjuvant must be injected to obtain synthesis of antibodies neutralizing this toxin.

In a previous publication (Dumarey and Boquet, 1972) it was concluded that

Table 7. Preimmunization[a] with α[b] and γ[c] Toxoids (Formaldehyde) Plus Freund's Adjuvant (Number of LD_{100} Neutralized by 1 ml of Serum)

Number of bleeding	Agent neutralized	Number of rabbit								Average
		49	51	53	55	57	59	61	63	
I	*N. nigricollis* venom	<10	10	10	Dead	10	10	10	10	8
		0[d]	*P*[d]	*P*		*P*	*0*	*P*	*P*	
II		10	35	20	–	20	20	10	30	21
		P	*P*	*P*		*P*	*P*	*P*	*P*	
III		10	50	Dead	–	20	20	10	30	23
		P	*P*			*P*	*P*	*P*	*P*	
I	α toxin	10	40	30	–	30	30	10	40	27
		0	*P*	*P*		*P*	*0*	*P*	*P*	
II		20	90	25	–	25	25	40	85	44
		0	*P*	*P*		*P*	*P*	*P*	*P*	
III		20	62	Dead	–	37	20	20	87	41
		P	*P*			*P*	*P*	*P*	*P*	
I	γ toxin	<10	<10	<10	–	<10	10	<10	10	3
		P	*P*	*P*		*P*	*P*	*P*	*P*	
II		<10	20	<10	–	<10	20	<10	10	8
		P	*P*	*P*		*P*	*P*	*P*	*P*	
III		<10	20	Dead	–	10	20	<10	10	10
		P	*P*			*P*	*P*	*P*	*P*	

[a]Booster injections: *Naja nigricollis* venom (formaldehyde). Total dose of venom: 3600 μg.
[b]Dose of α toxoid injected: 75 μg.
[c]Dose of γ toxoid injected: 250 μg.
[d]*P*, precipitating antibodies; *0*, no precipitating antibodies.

the soluble α toxoid obtained by treating with formaldehyde is composed of a mixture of monomers, dimers, and small polymers composed of at most 10 units of modified protein. Monomers and dimers are immunogens when they are associated with Freund's adjuvant. The molecules occur as dimers in the solution of α toxoid prepared by treatment with glutaraldehyde. They are immunogens when incorporated into Freund's adjuvant.

Preimmunization with α antigen followed by a second immunization by injections of formylated *N. nigricollis* venom provoked a substantial synthesis of anti-α toxin antibodies. The increase in the level of anti-α toxin antibodies in the sera of animals preimmunized by the injection of a mixture of α and γ toxoids is distinctly less. This observation suggests that a competition or "antigen-induced suppression," according to Pross and Eidinger (1974), might be established between the two proteins.

The comparative study of the antitoxic power of sera from rabbits preimmunized by injections of α toxoid, γ toxoid or a mixture of both, or of *N.*

nigricollis venom demonstrated the value of measurement at several levels such as those described by Ipsen (1938), Christensen (1966), and Krag and Weis-Bentzon (1966). The experiment confirmed that the total antitoxic titer of an antivenom depends upon its content of antibodies specific for each of the toxic constituents of the venom used for the immunization.

The injection of rabbits with relatively low doses of α toxin transformed into toxoid with formaldehyde in the presence of tyrosine, which attached to the protein by methylene bridges as demonstrated by chromato-electrophoresis, stimulated synthesis of α toxin-specific antibodies more actively than did the toxoid alone prepared under the same conditions but in the absence of tyrosine. Although it is difficult to conclude definitely that the increase in the immunogenic power of the toxin was the result of the fixation of the amino acid into the protein, it appeared that the procedure employed markedly increased the production of neutralizing antibodies when low doses of antigen were injected.

Finally, the repeated injections of rabbits with a high polymer of the α toxin treated with formaldehyde or glutaraldehyde did not provoke the synthesis

Table 8. Preimmunization[a] with *Naja nigricollis* Venom[b] (Formaldehyde) Plus Freund's Adjuvant (Number LD_{100} Neutralized by 1 ml of Serum)

Number of bleeding	Agent neutralized	Number of rabbit								Average
		461	463	465	467	469	471	473	475	
I	*N. nigricollis* venom	<10	10	10	10	18	10	<10	<10	7
		P[c]	*P*	*P*	*P*	*P*	*P*	*P*	*P*	
I		28	28	17	<10	17	28	>20	10	21
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	
III		40	35	20	10	20	35	40	10	26
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	
I	α toxin	<10	<10	<10	<10	<10	<10	<10	<10	<10
		0[c]	*0*	*P*	*0*	*0*	*0*	*0*	*0*	
II		20	20	10	<10	20	20	20	<10	14
		P	*0*	*P*	*0*	*P*	*P*	*P*	*0*	
III		33	20	13	10	20	27	20	10	19
		P	*0*	*P*	*0*	*P*	*P*	*P*	*0*	
I	γ toxin	<10	10	10	10	10	<10	10	<10	6
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	
II		10	13	<10	<10	10	10	10	<10	7
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	
III		20	23	13	13	10	10	10	<10	13
		P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	

[a]Booster injections: *Naja nigricollis* venom (formaldehyde). Dose injected: 3350 μg.
[b]Total dose of venom: 3600 μg.
[c]*P*, precipitating antibodies; *0*, no precipitating antibodies.

of antibodies specific to the native protein, however, the animals so treated were not immunologically tolerant to this toxin.

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Relationship between the Amount of Habu Toxoid Injected into the Monkey and the Resulting Antitoxin Titer in the Circulation

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INTRODUCTION

Envenomation from bite by the Habu snake (*Trimeresurus flavoviridis*), a crotalid, can be cured effectively if antivenin is administered soon after the bite. However, victims often suffer from residual symptoms such as hemorrhage and necrosis starting at the site of bite when they fail to receive immediate medical treatment (Sawai *et al.*, 1962). Prophylaxis by active immunization with toxoid has long been required, particularly for the inhabitants of remote islands.

Habu venom is known to contain a major lethal toxin and two hemorrhagic principles designated as HR1 and HR2 (Ohsaka *et al.*, 1960; Kondo *et al.*, 1965a,b). In view of the important roles of these toxic principles in envenomation (Kondo *et al.*, 1965b), any preparation of Habu toxoid should stimulate the recipient to produce antitoxins to all these toxic principles.

We have previously reported that a mixed toxoid composed of HR1 and HR2 toxoids was highly immunogenic for guinea pigs and also for monkeys; the animals immunized with the mixed toxoid proved to be satisfactorily protected against challenge with crude venom (Kondo *et al.*, 1970; Kondo and Murata, 1972; Kondo *et al.*, 1973). The toxoid was also immunogenic for human beings (Kondo *et al.*, 1971a; Someya *et al.*, 1972). The production of anti-HR1 was

rather poor in men and monkeys, as compared with that in guinea-pigs or rabbits (Kondo and Murata, 1972). These facts raised a fundamental question as to whether or not the potency of the toxoid to be used for immunizing human beings can be expressed in terms of guinea-pig units.

This paper describes our recent findings demonstrating a clear-cut relationship between the doses of the toxoid as expressed in guinea-pig units injected into monkeys and the antitoxin titers developing in them.

MATERIALS AND METHODS

Preparation of Habu Toxoids. The venom of *T. flavoviridis* contains two immunologically distinct hemorrhagic principles, HR1 (Omori-Satoh and Ohsaka, 1970) and HR2 (Takahashi and Ohsaka, 1970b). To separate HR1 from HR2, crude venom was subjected to gel filtration on Sephadex G-100 (Omori-Satoh *et al.*, 1967). The HR2 fraction was further purified by chromatography on Amberlite CG-50 (Takahashi and Ohsaka, 1970a). The preparations of HR1 and HR2 were treated separately with formalin and dialyzed to prepare HR1 and HR2 toxoids, respectively; the two toxoids thus prepared were combined to form a mixed toxoid (Sadahiro *et al.*, 1970).

Table 1 shows the composition and potencies of various preparations of mixed toxoid. Among the preparations was varied not only the ratio of HR1 to HR2 toxoid contents but also the aluminum content.

Preparation of a Reference Habu Toxoid. Crude venom was detoxified with formalin and dialyzed. The dialysis residue was absorbed onto aluminum phosphate gel (Kondo *et al.*, 1971b) and the mixture was lyophilized. One arbitrary

Table 1. Composition and Potencies of Various Preparations of Mixed Toxoid

Toxoid	Protein content, mg/ml	Ratio of HR1- to HR2-toxoid	Alminum content, mg/ml	Potency of toxoid guinea pig units, U/ml	
				HR1-toxoid	HR2-toxoid
12	1.0	1:1	1.35	63	1.1
13	0.5	2.7:1	0.34	38	6.3
13-B	0.5	2.7:1	1.35	73	5.3
14	0.5	2.7:1	0.34	11	0.7
15	0.5	2.7:1	0.8	29	1.9
16	0.2	2.7:1	0.8	11	0.6
17[a]	1.0	–	0.9	4.9	0.2

[a] This toxoid supplied by Dr. Sawai was prepared by directly formalinizing crude venom which contained HR1 and HR2 at an activity ratio of about 7:3.

unit was assigned to each of the toxoid components contained by 1 mg protein of the lyophilized product.

Potency Test in Guinea Pigs. At least two dilutions in saline of each of the reference and test toxoids were injected into guinea pigs (body weight 300–400 g); eight to 10 animals were used per dilution. After 4 weeks, each guinea pig received another injection with the same dilution. The circulating antitoxin titers in respect to both anti-HR1 and anti-HR2 were determined two weeks after the 2nd injection. From these titers, the potencies of the test toxoid in respect to both toxoid components were calculated and expressed in values relative to those of the reference toxoid (Kondo *et al.*, 1971b).

Immunization of Monkeys. Cynomolgus monkeys (*Macaca irus*) of both sexes weighing 1.8–3.6 kg were used. Each monkey received three injections of a toxoid preparation at four week intervals and bled two weeks after each injection. In some experiments the immunized monkeys were challenged with crude venom two weeks after the last injection.

Anti-HR1 and anti-HR2 titers were determined by the rabbit intracutaneous method; antilethal titer by the mouse intravenous method.

RESULTS AND DISCUSSION

Immune Responses of Various Animals to Habu Toxoid. The immune responses of various animals to a mixed toxoid (No. 12) are shown in Table 2. Apparently the immune response to each component of the mixed toxoid was different depending on animal species; production of anti-HR1 was rather poor in man and the monkey.

Immune Responses of Monkey to Various Toxoid Preparations. Monkeys were immunized with several preparations of mixed toxoid of different potencies.

Table 2. Comparison of Immune Responses to a Mixed Toxoid (No. 12) in Different Animal Species[a]

Three doses each, ml	Circulating antitoxin titers, U/ml							
	Guinea pig		Rabbit		Monkey		Human	
	Anti-HR1	Anti-HR2	Anti-HR1	Anti-HR2	Anti-HR1	Anti-HR2	Anti-HR1	Anti-HR2
2.0	–	–	25	9	21	32	–	–
1.0	24	25	11	5	10	24	–	–
0.5	17	12	7	4	8	21	4	11
0.25	17	3	–	–	–	–	3	7
0.1	–	–	–	–	–	–	3	8

[a] Reprinted from H. Kondo *et al.*, 1971.

The results (Fig. 1) demonstrated a linear relationship between the injected doses of various preparations of mixed toxoid, expressed in terms of guinea-pig units, and the resulting circulating antitoxin titers. Such correlation was pronounced with the HR1 toxoid component but not with the other component contained in the mixed toxoids. The lack of such linear relationship with the HR2-toxoid component may probably have been due to inadequate doses used.

Resistance of the Immunized Monkey to Challenge with Crude Venom. The monkeys immunized with various preparations of mixed toxoid were challenged with crude venom at different amounts. Figure 2 represents the relationship between the circulating antitoxin titers and the resistance of the monkeys. The monkeys having antitoxin titers of about 5 units tolerated a challenge with 5 mg of venom, which is equivalent to 2 MLD for the monkey. The animals with antitoxin titers higher than 10 units tolerated a challenge with 10 mg of the venom, the amount being close to that ejected by a single bite (Kondo *et al.*, 1972). In an immunized monkey, no local symptom characteristic of envenomation from Habu bite was observed after the challenge with 5 mg of crude venom (Fig. 3), while the nonimmunized animals developed severe local symptoms and died after challenge with the same amount of the venom.

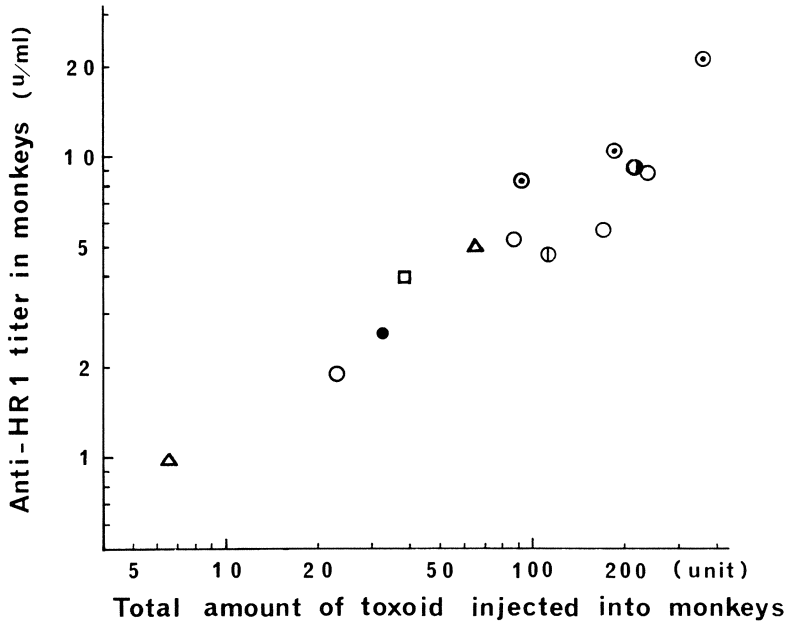


Fig. 1. Anti-HR1 titers in response to the amounts of Habu toxoid injected into monkeys. Each value represents the geometric mean of anti-HR1 titers obtained from 5 to 8 monkeys. (⊙) No. 12; (◫) No. 13; (◑) NO. 13-B; (●) No. 14; (○) No. 15; (△) No. 16; (□) No. 17 (S. Sadahiro *et al.*, 1976).

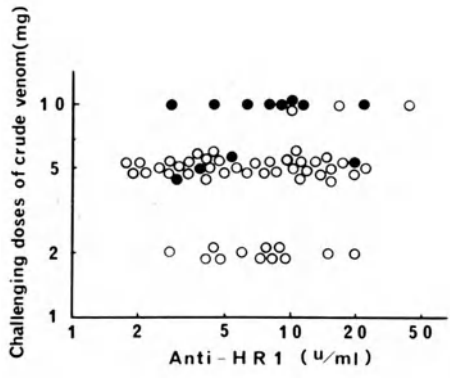


Fig. 2. Resistance of monkeys to the challenge with crude venom in response to their anti-HR1 titers. (black circles) died; (white circles) survived with or without slight local symptoms (reprinted from H. Kondo *et al.*, 1971).

These facts clearly demonstrate that the circulating antitoxin titers developing in the immunized monkeys correlate fairly well with the resistance of the animals to crude venom.

Although the minimum amount of the toxoid required for immunizing man to secure full protection against envenomation from Habu bite remains to be determined, the following information may provide a basis for estimating such an amount. Figure 4 shows the relationship between the circulating antitoxin titers in immunized guinea pigs and their resistance to the venom. In guinea pigs, antitoxin titers higher than 10 units were necessary to protect them against

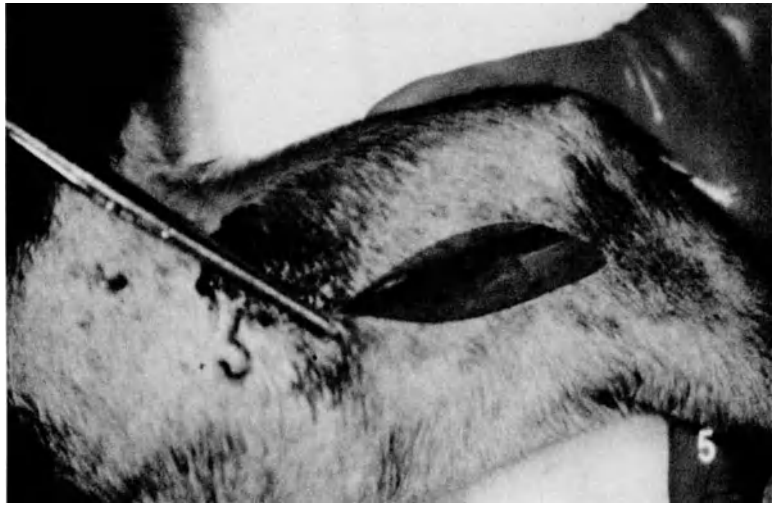


Fig. 3. Absence of local symptoms in an immunized monkey challenged with 5 mg of crude venom. Immunization: crude toxoid; Antitoxin titer before challenge: Anti-HR1 5 U/ml, Anti-HR2 22 U/ml (reprinted from H. Kondo *et al.*, 1971).

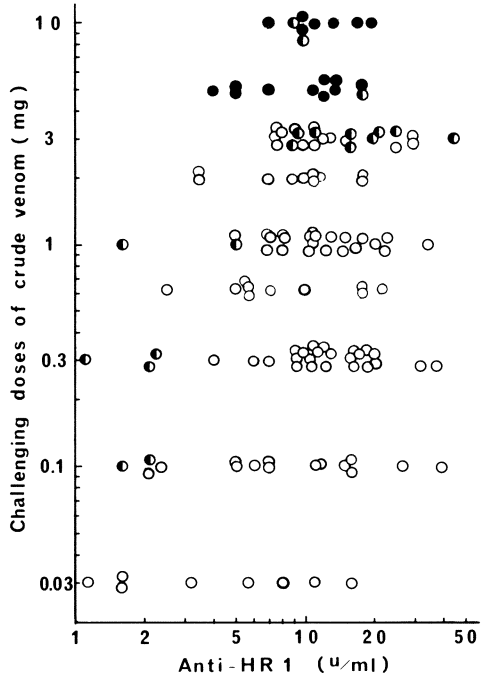
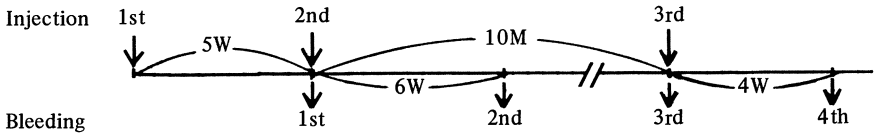


Fig. 4. Resistance of guinea-pigs to the challenge with crude venom in response to their anti-HR1 titers. (black circles) died; (half-black circles) survived with severe symptoms; (white circles) survived with or without slight local symptoms (reprinted from S. Kondo *et al.*, 1971).

the challenge with 3 mg of venom. As Fig. 2 shows, titers of 2-5 units were sufficient for monkeys to resist the challenge with the same amount of venom. From these results it is apparent that large animals (the monkey) show higher resistance to the venom than small ones do (the guinea pig), provided that the circulating antitoxin titers are the same. Experiences accumulated in hospitals indicate that the administration of 6000 units of Habu antivenin is quite effective for the treatment of envenomed cases. Considering that the total volume of the circulating blood of an adult (60 kg) is about 4600 ml, we can safely state that any person having a circulating antitoxin titer of about 1.3 units/ml should fully be protected against envenomation from Habu bite.

Immune Response of Man to Habu Toxoid. Table 3 records the immune responses of volunteers to mixed toxoids. Each volunteer received three injections of 0.5 ml of Habu toxoid at intervals of 5 weeks between the 1st and the 2nd, and 10 months between the 2nd and the 3rd injections. Only the anti-HR1 titers are shown in this table. Most volunteers attained anti-HR1 titers of 1 unit or higher after the 3rd (booster) injection.

Table 3. Production of Circulating Anti-HR1 in Volunteers Stimulated with Mixed Toxoid Preparations



Mixed toxoid used		Proportion of volunteers having anti-HR1 titers (a) less than 1 U/ml and (b) higher than 1 U/ml			
		1st bleeding	2nd bleeding	3rd bleeding	4th bleeding
Lot no. 13[a]	(a)	16 (57%)	4 (20%)	8 (31%)	2 (9%)
	(b)	12 (43%)	16 (80%)	18 (69%)	20 (91%)
Lot no. 15[b]	(a)	17 (85%)	1 (8%)	0	0
	(b)	3 (15%)	12 (92%)	16 (100%)	13 (100%)

[a] Toxoid no. 13 contained protein in 0.5 mg/ml and aluminum in 0.34 mg/ml.
[b] Toxoid no. 15 contained protein in 0.5 mg/ml and aluminum in 0.8 mg/ml.

SUMMARY

The ratio of the production of anti-HR1 to that of anti-HR2 stimulated with a mixed toxoid varied depending on the animal species.

We found a linear relationship between the immune responses of monkeys and the injected doses of the toxoids expressed in terms of guinea-pig units. The finding enabled us to determine, in guinea pigs, the potency of Habu toxoid for human use relative to that of the reference toxoid.

The potency test described herein may facilitate controlling the potency of Habu toxoid. A mixed toxoid we prepared stimulated human volunteers to produce antitoxins to HR1 and HR2, both to satisfactorily high levels.

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Epidemiological Study of Habu Bites on the Amami and Okinawa Islands of Japan

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INTRODUCTION

Seven years after cobra antivenin was originally made by Calmette in 1895, investigations aiming at producing antivenin against the venom of Habu (*Trimeresurus flavoviridis;* see Fig. 6) were started in 1902 by Dr. Kitajima at the Institute for Infectious Diseases, established by Dr. S. Kitasato, Father of Bacteriology in Japan. In the same year, a branch laboratory was built at Naze, Amami Island to supply Habu venom. On 16 May 1905, Dr. Kitajima travelled to Amami to test the antivenin that he had produced for effectiveness. He reported that the fatality rate from the bites of Habu on the Amami Island was reduced to one-third by the application of antivenin.

Since then studies to improve the potency of the antivenin have been carried out by several workers. In the mean time, a new type of purified and freeze dried antivenin (Sawai *et al.*, 1961a), EDTA (Sawai *et al.*, 1961b), glycyrrhizin (Sawai *et al.*, 1961c), tetracycline, and dihydrothioctic acid (Sawai *et al.*, 1963a, b) have been applied to improve the treatment of Habu bites. In spite of these efforts, the results of a survey on snake bites on the Amami and Okinawa Islands showed that severe necrosis and lethal cases were evident in 6%–10% of all the cases each year (Tateno *et al.*, 1965; Sawai *et al.*, 1967). The results of clinical analysis of severe bites suggested that a prophylactic toxoid might be useful in decreasing the number of severe cases, since antivenin treatment has a definite limit in counteracting accidents by the Habu.

The present report deals with an epidemiological study of habu bites conducted during the 9 year period from 1962 to 1970, the results of which served as the starting point of our recent work on snake venom and envenomation.

METHODS

Investigation cards of individual patients of bites were made according to the information collected at health stations, hospitals, and dispensaries. Most of the patients listed in the cards were visited by us to study prognosis of the bites. From these cards, statistical and clinical analysis of the bites were made.

RESULTS

Epidemiology of Habu Bites. The average annual number of cases of bites were 273 in the Amami Islands and 336 in the Okinawa Islands from 1962 to 1970. There were definite regional differences in frequency of bites (Fig. 1). Table 1 reveals that the highest morbidity was 4.6 per 1000 inhabitants in

Table 1. Regional Patterns of Habu Bites (1962–70)

Islands	Total cases[a]	Bites/year[a]	Mortality, %	Morbidity/1000
Amami Islands	2461 (17)	273 (1.9)	0.7	2.2
Amami (main island)	951 (11)	106 (1.2)	1.1	1.2
Tokunoshima	1510 (6)	168 (0.7)	0.4	4.6
Okinawa Islands	3027 (33)	336 (3.7)	1.1	0.4
North region	1322 (20)	147 (2.2)	1.5	1.2
Central region	556 (5)	62 (0.6)	1.0	0.2
South region	1149 (8)	128 (0.9)	0.7	0.3
Totals	5488 (50)	610 (5.6)	0.9 (average)	0.7 (average)

[a]Number of fatal cases is indicated in parentheses.

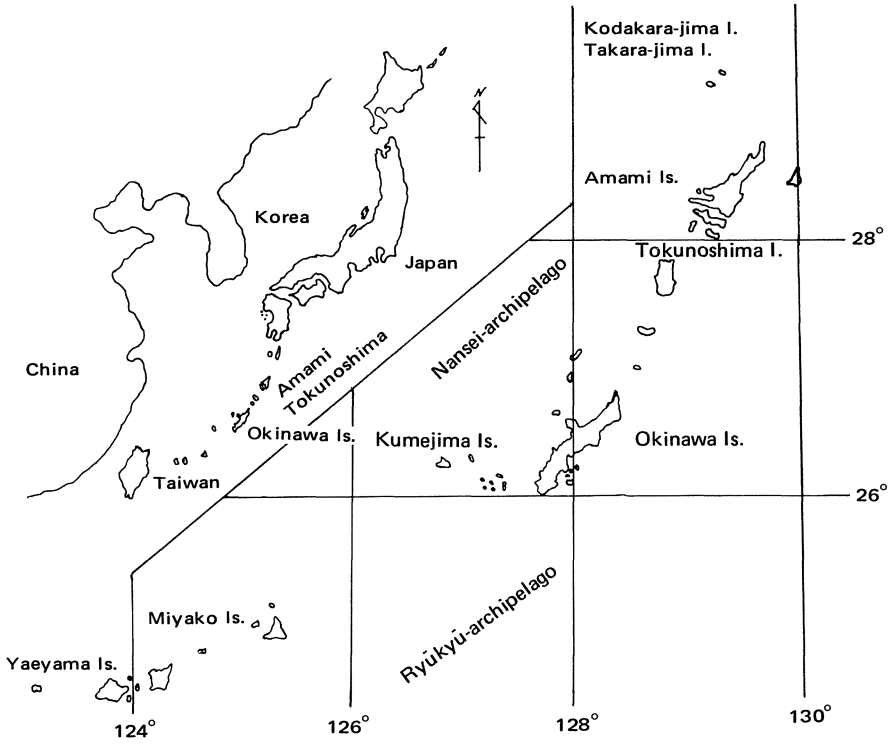

Fig. 1. Map of the Amami and Okinawa Islands in Japan.

Tokunoshima, and the lowest was 0.2 in the central region of Okinawa. There were seasonal and diurnal variations in Habu bites. Figure 2 reveals that during the warmer season from April to October, 97% of bites occurred. Although the Habu is nocturnal, 64% of bites occurred during daylight hours after 6 a.m. (Table 2). From Table 3, it can be determined that 86% of the total bites were widely distributed between the ages of 10-60, and bites in males were two times as frequent as those in females. The highest mortality rate occurred at age 0-to 9 (6.75%). Table 4 reveals the locations where the bites occurred; 42% of the total bites occurred in agricultural fields, 22% in human residences, and 14% on roads. Those facts suggest that Habu and human populations are in close proximity to each other. However, it is noted that the highest mortality rate (2.5%) was recorded among those cases bitten in their residences. With regard to the site of bite in the body, most bites occurred in extremities, as shown in Table 5; 54% of the total bites occurred in lower extremities, of which 25% of the bites occurred in the feet and 22% in the lower legs; whereas 41% of the total bites occurred in the upper extremities, of which 21% were in the fingers and 13% in the hands. However, the highest mortality rate, 4.8% was noted among those

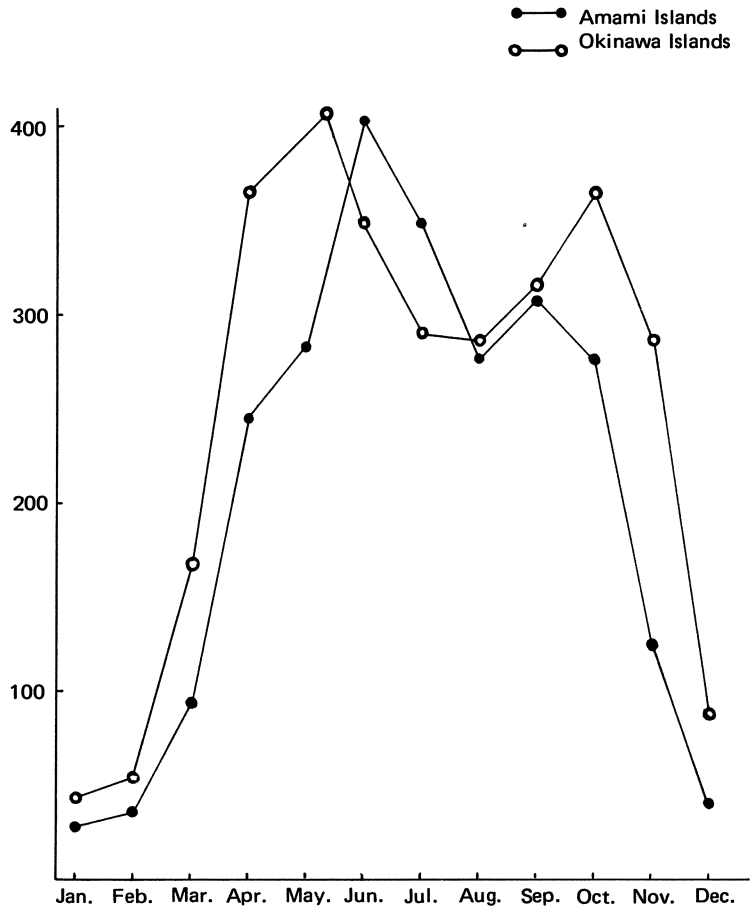

Fig. 2. Monthly Habu bites.

Table 2. Frequency Distribution of Habu Bites by Time of Day

Time	Total bites[a]	Bites/year	Percentage of bites
Midnight–6 a.m.	416 (9)	46	9.0
6 a.m.–noon	1519 (8)	169	31.5
Noon–6 p.m.	1567 (12)	174	32.5
6 p.m.–midnight	1321 (17)	147	27.4
Unknown	665 (4)	–	–
Total	5488 (50)		

[a]Number of deaths is indicated in parentheses.

Table 3. Frequency Distribution of Habu Bites by Age and Sex

Age	Male[a]	Female[a]	Total[a]	Mortality, %	Crippled	Percentage
0–9	139 (8)	83 (7)	222 (15)	6.75	14	6.3
10–19	694 (3)	281 (2)	975 (5)	0.5	42	4.3
20–29	389 (1)	130 (0)	519 (1)	0.2	20	3.85
30–39	574 (1)	252 (0)	826 (1)	0.2	40	4.8
40–49	542 (1)	354 (1)	896 (2)	0.2	27	3.0
50–59	500 (3)	322 (5)	822 (8)	1.0	44	5.35
60–69	371 (3)	273 (7)	644 (10)	1.55	29	4.5
70–79	201 (5)	128 (1)	329 (6)	1.8	22	6.7
⩾80	45 (1)	31 (1)	76 (2)	2.6	5	6.6
Unknown	100 (0)	40 (0)	140 (0)	–	5	–
Unknown (sex)	45		39			
Total	3555 (26)	1894 (24)	5488 (50)	0.9 (average)	248	4.8 (average)

[a]Number of fatal cases is indicated in parentheses.

who were bitten on the head, although the rate of bite on the head is low; the next highest mortality rate, 2% was seen in the bites of the upper arms and the lower legs.

Clinical Aspects of Habu Bites

Treatment of Habu Bites. Owing to the poor medical facilities and scarcity of physicians, the antivenin sera for emergency use are usually kept by the village master, at schools, village offices, post offices, and police stations in remote places in the Amami Islands. Victims receive the antivenin treatment as first aid and then come to the hospitals by vehicles. On the Okinawa Islands, it is the rule

Table 4. Frequency Distribution of Habu Bites by Location

Location	Cases[a]	Mortality, %	Crippled	Percentage
In residence	951 (24)	2.5	62	6.5
On road	823 (5)	0.6	34	4.1
In field	1978 (12)	0.6	84	4.2
In groves	251 (3)	1.2	9	3.6
In mountain	387 (2)	0.5	29	7.5
In other places	297 (2)	0.7	18	6.1
Unknown	801 (2)	0.2	12	1.5
Totals	5488 (50)	0.9 (average)	248	4.5 (average)

[a]Number of fatal cases is indicated in brackets.

Table 5. Frequency Distribution of Habu Bites by the Site of the Body

Body site	Bites[a]	Mortality, %	Crippled	Percentage
Upper Extremities	2124 (12)	0.6	142	6.6
Fingers	1096 (2)	0.2	5	0.45
Hands	673 (4)	0.6	108	16.0
Fore arms	306 (5)	1.6	21	6.9
Upper arms	49 (1)	2.0	8	16.3
Lower Extremities	2727 (31)	1.1	103	3.8
Toes	242 (0)	0	0	0
Feet	1284 (2)	0.15	8	0.6
Lower legs	1062 (21)	2.0	89	8.4
Femurs	139 (8)	5.75	6	4.3
Heads	146 (7)	4.8	1	0.7
Other sites	90 (0)	0	0	0
Unknown	401 (0)	0	2	0.5
Totals	5488 (50)	0.9 (average)	248	4.5 (average)

[a]Number of fatal cases is indicated in brackets.

Table 6. Time from the Bites to Death

Time	Number of deaths	
0–6 hr	11	74.5%
6–12 hr	9	
12–18 hr	8	
18–24 hr	7	
24–30 hr	5	14.9%
30–36 hr	0	
36–42 hr	0	
42–48 hr	2	
3 days	1	10.6%
4 days	1	
5 days	1	
6 days	0	
7 days	1	
8 days	0	
9 days	1	
Unknown	3	
Total	50	

Table 8. Time from Bites to Medical Treatment

Time	Death	Crippled, %
0–30 min	22 } 80%	97 } 74.5%
30–60 min	10 }	58 }
1–2 hr	4 }	42 }
2–3 hr	1 }	} 25.5%
3–4 hr	0 }	}
4–5 hr	1 }	11 }
5–6 hr	1 }	
6–7 hr	1 }	
Unknown	10	40
Totals	50	248

that the victims visit dispensaries or hospitals directly, and receive incision and suction of bite wound together with antivenin. In severe cases when generalized symptoms such as nausea, vomiting, cyanosis, and hypotension occur, victims are treated by application of fluid transfusion, steroid, or noradrenalin. The victim expires from intoxication if the treatment is not successful. As shown in Table 6, deaths occurred within 24 hr after the bite in 35 (74.5%) out of 50 fatal cases, as recorded during the 9 years from 1962 to 1970. Seven (14.9%) of the patients expired within 48 hr and 5 (10.6%) between 3 and 9 days.

Lyophilized monovalent antivenin is distributed free of charge. It is a salt-precipitated equine globulin in a 20-ml vial/dose.

Prognosis of Habu Bites. Pain and local edema were very common at the locus of bites. But local necrosis or generalized symptoms or both appeared in severe cases. As shown in Table 7, out of 5488 cases of bite, generalized symptoms without local necrosis occurred in 544 (9.9%) cases, whereas necrosis without generalized symptoms appeared in 158 (2.9%) cases. On the other hand, both necrosis and generalized symptoms appeared in 212 (3.9%) of which 50

Table 7. Prognosis of Habu Bites

Severe cases	Total cases	Crippled	Dead
Total cases	5488		
Severe cases	544 (9.9%)	248 (4.5%)	50 (0.9%)
With generalized symptoms	174 (3.2%)	0	0
With local necrosis	158 (2.9%)	109	0
Combined	212 (3.9%)	139	50

(0.9%) died. The total necrotic cases were 373 (6.8%), of which 248 (4.5%) left some motor disturbances after the wound had healed.

Case Reports

Case 1. A seven-year-old female was bitten by a Habu on her right lower leg at 22:00, on 19th July, 1969, while walking along a road at Amagi-town, Tokunoshima Island. After application of a tourniquet, she was admitted to the Takehara Hospital at Hedono 30 min after the bite. Two fang marks were found at the locus of bite. Edema spread as far as the abdomen. On incision, necrosis was marked at the locus of bite. In spite of the treatment by application of antivenin, fluid transfusion, and steroid hormone, the condition of the patient deteriorated with such symptoms as vomiting, cyanosis, and hypotension. It was 27 hr after the bite when she expired at 1 a.m. on 21 July.

Case 2. A sixty-three-year old male was bitten on the left lower leg by a Habu at 10:00 on 29 May, 1966, while walking along a road at Setouchi-town, Amami Islands. Having received antivenin for emergency use 30 min after the bite, he was admitted to Kawai Hospital at Koniya at 12:30. The patient was treated by application of antivenin, fluid, transfusion, and steroid hormone, but edema spread over the entire leg. Vomiting continued for several days. Necrosis was marked when two incisions were made at the locus of bite on June 1. On June 10, the tissue of *M. tibialis anterior* was completely removed and tested for

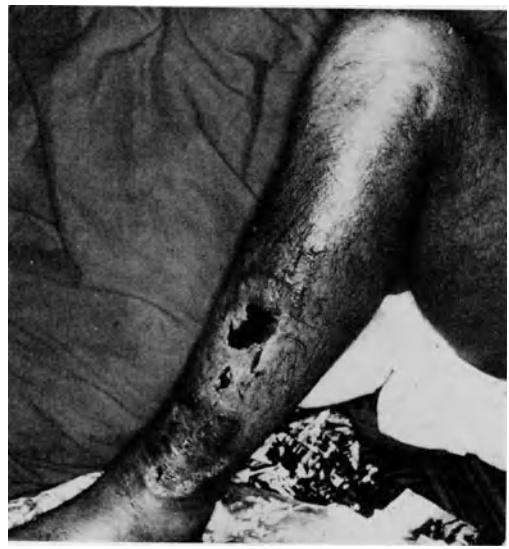

Fig. 3. Case 2, four days after the bite on the left lower leg.

Fig. 4. Case 2, twenty days after the bite on the left leg.

histologic lesions. Motor disturbance of the foot joint persisted after the wound had healed (Figs. 3, 4, and 5).

DISCUSSION

Sowder and Gehrer (1963) reported that 277 cases of snake bites occurred in the state of Florida, U.S.A., in 1962, and the mortality per 100,000 was 5.2. As compared with these results, the average morbidity rate on the Amami and Okinawa Islands is more than ten times higher than that in Florida. The reasons for the high morbidity are that the population of Habu in the islands is very large and Habu and human beings are in close proximity to each other. The frequencies of bites by age group were widely distributed between 10 and 69 years on the islands, whereas 50% of the total bites occurred in persons younger than twenty in Florida. Habu bites occurred frequently in the agricultural field and human residence, whereas 50% of bites occurred in open ground in Florida.

As shown in Table 8, 50 deaths and 248 permanent cripples have been recorded among cases of Habu bites in nine years, although all of the patients received antivenin treatment. These results suggested that there is a limit in the treatment by antivenin, although the effectiveness of the antivenin is dependent largely upon the amount of venom introduced into the victims, the site of bite of the body, location of cases, and the period of time between the bite and antivenin treatment.

Fig. 5. Case 2, four months after the bite.

Deaths from Habu bites may be ascribed to acute circulatory failure that leads to shock. Therefore, symptomatic treatment is very important when the condition of the patient does not improve after application of antivenin. On the other hand, surgeons who believed that the severe necrosis seen in the lower leg was induced by intensive edema used to perform fasciotomy to remove the edema. Thus studies on the toxoid of Habu venom for prophylaxis should be promoted to prevent severe necrosis.

Fig. 6. Habu (*Trimeresurus flavoviridis*).

SUMMARY

The total cases of Habu (*T. flavoviridis*) bites on the Amami and Okinawa Island were 5488 in the nine-year period from 1962 to 1970. The highest morbidity per 1000 inhabitants was 4.6 in Tokunoshima Island, and the lowest was 0.2 in the central region of Okinawa Mainland. In the warmer months from April to October, 87% of the total bites occurred. 86% of the total cases were widely distributed in age groups between 10 and 69, and bites in males were two times as frequent as those in females. 42% of the total bites occurred in the agricultural field, 22% in human residences, and 14% in roads. 54% of the total bites occurred in the lower extremities, and 41% in the upper extremities. It has been found that necrosis occurred in 373 out of the total 5488 cases of bites, of which 248 cases left some motor disturbances after the wound had healed; whereas 212 cases were accompanied with generalized symptoms, of which 50 cases were fatal.

ACKNOWLEDGMENT

The authors are indebted to the doctors in the Amami and Okinawa Islands for their kind cooperation. Part of this work was aided by the funds supplied from the Ministry of Education and Kagoshima and Okinawa Prefectural Governors.

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Snake Bites in India

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INTRODUCTION

Since 1957, Sawai *et al.* (1967) have extensively investigated snake bites by Habu (*Trimeresurus flavoviridis*) on the Amami and Okinawa Islands in Japan. In 1968 and 1969, Sawai and Tseng (1973) also investigated the actual state of snake bites in Taiwan (Formosa). In 1971 and 1972, Sawai *et al.* (1972) planned an investigation on venomous snake bites in Southeast Asia and India supported by a grant in aid for the Oversea Scientific Survey from the Ministry of Education of Japan, and carried out investigation on snake bites in Taiwan, the Philippines, Thailand, Malaysia, Indonesia, and India. The purpose of these studies was to make comparisons of the situations of venomous snake bites in different areas in Southeast Asia and India and to contribute to the improvement in the methods for treatment and prophylaxis adapted in each area. This report concerns the investigation carried out in 1972 on snake bites in India.

METHODS

During the period from October to December, 1972, we visited the Central Government of India and government hospitals in New Delhi, the offices of

Health Services, hospitals, and dispensaries in the states of Uttar Pradesh, Maharashtra, Tamil Nadu, Kerala, and West Bengal, and collected the statistical data on snake bites and historical records of the patients. Epidemiological and clinical analysis were made with these data.

RESULTS

Epidemiological Aspects of Snake Bites

Incidence of Cases of Snake Bite in India. The statistical data given by the government of India indicated that the total number of patients that visited the government hospitals and dispensaries in 1969 were 24,000 of which 1100 (4.6%) cases were fatal. However, the data did not include those who were admitted to mission or private hospitals or treated by local doctors. Sixty-eight or 5% of 1351 patients died from snake bites in the public hospitals and dispensaries in Uttar Pradesh in 1969, although the total death rate due to snake bites registered in the state in the same year were 84 (0.1 per 100,000 population). Deaths in the public hospitals and dispensaries in Maharashtra were 100 in 1969; 993 patients or 89.9% died outside of those institutions in the same year. The mortality rate per 100,000 was 2.1. Cases of snake bites in civil hospitals and dispensaries in Kerala were 1484 and the fatal cases numbered 97 or 6.5% in 1969. Total deaths due to snake bites registered in the state were 264 (1.3 per 100,000) in the same year. Therefore, it is clear that only 37% of the total deaths occurred in the governmental institutions. In Tamil Nadu, fatal cases were 31 or 3.2% of 961 patients, whereas 384 or 5% of 7655 patients died in public hospitals or dispensaries in the West Bengal State in 1969.

Frequency Distribution of Snake Bites by Age and Sex. Table 1 indicates the number of patients admitted to the hospitals or dispensaries in Uttar Pradesh, Kerala, and West Bengal in 1969. As shown in this table, 66.1% of the bites occurred in males and the other 33.9% in females. It also indicates that the largest number of bites (28%) occurred in an age group between 10 and 19, 23% between 20 and 29, and 20% between 30 and 39.

Frequency Distribution of Snake Bites by Months. Incidence of snake bites by months was studied in each of the north (New Delhi), west (Maharashtra), south (Madras and Kerala), and east (West Bengal) areas. As shown in Fig. 1, there were marked seasonal differences in frequency of snake bites, according to the data from two hospitals in New Delhi. The peak incidence occurred in September, when 92 (26.5%) of the total of 347 bites were recorded. During the warmer months of the year (July through October) 274 bites (79%) were recorded, whereas during cooler months (December through February) the average monthly rate of bites was lower than 1%. Similarly in West Bengal,

Table 1. Frequency Distribution of Snake Bites by Age and Sex in Uttar Pradesh, Tamil Nadu, and West Bengal in 1969

Age	Male	Female	Total	Percentage
0–9	324	198	522	7.8
10–19	1192	664	1856	27.8
20–29	1097	454	1551	23.2
30–39	884	451	1335	20.0
40–49	565	275	840	12.6
50–59	242	151	393	5.9
>60	107	69	176	2.6
Totals	4411 (66.1%)	2262 (33.9%)	6673	

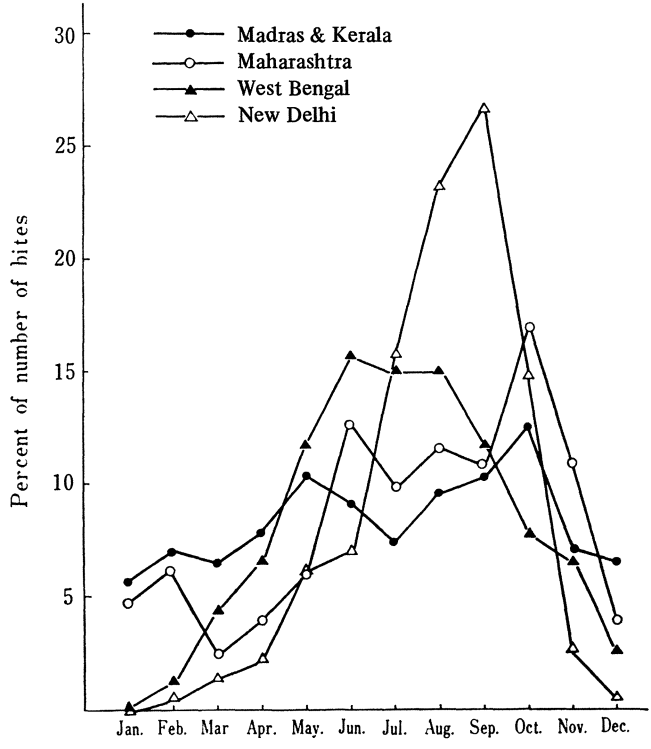

Fig. 1. Seasonal patterns of snake bites in India.

Table 2. Frequency Distribution of Bites by Time of Day in India

Time	Bites	Percentage
Midnight–6 a.m.	94	13.9
6 a.m.–noon	148	21.95
Noon–6 p.m.	159	23.6
6 p.m.–midnight	273	40.5
Unknown	516	
Total	1190	

805 (69%) of 1341 bites in hospitals were seen during warmer months, from June through September. In the Bombay area, 258 (73%) of 353 bites in two hospitals were recorded during the six-month period from June through November, whereas no characteristic seasonal pattern of bites was seen in Madras and Kerala.

Frequency Distribution of Bites by Time of Day. From Table 2, it is noted that between 6 p.m. and midnight almost 40% of the bites were reported and almost the same number of the bites occurred during the day time.

Sites of Bites on the Body. From Table 3, it is evident that 598 (72.7%) of the total bites occurred on the lower extremities, of which the highest frequency was seen on the feet (41.1%), whereas 207 bites (25%) were on the upper extremities, of which the highest frequency was seen on the fingers (13.7%).

Table 3. Sites of Bites on the Body

Body sites	Number of cases	Percentage
Upper extremities	207	25.2
Fingers	113	13.7
Hands	66	8.0
Forearms	24	2.9
Upper arms	4	0.5
Lower extremities	598	72.7
Toes	111	13.5
Feet	338	41.1
Lower legs	138	16.8
Thigh	11	1.3
Heads	5	0.6
Other sites	12	1.5
Unknown	368	
Total	1190	

Clinical Aspects of Bites

Clinical Analysis of Bites. There are four species of venomous land snakes responsible for bites in India. They are the Indian cobra (*Naja naja*), Indian krait (*Bungarus caeruleus*), Russell's viper (*Vipera russelli*), and saw-scaled viper (*Echis carinatus*). Identification of the snakes involved in bites, however, is sometimes quite difficult, because of poor information obtainable from patients or physicians about the snakes that made the attacks. As shown in Table 4, the snakes responsible for 1088 cases of bite or 91.4% of the total that were hospitalized were not named, whereas those responsible for only 73 bites or 6.1% were identified.

Accordingly, an effort was made to identify the snakes from the clinical symptoms described in the historical records of patients. In most cases of cobra bites, severe pain and edema appeared at the locus of bite and so did certain paralytic symptoms such as ptosis of the eyelids, difficulty in speaking, dyspnea, and unconsciousness. Edema is a very important sign appearing at the locus of cobra bites, which distinguish them from bites by krait. In krait bites, severe paralytic symptoms appear without any local reaction. In viper bites, edema, hemorrhage, and cellulitis at the locus of bites are more prominent in Russell's viper bites than in Echis bites. Bleeding from gums is very common in Echis bites, whereas anuria or oliguria occur infrequently in fatal cases of Russell's viper bites.

On the basis of the historical records on 75 fatal cases (mortality rate 5.7%) out of 1303, seven cases have been identified as cobra bites, three cases as krait bites, eleven cases as Russell's viper bite, and two cases as Echis bite. However, it was quite difficult to identify the snakes responsible for 30 patients who were

Table 4. Kinds of Snakes Responsible for Bites in India

Snakes	Bites		Percentage
Indian cobra, *Naja naja*	29		
Indian krait, *Bungarus caeruleus*	6	73	6.1%
Russell's viper, *Vipera russelli*	20		
Saw-scaled viper, *Echis carinatus*	18		
Nonpoisonous	29		
Unidentified	1088		91.4
Total	1190		

admitted to the hospitals in critical conditions such as in an unconscious state and who then expired within a few hours.

Treatment of Snake Bites. Most of the patients did not come to the hospitals but were treated by local doctors or medicine men. This is the reason why so many patients expired outside hospitals without receiving antivenin. Polyvalent antivenin against the venoms of cobra, krait, and two kinds of viper is produced at Haffkine Institute and Kasauli Central Laboratory. Application of artificial respirators and treatment with neostigmine (Banerjee *et al.*, 1972) were reported to be effective for bites by cobra and krait. Application of peritoneal dialysis is also effective upon viper bites, saving many patients.

Case Reports

Case 1, Cobra. A 18-year-old snake catcher was bitten by a cobra, when he was pushing the snake into a cloth bag, through cloth on the fleshy part of the left hand below the little finger with both fangs at 12:30 p.m., 2 June, 1972. Thirty minutes after the bite, he applied a tourniquet on the arm. Swelling developed on his arm half way up and he complained of much burning pain throughout the arm. At 6:30 p.m., he started having symptoms of ptosis of eye-

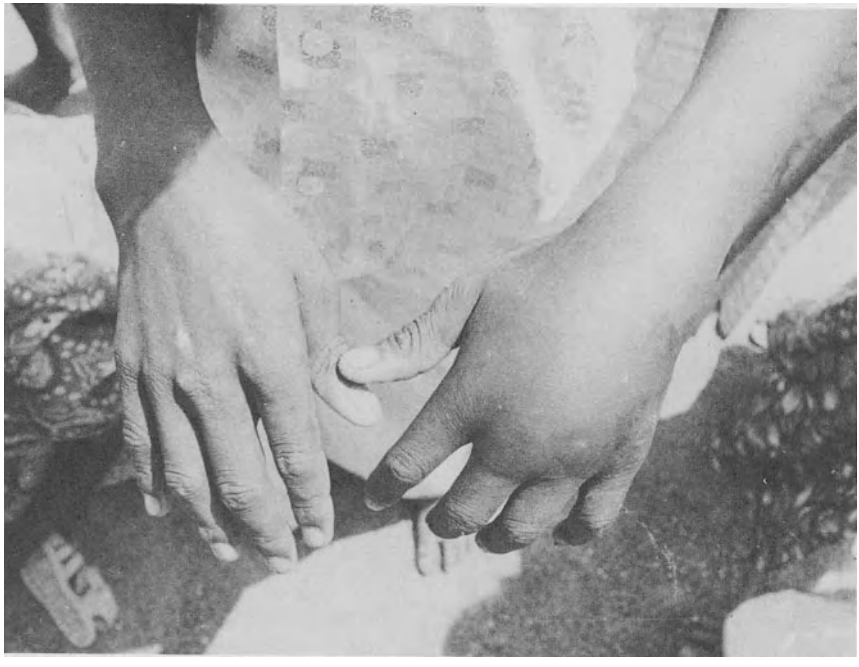

Fig. 2. Case 1. The left hand 24 hr after a bite by an Indian cobra.

lids and puffed lips. Immediately, he received 10 ml of polyvalent antivenin with 5-mg cortisone at a dispensary. Twelve days later, the hand was still swollen with cellulitis. Thirty days later, an area of the skin was lost but recovery was complete except for stiffness of the hand. (This case was reported by Mr. R. Whitaker.)

Case 2, Cobra. A 9-year-old boy was bitten on his left foot by a cobra at 3 p.m., 17 September, 1970 in his home. Immediately, a tourniquet and suction were applied. He was admitted to a hospital in New Delhi at 4:30 p.m. and intravenously received two vials of antivenin at an interval of 4 hr. In spite of the treatment, he became drowsy, unconscious, and dyspneic. Dyspnea was countered by the application of an artificial respirator, though difficulty of speaking still remained. On 18th afternoon, general symptoms had subsided but edema and cellulitis were marked at the locus of bite.

Case 3, Krait. A 40-year-old man was bitten on the left foot at 2:30 a.m., 28 October, 1968, while sleeping in his house. As he became drowsy, he was admitted to a hospital in New Delhi at 6:30 a.m. But his condition deteriorated and he expired at 8:30 from dyspnea and hypotension. The responsible snake was identified since neither pain nor edema or hemorrhage was noticed at the locus of bite in spite of the onset of severe paralytic symptoms.

Case 4, Russell's Viper. A 20-year-old man was bitten on the right index finger by a snake at 5 p.m., 20 November, 1972, while working in a field near Alibag, Maharashtra State. He visited the District Hospital with the dead snake

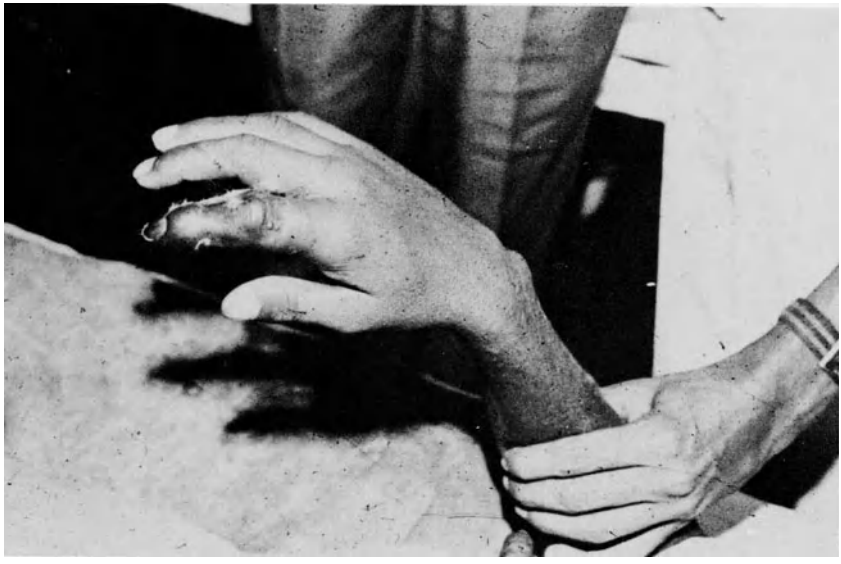

Fig. 3. Case 4. Necrosis of the right index finger after bite by a Russell's viper.

Table 5. Symptoms of Patients of Bites in India

Snakes	Pain	Swelling	Hemorrhage	Cellulitis	Paralysis
Indian cobra	+	+	(–)	+	+
Indian krait	±	±	(–)	(–)	+
Russell's viper	++	++	+[a]	++	(–)
Saw-scaled viper	+	+	++[b]	+	(–)

[a]Prolonged coagulation time of blood.
[b]Hemorrhage from gums.

which was identified by the physician as a Russell's viper. Pain and edema developed 30 min after the bite. Two vials of antivenin added with 500 ml of 5% glucose saline solution were injected at an interval of 4 hr, but 2 days later, cellulitis and necrosis developed at the locus of bite.

Case 5, Saw-Scaled Viper. A 60-year-old woman was bitten on the right foot by a saw-scaled viper at 7:00 p.m., 20 November, 1972, while working in a field near Alibag in Maharashtra State. She was admitted to the District Hospital at 12:30 p.m., 21 November, 1972. Bleeding from the gums was characteristic but local edema was very slight.

DISCUSSION

It has been estimated that about 10,000–15,000 persons are killed by snakes every year in India. However, such a large number of deaths seem to us incredi-

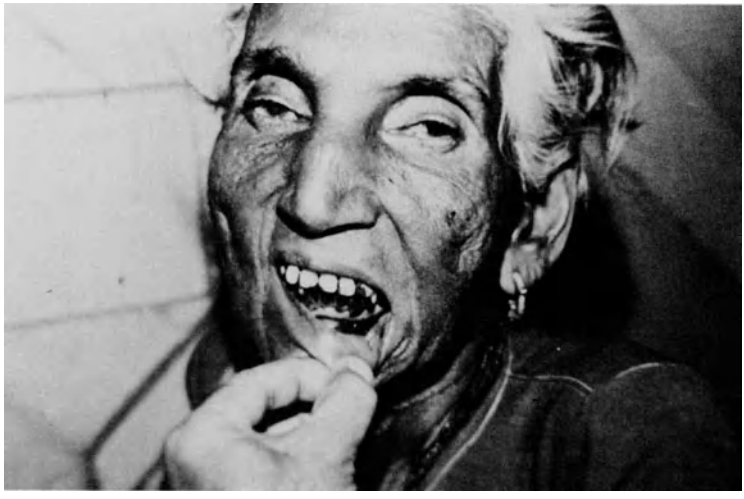

Fig. 4. Case 5. Bleeding from the gum after bite by a saw-scaled viper.

ble, so far as we investigated the snake bites in hospitals and dispensaries in New Delhi and five states in India. A more exact number of deaths will be available, if information of the total deaths due to snake bites registered in each state can be obtained. It is quite difficult to know the exact number of persons bitten by snakes, because most of the bites occur in remote places and the victims are treated by medicine man or prayer (Mantras) and, therefore reports are not submitted on the snake bites. The death rate due to snake bites per 100,000 in Kerala was about 1.2, which is greater than that in the Philippines (0.8) and in Thailand (0.9). Our data suggest that the main species causing deaths is cobra in India as well as in the other two countries mentioned above.

The monthly records of snake bites in Kerala resembled those in Malaysia in that there were no definite seasonal differences. In this investigation, we found no report on sea snake bites, although Madras is inhabited with as many sea snakes as found in Malaysia. The scarcity of accidents from sea snakes may be due to the different method of fishing in India.

The polyvalent antivenin seems to be effective even if administered after the onset of paralytic symptoms in cobra bites. However, the use of polyvalent antivenin against four kinds of snake venoms seems to have discouraged physicians from studying the detailed symptoms from each kind of snake. We recommended the use of a toxoid for cobra venom because of the situation that many patients expire before they receive antivenin treatment.

SUMMARY

Epidemiological and clinical studies of snake bites in India were carried out in 1972. Statistical data on snake bites were given by the State Government of New Delhi, Uttar Pradesh, Maharashtra, Tamil Nadu, Kerala, and West Bengal. Analysis of clinical signs and symptoms of snake bites were made on the protocols obtained from hospitals and dispensaries in these states.

The over-all snake bite mortality in India was not known, but was 0.1 per 100,000 population in Uttar Pradesh, 2.1 in Maharashtra, and 1.3 in Kerala. There was seasonal variation in incidence of snake bites in all the states except Madras and Kerala. More bites are reported during warmer months of June through November. Snake bites occurred in males two times more frequently than in females. The largest number of bites (28%) occurred in persons of age group of 10-19, 23% in the age group of 20-29, and 20% in the age group of 30-39. There are four kinds of venomous land snakes responsible for bites: Indian cobra, Indian krait, Russell's viper, and saw-scaled viper. However, only in a very few bites (6.1%) were the responsible snakes identified because of the lack of knowledge of both patients and physicians.

Many patients expired without visiting hospitals. The toxicities of the venoms of cobra and krait are so high that most of the patients die within 24

hr. On the other hand, the time to death is prolonged to 3–7 days in cases of viper bites. Polyvalent antivenin for the venoms of cobra, krait, Russell's viper, and saw-scaled viper is used for the specific treatment. In some cases, symptomatic treatment such as application of artificial respirators and peritoneal dialysis were effective to save the patients from death. It is suggested that prophylactic immunization with cobra venom toxoid would be useful to decrease fatal cases.

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Snake Venom Poisoning in the United States

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In the United States, approximately 45,000 bites are inflicted by snakes each year, of which about 8000 are caused by venomous snakes, and 6800 of these are reported (Parrish, 1966; Russell, 1969). During the past five years, fewer than 12 people have died each year from snake venom poisoning. Before the advent of antivenin and antibiotics, more than 100 persons a year died from rattlesnake bites in the United States. Most of the present deaths occur in small children, untreated or mistreated cases, and in members of religious sects who handle rattlesnakes during worship services.

In about 20% of the bites by venomous snakes in the United States there is no evidence of envenomation; that is, the snake bites but does not inject venom or it bites very superficially, the venom being ejected onto the skin. It is therefore, important that in the admitting diagnosis the attending physician differentiate between snake bite (usually limited to bites by nonvenomous snakes), venomous snake bite without envenomation, and snake venom poisoning. Identification of the offending reptile should be noted, when obtainable.

There are about 120 species of snakes in the United States, approximately 20 of which are venomous. The crotalids, which include the rattlesnake, cottonmouth (water moccasin), copperhead, pigmy rattlesnake, and the massasauga, are responsible for about 99% of the bites and deaths. Bites by the elapids, the Eastern coral snake and the relatively unoffensive Arizona coral snake are uncommon, and about five bites per year are reported for imported species.

It is well known that the venoms of snakes are complex mixtures, chiefly proteins, many of which have enzymatic activities. During recent years it has been shown that the lethal and sometimes more deleterious effects of snake venoms are caused by certain peptides and proteins of relatively low molecular weight (Russell and Brodie, 1974). The role of these peptides in snake venom poisoning has too often been overlooked by some physicians, who have mistakenly believed that snake venoms are only "enzymes," and thus departed on therapeutic measures which have not only been inconsistent with our knowledge of the chemistry and pharmacology of these complex poisons, but which have led to serious clinical errors.

Equally as misleading has been the division of snake venoms into such artificial groups as neurotoxins, cardiotoxins, coagulants, and the like. Although perhaps serving some purpose in classification, particularly for the pharmacologist, these arbitrary divisions, usually based on an admixture of pharmacological, chemical, and immunological experiments sometimes sprinkled with intuitive hunches, have resulted in grave errors in treatment. The physician attending a patient with snake venom poisoning must remember that he is faced with a case of multiple poisoning, perhaps three or more toxic reactions, with changes that may occur simultaneously or consecutively. It should also be remembered that the effects of various combinations of the venom components, and of the metabolites formed by their interactions, can be complicated by the response of the patient. The release of autopharmacologic substances by the envenomated patient may complicate the poisoning and make treatment more difficult. These important clinical factors are not always obvious from studies on various physiopharmacological or chemical preparations; unfortunately, the squid axon has not always turned out to be a very sophisticated human being.

Snake venom poisoning is a medical emergency requiring immediate attention and the exercise of considerable medical judgment. To be effective, treatment must be instituted immediately and must include measures (1) to remove as much venom as possible from the wound; (2) to retard absorption of the venom; (3) to neutralize the venom; (4) to mitigate the effects of the venom; and (5) to prevent complications, including secondary infection. The first aid treatment of snake venom poisoning has been reviewed elsewhere (Russell, 1967; Dowling, *et al.*, 1968; Russell, 1974). The present report is limited to a review of the medical treatment. The advice is based on the authors' experiences with more than 500 cases of snake venom poisoning, involving 34 species of snakes.

On admission to the hospital the appropriate laboratory tests should be done. The choice of tests depends on the type of venom poisoning. In general, however, the following tests should be carried out immediately: typing and cross matching, bleeding, blood clotting and clot retraction times, a complete blood count, hematocrit, platelet count, and urinanalysis. The authors routinely

obtain sedimentation rate, prothrombin time, carbon dioxide combining power, and determinations of blood urea nitrogen, sodium, potassium, and chloride. In severe poisonings it is advisable to obtain an electrocardiogram. Serum bilirubin, red cell fragility, and renal function tests are optional. In severe envenomations by rattlesnakes, the hematocrit, blood count, hemoglobin concentration, and platelet count should be done several times a day for the first four days following the bite, and all urine and stool samples should be examined for blood.

Measurement of the circumference of the injured part four inches above the bite and at some point distant to that should be recorded every 15-30 min during the first few hours after the injury, every 30-60 min during the next 24 hr, and at least four times a day during the next four-day period. Regardless of the snake involved, facilities and drugs for combatting shock must be readily available. In severe envenomations, oxygen, positive-pressure breathing apparatus, and a tracheostomy set should be in readiness.

The importance of the early administration of antivenin, preferably intravenous for many types of venom poisoning, cannot be overemphasized. The choice of antivenin, the route of injection, and the amount to be given will depend upon the species and size of snake involved, the site of envenomation, the size of the patient and a number of other factors. In rattlesnake bites, antivenin (*Crotalidae*) polyvalent (Wyeth) is the antitoxin of choice. An antivenin for a North American coral snake is also available. In the United States, when the offending species is an imported one, the physician consults his nearest Poison Control Center for guidance on the availability and choice of antivenin.

In minimal rattlesnake venom poisoning, and after the appropriate skin or eye tests for sensitivity, one to four units (vials or packages) of antivenin will usually suffice. In moderate cases 4–7 units may be required, whereas in severe cases 10–20 units may be needed. Poisoning by water moccasins usually requires lesser doses, whereas in copperhead bites antivenin is usually limited to use in children and the elderly. The choice of route of administration will depend on several factors, including the length of time that has elapsed between the bite and antivenin administration. The longer the delay, the more urgent the need for intravenous antivenin. In cases of shock the antivenin should always be given intravenously. Under no circumstances should antivenin be injected into a finger or toe. Recent studies indicate the value of antivenin given within four hours of the bite. It is of less value if administration is delayed eight hours, and of questionable value after 24 hours, except perhaps in poisoning by certain elapids.

A decrease in circulating blood volume and flow is a common finding in all severe and in most moderate cases of snake venom poisoning, particularly after rattlesnake envenomation. Concomitant with these changes the red blood cells may undergo lysis and further embarrass the circulation. Although plasma or plasma expanders can be given, fresh whole blood is preferred, because the patient may also be unable to maintain an adequate number of platelets. Ex-

change transfusions should be considered when clotting time is at infinity and the blood picture displays no evidence of improvement. The loss of blood and intracellular fluid which may occur can cause changes in fluid and electrolyte balance and require immediate attention. However, parenteral fluids must be given cautiously, since these may aggravate swelling, burden cardiac function and precipitate pulmonary edema, particularly in severe envenomations.

A broad-spectrum antimicrobial should be given if there is severe tissue involvement, since the nature of the injury predisposes to infection. Antimicrobial drugs are not advised for trivial cases. The appropriate antitetanus agent should always be given. Aspirin or codeine are usually sufficient for pain but morphine or Demerol may need to be used if the pain is severe. At the first signs of respiratory distress, oxygen should be given and preparations made to apply intermittent positive-pressure artificial respiration. Tracheal intubation or tracheostomy may be indicated, particularly if trismus, laryngeal spasm, or excessive salivation is present. The routine emergency measures for the treatment of renal shutdown should be followed if this becomes a problem. Renal dialysis may be necessary. Peritoneal dialysis has been of little value in the authors' experience; however, it is said to have been used successfully in severe cobra venom poisoning. Mild sedation is indicated in all severe bites and when respiratory depression is not a problem. Sedation will usually reduce the amount of analgesic necessary to control pain.

Antihistamines are of no value except to control manifestations of sensitivity to antivenin; they might be given intravenously before administering antivenin to prevent an anaphylactic reaction. There is experimental and clinical evidence to indicate that steroids are not of value during the acute stages of rattlesnake venom poisoning, and may actually be contraindicated. They are of use in treating the subsequent occasional reaction to horse serum antivenin. Recent clinical experiences with hyperbaric oxygen indicate its value in some cases. Isolation-perfusion of an extremity and intra-arterial infusion with antivenin have been tried with indifferent results.

The wound should be cleansed and covered with a sterile dressing. The injured part should be immobilized in a physiologic position. Skin temperature should be kept between 10 and 15° C for 24–96 hours. Under no circumstances should an extremity be placed or packed in ice or left in ice for an extended period. Surgical debridement of the blebs, bloody vesicles, and superficial necrosis may have to be performed between the fourth and tenth days following the bite. Most of these changes appear between the second and fifth days, and have usually reached maximal development by the third to sixth days. If surgical debridement is attempted too early, the underlying tissues will continue to lose fluid and blood and be more susceptible to infection. If debridement is done too late, the clots and superficial necrotic tissues become difficult to remove. Debridement may need to be done in stages. Once it has been initiated, the

patient should be evaluated for joint motion, muscle strength, sensation, and girth measurements. Immobilization should be interrupted by frequent periods of gentle exercise, progressing from passive to active.

Follow-up care should include sterile whirlpool treatment, debridement as necessary, daily cleansing of the wound followed by several 15-min soaks in 1:20 Burow's solution, and daily painting of the affected area with an aqueous dye containing brilliant green 1:400, gentian violet 1:400, and *n*-acriflavin 1:1000. A polymyxin-bacitracin-neomycin ointment can be applied at bedtime. Daily exposure to continuous oxygen in a plastic bag is of value. When the injury is to a lower limb, it should be covered with Telfa, which should be reasonably firm when the patient is ambulatory. Fasciotomy should be avoided and is rarely called for. The authors have not observed the need for this method of therapy in the early management of more than 500 cases of snake venom poisoning. It may need to be done when early antivenin treatment has been inadequate and there is substantial evidence of vascular impairment.

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Unusual Complication of Snake Bite: Hypopituitarism after Viper Bites

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INTRODUCTION

A total of 600 cases of snake bite were treated in the Little Flower Hospital, Angamally during a period from June 1966, to July, 1974. Three of these people were seen later with manifestations of panhypopituitarism. This occurrence as a complication of snake bite is being reported, perhaps for the first time.

CASE REPORTS

Case 1. Mr. X aged 40 was brought to the hospital in 1968, 2 days after the snake bite. From the description, the agent was probably viper. He was bleeding from the site of bite and from the gums. He also had hematuria. Usual treatment with antivenom, cortisone, antibiotics, and intravenous fluids was given. Bleeding was controlled, but he went into anuria from the fifth day on. He was shifted to another hospital for hemodialysis. During the stay there a lumbar puncture was done on him because of intense headache. The cerebrospinal fluid was hemorrhagic. He was discharged after 21 days by which time his kidneys had resumed normal function and headache had disappeared. In December 1969, he came back to our hospital for weakness and loss of libido. He complained of weakness in producing and sustaining an erection. Physical examination revealed a moderately well-built man with very little hair on his face, no hair in the axilla and scanty hair over the pubis. Genitalia looked normal. Blood pressure was

Fig. 1

90/70; ankle jerks were normal. X-ray skull and routine investigations were within normal limits. He was sent to a nephrologist for investigations. Low protein-bound iodine and low urinary 17 ketosteroids were reported. The clinical findings and the above values together confirmed the clinical impression of panhypopituitarism. He was started on oral administration of cortisone, thyroxin, and parentral testosterone propionate. With this treatment he was able to lead a normal life. One injection produced an abscess and he has been lost to follow up after that (Fig. 1).

Case 2. Mr. Y, 38 years old, was brought to the hospital in November, 1973, 12 hr after snake bite with bleeding from the gums and malena. His blood pressure was less than 80-mm systolic. By usual management and blood transfusion, his bleeding was corrected. Since he had been given large doses of cortisone, he was asked at the time of discharge to taper the dose. A month later he reported in the out-patient department saying that he was quite well but he

could not reduce cortisone to less than 1 tablet a day. He kept on taking one tablet a day for one month. This time he complained that he had no sexual urge. He blamed his general weakness for the possible cause of the impotence. He could produce a weak erection but could not sustain it to have an intercourse. His beard had thinned out and he had shaved off his moustache because it was looking ugly due to the dropping of hairs. General examination revealed scanty facial and pubic hair, no axillary hair, and a normal-sized penis and testicles. The only positive findings on systematic examinations was a low blood pressure of 94/70 mm. He is keeping well with replacement therapy with cortisone and thyroxine. He is due to go for detailed endocrinological studies at Vellore very soon (Fig. 2).

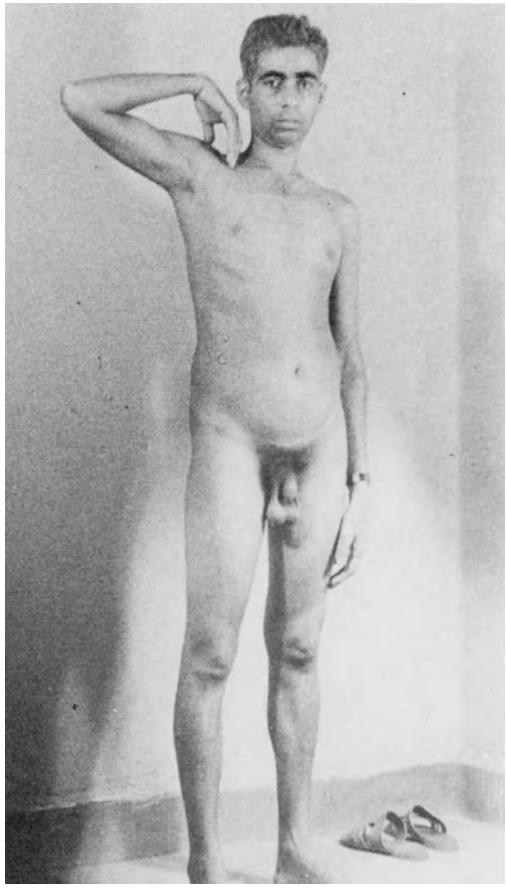

Fig. 2

Case 3. Mrs. Z, 21 years of age, was brought to the hospital in August, 1973 with a history of being bitten on the leg while working in the plantations. She was having bleeding from the site and hematuria. The blood when tested was found to be not coagulable. In spite of antivenom, cortisone, and intravenous fluids, she went into uremia. This was tided over by conservative management without applying any dialysis. Six weeks later she reported to the Gynaecology Department with a complaint of amenorrhea since the snake bite. After taking some tablets she had bleeding for one day on one occasion. Past history, puberty at the age of 15 and periods once in 2-3 months till the snake bite. On leading questions she mentioned that her axillary and pubic hair had dropped completely. Her breasts had not decreased in size but to quote her own words "she was and felt like a girl of 14." General examination–absence of axillary and pubic hair, low blood pressure of 85/60, vaginal examination, uterus smaller than normal. Routine blood tests and skull x-ray were normal. She has been

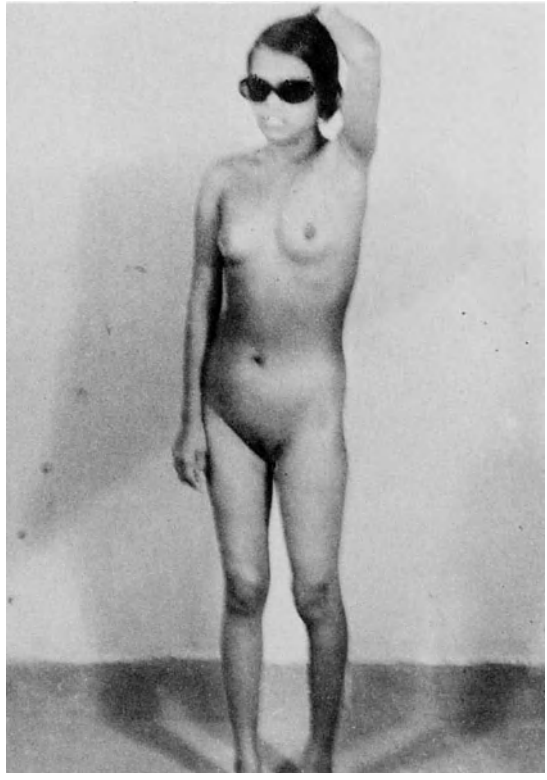

Fig. 3

worked up in detail by Professor T. S. Koshy of the endocrinology department of C.M.C. Hospital, Vellore. He concludes that biochemically it fits in fully with a diagnosis of panhypopituitarism. The summary of the tests done is shown in Table 1. Note that uptake had very low values. Scan shows scanty uptake in the thyroid which is comparatively smaller in size. In the words of Dr. T. S. Koshy "the basal plasma cortisol at 8 a.m. and 11 p.m. were both far below normal levels. Similarly, protein-bound iodine was undetectable, and the values of 24-hr urine for 17 KGS were very low on both basal samples. This indicates that her basal adrenocortical activity as well as thyroid function were very much diminished. We used the ACTH stimulation test; immediately the plasma cortisol went up to 20 μg, indicating that her adrenal cortex was susceptible to stimulation. This indicates that the fault was in the putuitary. This point was further confirmed by the metapyrone tests, in which the 17 ketogenic steroids did not go up appreciably after the drug administration. In fact, the test had to be abandoned

Table 1. Laboratory Data on Patient No. 3

Blood sugar: AC 76 mgm%; PC 62 mg%		
Electrolytes: Na. 143 meq/liter, K 4.3 meq/liter		
Basal plasma cortisol: 8 a.m. 4 μg		
11 p.m. 1 μg		
Protein-bound iodine Nil		
24 hr urine for:		
Basal sample I:	17 Ketosteroids	1.8 mg/24 hr
	17 Ketogenic steroids	3.3 mg/24 hr
	Creatinine	436 mg/24 hr
	Total volume	1505 ml
Basal sample II:	17 K.S.	1.4 mg/24 hr
	17 K.G.S.	3 mg/24 hr
	Total volume	1720 ml
ACTH stimulation test with 40 units:-		
a. Plasma Cortisol 1 hr after start:		20 μg
b. Urine 24 hr for 17 K.S.		
	17 K.G.S.	5 mg
Metapyrone test: (only 3 doses– 750 mg each given)		
Urine 17 KGS:	0–24 hr	6 mg
	24–48 hr	9.2 mg
Radioactive iodine uptake studies:		
Uptake	2 hr	8.9%
Uptake	4 hr	9.9%
Uptake	6 hr	11.4%
Uptake	24 hr	21.2%
Uptake	48 hr	20.1% (below normal levels)
TSH stimulation test done:		
Serum I–131 0.64% per liter of serum		
PBI–Nil		

after only 3 doses of metapyrone given because she went into adrenal crisis. The radioactive iodine uptake studies also confirmed that the early uptakes as well as 24 and 48-hr uptakes were lower than normal. PBI-131 was not detectable. This therefore, confirms the diagnosis of "panhypopituitarism."

DISCUSSION AND SUMMARY

These three cases present clinically classic features of panhypopituitarism. The 3rd case has been worked up in detail by an endocrinologist and he agrees with the diagnosis of the same. The 1st case has been studied by a nephrologist. With the clinical features, low PBI and low 17 ketosteroids he was willing to call it panhypopituitarism. The 2nd case has the clinical features of hypogenitalism and low B.P. but since he resembles case 1 we can also call him panhypopituitary though his biochemical data are not ready.

Diffuse intravascular coagulation is known to occur after viper bite. Such obstruction in the blood vessel supplying the pituitary stalk can produce infarction in the pituitary gland. Perhaps the severe stress given by the snake bite, the fall of blood pressure, the foreign protein (antivenom) being given for treatment, and the big doses of steroids make the pituitary gland susceptible to infarction. This may be comparable to the pituitary gland undergoing necrosis resulting in Sheehan's syndrome after delivery or abortion.

Diffuse intravascular coagulation is known to occur in the rabbits intravenously given foreign proteins. The first preparatory injection, which may be of a quite different endotoxin or even a particulate matter such as kaolin or agar has only to be spaced 6-72 hr after the first injection to produce the dramatic effects. The generalized Shwartzman phenomenon can follow a single injection. This occurs after recticulo-endothelial blockade, inhibition of fibrinolysis, treatment with cortisone and, in some species at least, during pregnancy. A snake bite patient getting large doses of antivenom over a long period of 24-72 hr and large doses of cortisone and with a fall of blood pressure sometime ending in diffuse intravascular coagulation has many features in common with those of the animals showing generalized Shwartzman phenomenon.

Diffuse intravascular coagulation also produces bleeding; bleeding at one site and clotting at another site. The latter could be due to "consumption coagulopathy" or various other anticoagulant actions of the venom. In our first case there was blood in the DSF. There could have been bleeding as well as thrombosis in the pituitary gland and its blood vessels.

In summary, three cases of snake bites, most probably by vipers, with resultant bleeding disorder came back to the hospital with clinical features of panhypopituitarism. Their case histories and biochemical data have been presented. Probable causes of the occurrence of such disorder have been discussed.

ACKNOWLEDGMENT

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Neostigmine in the Treatment of *Elapidae* Bites

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INTRODUCTION

A high rate of mortality in the patients brought to the hospital with neuroparalytic snake venom poisoning, a close resemblance of the clinical picture to that of myasthenic crisis or curare poisoning, and a dramatic response to neostigmine therapy in our first case prompted us to undertake a therapeutic evaluation of anticholinesterase in *Elapidae* venom poisoning. In a preliminary communication, (Banerjee *et al.*, 1972), we reported an unequivocal benefit of neostigmine in such cases. The present communication describes the results of an extended study in 32 cases of *Elapidae* bite treated in our hospital during the trial period 1971-1973.

MATERIALS AND METHODS

Clinical Materials. Thirty-two cases admitted in the hospital with (1) definite history of snake bite, (2) characteristic fang marks, and (3) signs of neuromuscular paralysis or cardiotoxicity, were studied.

Methods of Study. Therapeutic evaluation was carried out by comparing the results of a trial regimen of neostigmine-atropine to that of a control group treated by the conventional methods only. For the purpose of objective assessment, the patients were treated in six independent medical and pediatric units of the hospital and the results of the treatment were analyzed at the end of the

study period of two years. The patients were randomly selected in the two treatment groups, viz., the control and the trial groups, by the respective treating units.

Treatment Groups

Control Group. Ten patients were treated with intravenous administration, according to the recommended schedule, of 40–60 ml of polyvalent antivenin antiserum obtained from the Central Research Institute, Kasauli, India. Besides antiserum, the patients received intensive symptomatic care by oxygenation through endotracheal intubation or tracheostomy, with the positive-pressure respirator of Bird's type, corticosteroid, fluid, and electrolyte infusion, and by administration of pressure amines, dieuretic, and antibiotic as indicated.

Trial Group. Twenty-two patients were given a protocol regimen of neostigmine–atropine therapy, besides the conventional treatment as given in the control group. According to the schedule, the patients received five intravenous injections each of 0.5 mg neostigmine, at half-hour intervals, followed by injections at the same doses at increasing intervals of 2–12 hr, according to the state of recovery. Each dose of neostigmine was preceded by an intravenous administration of 0.6 mg atropine sulfate so as to ensure a rise in the pulse rate by about 20 beats per minute. The treatment was continued until the termination of the illness either in complete recovery or death.

RESULTS

Clinical Features. On the basis of the clinical presentations, 29 cases were diagnosed as the neurotoxic type, showing various degrees of focal, and generalized neuromuscular paralyses. The types and the incidence of various neurotoxic manifestations are shown in Table 1. The largest proportion of the cases (72.4%) presented ptosis and respiratory paralysis. About 38.0% of the cases developed ophthalmoplegia of various degrees. In about one-third of the patients (34.4%), palatal and pharyngeal paralyses were observed; 38.0% of the cases developed coma. The flaccid limb paralysis was noted in 24.1%. The onset of the neurotoxic signs was observed within 20 minutes to 15 hr after the bite. Three cases presented sudden hypotension, cardiac arrythmia and cardiac asystole within 30 min to 2 hr after the bite. The cases were diagnosed as cardiotoxic type.

Therapeutic Results. Results of treatment of 32 cases by the two respective regimens are shown in Table 2.

1. The mean intervals between the bite and the onset of the treatment in the control and the trial groups of neurotoxic cases were 2 hr and 40 min and 4 hr

Table 1. Clinical Presentations in Thirty-Two Cases of *Elapidae* Bite

Symptomatology	Number of cases	Percentage
Neurotoxic group (29 cases)		
Ptosis	21	72.4
Respiratory paralysis	21	72.4
Ophthalmoplegia	11	37.9
Coma	11	37.9
Palatal paralysis	10	34.4
Pharyngeal paralysis	10	34.4
Flaccid limb paralysis	7	24.1
Convulsions	3	10.3
Cardiotoxic group (3 cases)		
Hypotension	3	100
Cardiac arrest	3	100
Coma	3	100

and 15 min, respectively. In the cardiotoxic cases, the patients received treatment within 2 hr after the bite.

2. Survival rate: in the control neurotoxic group the survival rate was only 22.3% (2 out of 9 cases), while in the trial group it was found to be as high as 95.0% in the recovery of 19 out of 20 patients. All the three patients presenting cardiotoxicity, however, ended fatally irrespective of the treatment regimen.

3. Speed of recovery: the clinical course as assessed by the speed of recovery was observed to be significantly faster in the trial group. A mean recovery period of 20.5 hr with a range of 10 min to 48 hr for a complete recovery was observed with neostigmine therapy. In the control group, the progress in the two surviving cases was considerably slower, being 3 and 7 days.

Table 2. Therapeutic Results in Thirty-Two Cases of *Elapidae* Bite

Treatment group	Number of cases	Survival rate		Recovery period, Mean, range
		Number of cases	Percentage	
Neurotoxic group				
Control	9	2	22.3	3 and 7 days
Trial (Neostigmine regime)	20	19	95.0	20.5 hr, (10 min–48 hr)
Cardiotoxic group				
Control	1	–	–	–
Trial	2	–	–	–

4. Specificity of neostigmine response: the specificity of neostigmine therapy was clearly observed in the response pattern of four cases, viz., case numbers 1, 4, 6, and 9 (Fig. 1). In case numbers 1 and 4, the patients presenting respiratory paralysis and coma did not show any sign of improvement during the initial 6–8 hr after intensive conventional treatment. However, on introduction of the neostigmine regimen, the response was noted almost immediately in case 1 and within $1^1/_2$ hr in case 4. The patients made complete recovery in 10 min and

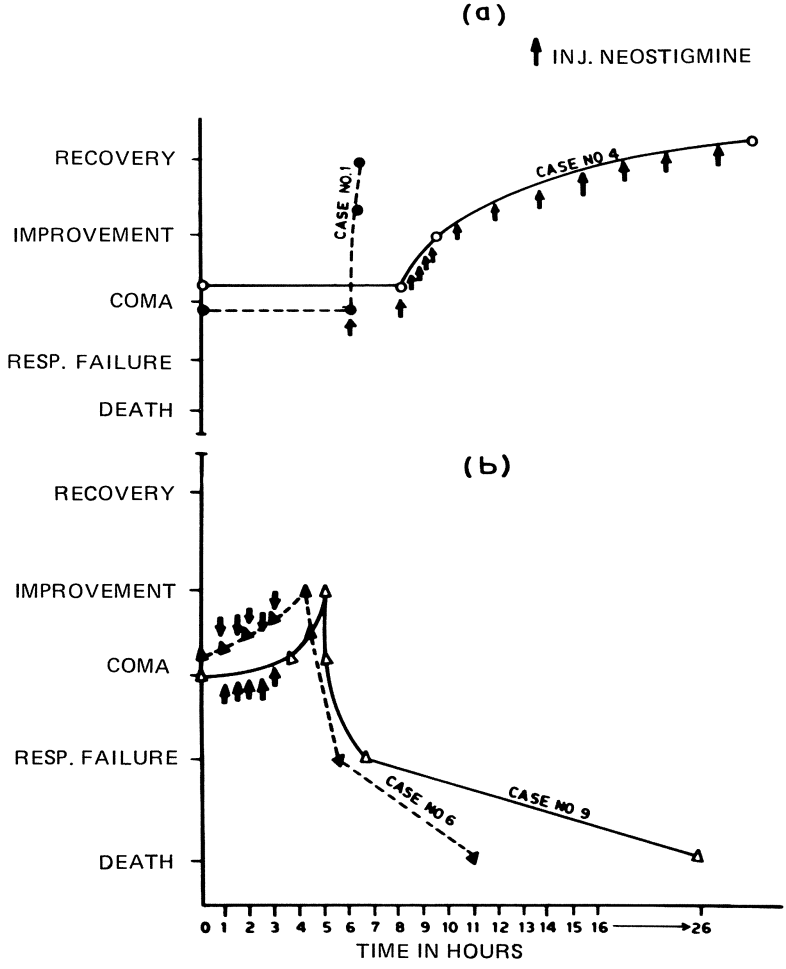

Fig. 1. Therapeutic specificity of neostigmine showing the effects of administration (cases 1 and 4) and withdrawal (cases 6 and 9) of the drug in neuroparalytic snake venom poisoning. Arrows indicate the injections of neostigmine.

12 hr, respectively. Contrary to these observations, cases 6 and 9 showed marked improvement by the initial neostigmine therapy. Neostigmine was then discontinued after the initial five injections. In both the cases, the patients relapsed into respiratory paralysis and expired after 6 and 20 hr, inspite of the intensive symptomatic treatment.

5. Side effects and toxicity of neostigmine: the trial regimen of neostigmine-atropine was found to be completely safe and no side effect or toxicity whatsoever was observed.

DISCUSSION AND CONCLUSION

The results of the present study provide a therapeutic evaluation of the current practice of passive immunotherapy and a trial regimen of supplementary neostigmine in the treatment of *Elapidae* venom poisoning.

The limitation of passive immunotherapy with any antiserum is well known. The therapeutic effect of any such treatment is restricted to immunological neutralization of the circulating toxin of the specific antigenicity and has no pharmacological action against the toxic effects brought about by the absorption of the toxin by the target tissue. With the polyvalent antivenin antisera in current use, this problem is particularly important because of the low molecular size, poor antigenicity, and rapid lethal absorption of the major offending toxin, the neurotoxin (NT), in cases of elapid venom poisoning (Carey and Wright, 1960; Moroz *et al.*, 1967). In the present study, such limitation of antiserum therapy has been confirmed in the progressive neuroparalytic symptoms and a high mortality (77.7%) in the control group of cases treated with the antiserum and symptomatic care only. The results therefore, leave room for making search of some specific pharmacological agents to counteract the toxic effects of the venom at the tissue level.

The elapid venoms have long been recognized for their characteristic neurotoxic and cardiotoxic actions. Although for over 40 years the neurotoxicity of the venom has suggested a curarelike action for some of its toxic fractions, no therapeutic evaluation of anticholinesterase has been reported so far. The present study convincingly shows the therapeutic effectiveness of neostigmine in a higher survival rate (95.0%) and a faster pace of recovery in the trial group of neurotoxic cases. The specificity of neostigmine is also proved by the response pattern obtained either by its withdrawal or introduction at appropriate clinical stages in some of the selected cases.

This therapeutic action of neostigmine can now be well explained on the basis of the current toxicological information.

It is now largely established that the major toxins in elapid venom fall into one of the three groups, two of which are neurotoxins and the third a cardiotoxin.

Of the two neurotoxins, the first group, to which the cobra neurotoxin, α-bungarotoxin and most of the elapid venoms belong, produce an antidepolarizing neuromuscular block by acting on the postjunctional membrane of the motor endplate, similar to *d*-tubocurarine (Chang and Lee, 1963; Lee and Tseng, 1966; Lee *et al.*, 1967, 1969). The blockade produced by venoms of this group can be reversed by neostigmine (Su *et al.*, 1967). The reported results of the electrophysiological studies also indicate that the effect of cobraneurotoxin as well as α-bungarotoxin on neuromuscular transmission is essentially the same as that of *d*-tubocurarine (Chang and Lee, 1968).

The second group of neurotoxin, as found in β-bungarotoxin, produces the neuromuscular block by acting presynaptically on the motor nerve ending, leaving the sensitivity of the endplate to acetylcholine unaffected (Chang and Lee, 1963; Lee *et al.*, 1969). In this respect, the toxin is similar to that of botulinium toxin and is nonresponsive to anticholinesterase.

The third group of elapid toxin is the cardiotoxin (CT), which is now known to be identical to the earlier-reported fraction of DLF (Slotta and Vick, 1969). The primary action of CT is depolarization of the cell membrane of the skeletal, cardiac, and smooth muscles, nerves and the neuromuscular junction (Lee *et al.*, 1968), resulting in profound shock, hypotension, respiratory and cardiac arrest. Neostigmine would necessarily have no action whatsoever against the toxin.

In the light of such information, the observed therapeutic benefit of neostigmine in 95% of the treated neurotoxic cases in the present series would be explained by its anticurare action against the predominant cobra neurotoxin and α-bungerotoxin, while the solitary fatal neurotoxic case (Case 29) could be one due to β-bungarotoxin. The treatment is ineffective in cardiotoxic cases.

In conclusion, the present study establishes the role of neostigmine as a specific therapeutic agent in the treatment of cases of neurotoxic snake venom poisoning. The fact that cobra neurotoxin and α-bungarotoxin form the major toxic component in most of elapid venom poisoning and that as yet we have no laboratory test to make a specific toxicological diagnosis in the clinical management, suggest that neostigmine-atropine should be employed as a routine supplementary treatment in managing cases of neuroparalytic snake venom poisoning.

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Renal Involvement in Snakebite

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INTRODUCTION

Renal lesions in snakebite vary widely. Glomerulonephritis is occasionally observed (Acharya *et al.*, 1972), sometimes associated with nephrotic syndrome (Steinbeck, 1960). Renal infarction has been described (Raab and Kaiser, 1966). However, renal manifestation in man is mostly in the form of renal failure either due to acute tubular necrosis (Amorim and Mello, 1954) or cortical necrosis (Silva *et al.*, 1966; Oram *et al.*, 1963). Recently we reported acute renal failure in patients bitten by sea snakes and Russell's vipers with histological and laboratory details (Sitprija, *et al.*, 1971; Sitprija *et al.*, 1973). The purpose of this article is to review the available data in the literature on renal involvement in snakebite and to present our additional experience from further study.

Literature Review. The effects of snake venoms on the kidney are quite complex. Amorim and Mello (1954) reported pathological findings at autopsy in three patients bitten by crotal snakes. Tubular necrosis was shown in all cases. Danzig and Abels (1961) described a patient bitten by a crotal snake, who collapsed, convulsed, and developed acute renal failure. The patient was successfully treated by hemodialysis. There was no pathological diagnosis, but the clinical course resembles that of tubular necrosis. Similar findings and results were experienced by Frazier and Carter (1962). In a series of 15 patients bitten by poisonous snakes reported by Visuvaratnam *et al.* (1970), renal failure was observed in four cases. The clinical symptoms included drowsiness and restlessness. There was hemoglobinuria. The snakes were, however, not identified. Silva *et al.* (1966) noted acute tubular necrosis in three patients bitten by *Crotalus terrificus* and cortical necrosis in one patient envenomated by *Bothrops*

Table 1. Summary of Clinical Data on Renal Failure Due to Snakebite

Patient	Snake	Clinical findings	BUN mg/100 ml	Creatinine mg/100 ml	Oliguria	
					Onset	Duration
58 M	*Crotalus terrificus*	Hematuria	–	–	–	–
30 M	*Crotalus terrificus*	Dizziness, hypotension, hematuria, hemoglobinuria	–	–	–	–
12 M	*Crotalus terrificus*	Hematuria, hemoglobinuria	–	–	–	–
11 M	Rattlesnake	Collapse, convulsions, hematuria	150 (NPN)	–	–	–
33 M	*Crotalus ruber*	Opisthotonos, pinpoint pupils, hematemesis, hematuria	138	–	2nd day	18 days
50 F	*Echis carinatus*	Hematemesis, ecchymosis	206	–	2nd day	21 days
(3 cases)	*Crotalus terrificus*	Intravascular hemolysis	–	–	–	–
(1 case)	*Bothrops jararaca*	Afibrinogemia	–	–	–	–
20 M	*Cryptophis nigrescens*	Muscular pains and weakness, myocrosis, myoglobinuria	94	–	–	–
22 M	Russell's viper	Hypotension, general paresis, ptosis, hemoglobinuria	69	–	no oliguria	
49 M	Unidentified	Drowsiness, restlessness, ptosis, hemoglobinuria	338	–	–	11 days
58 M			308	–	–	13 days
63 M			242	–	–	8 days
32 M			182	–	–	4 days
30 M	*Agkistrodon hypnale*	Local swelling	100	–	8th day	18 days
(3 cases)	Sea snakes	Muscular pains and paresis, ptosis, trismus, dilated pupils, myonecrosis, myoglobinuria	–	–	–	–
19 M	Sea snake	Muscular pains and paresis, trismus, myonecrosis, myoglobinuria	80	5	1st day	6 days
22 M	Sea snake	Same	100	5.1	1st day	10 days
49 M	Russell's viper	Hematemesis, bleeding from gum, hematuria	124	5.5	1st day	7 days
60 F	Russell's viper	Hematemesis, ecchymosis	103	5.5	1st day	6 days

Table 1. (*Continued*)

Duration of renal failure	Renal lesion	Outcome	Remark	Reference
–	Tubular necrosis	Death	–	Amorim and Mello, 1954
–	Tubular necrosis	Death	–	
–	Tubular necrosis	Death	–	
–	–	Recovery	Antivenom, corticosteroid, and hemodialysis	Danzig and Abels, 1961
–	–	Recovery	Hemodialysis	Frazier and Carter, 1962
–	Cortical necrosis	Improved	Hemodialysis	Oram *et al.*, 1963
–	Tubular necrosis	Recovery	Dialysis	Silva *et al.*, 1966
–	Cortical necrosis	Death	Dialysis	
–	Tubular necrosis	Death	–	Furtado and Lester, 1968
–	–	Recovery	Exchange transfusion	Peiris *et al.*, 1969
34 days	–	Recovery	Peritoneal dialysis	Visuvaratnam *et al.*, 1970
32 days	–	Recovery	Peritoneal dialysis	
28 days	–	Recovery	–	
19 days	–	Recovery	–	
–	Cortical necrosis	Death	Peritoneal dialysis	Varagunam and Panabokke, 1970
–	Tubular necrosis	1 Recovery 2 Deaths	Polyvalent antivenom and corticosteroid	Marsden and Reid, 1961
26 days	Tubular necrosis	Recovery	Hemodialysis	Sitprija *et al.*, 1971
24 days	Tubular necrosis	Recovery	Hemodialysis	
19 days	Tubular necrosis	Recovery	–	Sitprija *et al.*, 1973
17 days	Tubular necrosis	Recovery	–	

jararaca. Cortical necrosis has also been shown after the bite by *Agkistrodon hypnale* (Varagunam and Panabokke, 1970), and Echis carinatus (Oram *et al.*, 1963).

Peiris, *et al.* (1969) reported on clinical improvement by exchange transfusion in a patient bitten by a Russell's viper. In the clinical course renal failure was noted. Furtado and Lester (1968) have shown tubular necrosis at autopsy in their patient bitten by a small-eyed blacksnake (*Cryptophis nigrescens*).

Of nine cases of sea-snake bite studied by Marsden and Reid (1961) tubular necrosis was noted in three patients. Recently renal failure in sea-snake poisoning attributed to myoglobinuria resulted from myonecrosis has been described by Sitprija *et al.* (1971). Muscular symptoms including pains, paresis or weakness, trismus, and ptosis were remarkably improved by hemodialysis.

The clinical and laboratory data in the 24 reported cases of renal failure in snakebite are summarized in Table 1.

Clinical Pictures. Of 24 patients age and sex were described in only 17, whose age ranged from 11 to 63 years with an average of 34 years. Fifteen were male and two were female. The nature of the snakes was unknown in four patients. The blood pressure was within the normal range except for three patients. Two of these patients were bitten by crotal snakes (Amorim and Mello, 1954; Danzig and Abels, 1961) and the other by Russell's viper (Peiris *et al.*, 1969). Muscular symptoms including pains and paresis or weakness were noted in seven cases, five of which were sea-snake cases (Marsden and Reid, 1961; Sitprija *et al.*, 1971); the other two were a case of Russell's viper bite (Peiris *et al.*, 1969) and a case of small-eyed blacksnake bite (Furtado and Lester, 1968). Ptosis was observed in eight patients, three of whom were bitten by sea-snakes (Marsden and Reid, 1961), one by a Russell's viper (Peiris *et al.*, 1969), and in the other four by unidentified snakes. The size of pupils varied. In three cases of sea-snake bite pupils were dilated (Marsden and Reid, 1961), and in one case of crotal-snake bite pupils were pinpoint (Frazier and Carter, 1962). Drowsiness and restlessness were present in four patients. Convulsions were found in two cases; both were bitten by crotal snakes (Danzig and Abels, 1961; Frazier and Carter, 1962).

Bleeding symptoms included hematuria, hematemesis, and ecchymosis of the skin. Gross hematuria was most common, being noted in five patients bitten by crotal snakes and Russell's viper. Disseminated intravascular coagulation was present in three cases. Afibrinogenemia was seen in one patient bitten by *B. jararaca*. Evidence of mild to severe degree of intravascular hemolysis was observed in 10 cases.

In most cases the onset of renal failure was not known. In four cases oliguria was noted on the first day of the bite. In three cases oliguria was observed 2-8 days after the bite. The blood urea nitrogen was detected in 13 cases and ranged

from 69 to 338 mg/100 ml. In one case the nonprotein nitrogen was 150 mg/100 ml. The serum creatinine in four cases ranged from 5.0 to 5.5 mg/100 ml. The duration of oliguria in 11 cases varied from 4 to 21 days. In one patient oliguria was never observed. The duration of renal failure was documented in eight cases and ranged from 17 to 34 days. Hemoglobinuria was noted in 10 patients. Myoglobinuria was observed in six cases.

Management and Outcome. Symptomatic treatment was instituted in all patients. In four patients antivenom was also given. It did not appear from the data that antivenom therapy prevented renal failure. Twelve patients underwent either hemodialysis or peritoneal dialysis for renal failure. One patient had exchange transfusion.

Of twenty-four patients, sixteen recovered. One of these patients had cortical necrosis and made only partial recovery since the renal function remained impaired when the patient was discharged from the hospital. Eight patients expired, giving a mortality rate of 33%. Of the sixteen patients who survived, ten were treated by dialysis, another had exchange transfusion, and the other five had only conservative treatment. Survival rate bore good relationship to dialysis.

MATERIALS AND METHODS

Eight patients bitten by poisonous snakes were studied. Six were male and two were female ranging in age from 23 to 46 years. Five patients were bitten by green pit vipers, and three patients were bitten by Russell's vipers. The patients were admitted to the hospital 1-6 days after the bite. One patient was given antivenom (anti-Russell's viper) prior to hospitalization. In addition to the routine laboratory and hematological studies (Sitprija *et al.*, 1973), blood was drawn on the first hospital day to be examined for β1C globulin, plasma hemoglobin, and fibrin degradation products (FDP).

After intravenous pyelography, percutaneous renal biopsy was performed during the period from the 9th to 26th day after the bite, after the bleeding episode was over, and when the general condition permitted. Two portions of renal tissue were obtained in each biopsy. One portion was fixed in Zenker's formal solution and stained with hematoxylin and eosin and the periodic acid-Schiff reaction for light microscopy. The other portion was quickly frozen in a dry ice-isopentane mixture for immunofluorescent study. Staining was made for human IgG, IgM, IgA, β1C globulin, fibrin, and fibrinogen by applying the appropriate conjugates onto the sections which have been washed with buffered saline.

RESULTS

Clinical Observations. The clinical and laboratory data are summarized in Table 2. Bleeding symptoms attributed to disseminated intravascular coagulation were found in all patients (Sitprija *et al.*, 1973). Gross hematuria was a common renal manifestation. Renal failure was observed only in Russell's viper cases. The serum creatinine ranged from 12.2 to 14.6 mg/100 ml. In green pit viper cases the serum creatinine varied from 0.6 to 0.9 mg/100 ml. All patients displayed varying degrees of intravascular hemolysis with the plasma hemoglobin ranging from 2.0 to 6.2 mg/100 ml. The serum β1C globulin ranged from 66 to 81 mg/100 ml, rising to the normal level 1-2 weeks later.

Renal Pathological Changes

Russell's Viper Bite. Glomerular changes were not remarkable. There was only mild mesangial proliferation (Fig. 1). Intraglomerular fibrin deposit was not observed. In contrast, tubular changes were striking. Tubular necrosis was observed in all cases (Fig. 2). The lesions did not differ from those previously described (Sitprija *et al.*, 1973). Of interest is the vascular lesion. Necrotizing arteritis of the medium-sized arteries was noted (Fig. 3). There was deposition of eosinophilic material in the necrotic wall. Venous damage was also observed. The venous wall showed necrosis and the lumen was occluded by platelet thrombus and inflammatory cells (Fig. 4). Interstitial changes consisted of edema and cellular infiltration.

The immunofluorescent study revealed granular deposition of β1C globulin in the glomerular mesangium and in the arteriolar and capillary walls (Fig. 5).

Table 2. Laboratory Data[a]

Patient	Age	Sex	Snake	BUN	Creatinine	FDP	β1C globulin	Plasma hemoglobin
1	30	M	Green pit viper	10	0.8	2.8	74	2.1
2	24	M	Green pit viper	9	0.9	3.6	80	3.6
3	33	M	Green pit viper	12	0.6	4.4	69	4.2
4	24	F	Green pit viper	11	0.7	2.4	72	2.0
5	30	F	Green pit viper	11	0.6	1.2	81	4.4
6	23	M	Russell's viper	190	13.1	2.1	78	2.0
7	46	M	Russell's viper	150	14.6	4.1	70	4.0
8	41	M	Russell's viper	110	12.2	6.4	66	6.2

[a]Data in mg/100 ml.

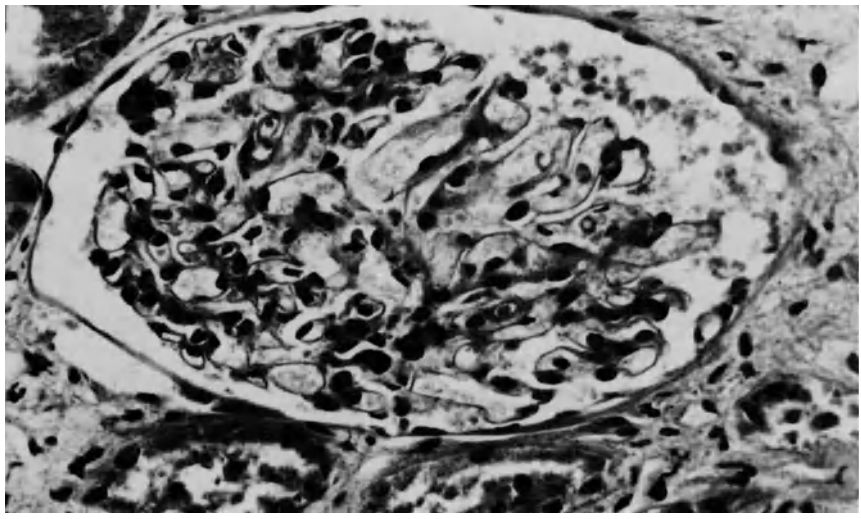

Fig. 1. A glomerulus showing a relatively normal number of cell component and mild mesangial cell proliferation. Some capillary lumens are dilated (hematoxylin-eosin, 222×).

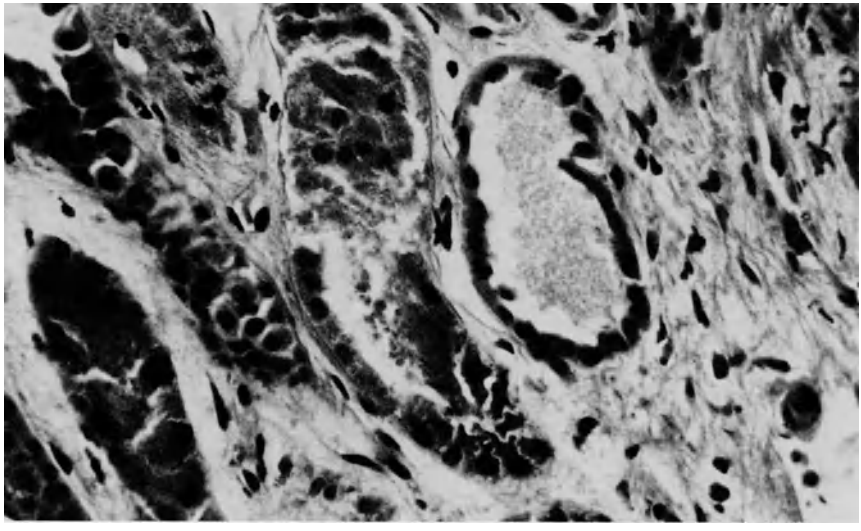

Fig. 2. Necrosis of proximal tubules and interstitial edema are demonstrated (hematoxylin-eosin, 222×).

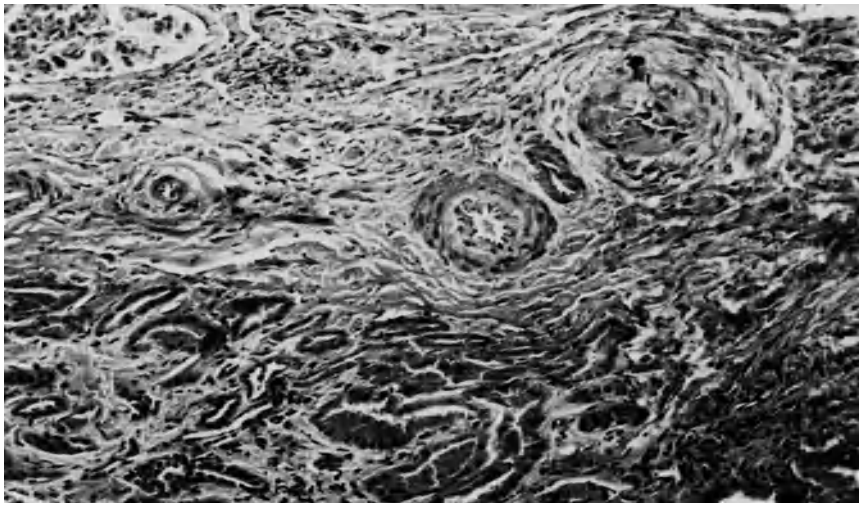

Fig. 3. A renal cortical area showing two segments of the interlobular artery. One of them shows necrotizing arteritis with deposition of eosinophilic homogeneous material in the necrotic wall (hematoxylin–eosin, 88×).

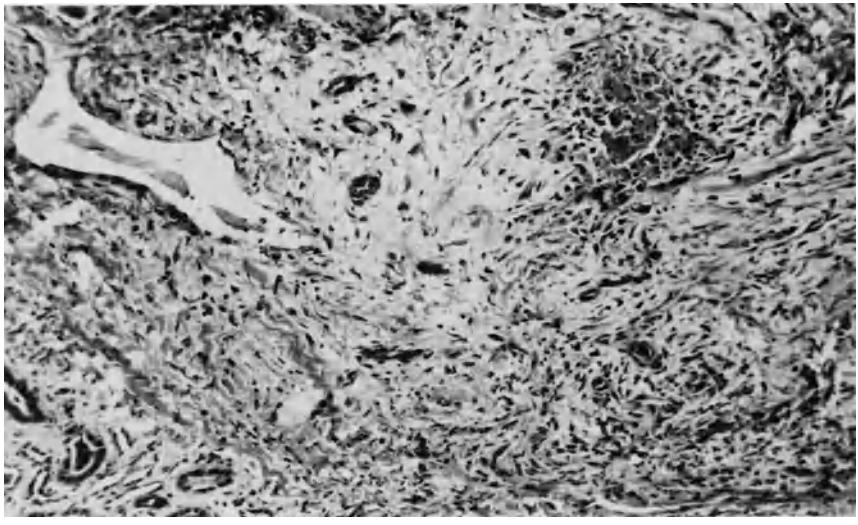

Fig. 4. A renal corticomedullary area showing necrotizing thrombophlebitis of an interlobular vein. Tubular atrophy and interstitial fibrosis are noted (hematoxylin–eosin, 88×).

Fig. 5. A glomerulus with an arteriole showing granular deposition of β1C globulin in the arteriolar wall, in mesangial areas and in the glomerular capillary wall (fluorescent anti-human β1C, 222×).

Deposition of β1C globulin was also observed in the wall of the interlobular artery (Fig. 6). There was no deposition of immunoglobulins, fibrin or fibrinogen in glomeruli or blood vessels.

Green Pit Viper. There was mild mesangial proliferation with β1C globulin deposit in the glomeruli and arteriolar walls. Fibrin deposit could not be demonstrated. The interstitium, tubules and blood vessels showed no pathological changes.

DISCUSSION

Clinical Symptoms. All patients had common bleeding symptoms attributed to disseminated intravascular coagulation. Intravascular hemolysis was of minor degree. Of interest is the decrease in serum β1C globulin. Although the decrease in this complement fraction might be related to disseminated intravascular coagulation since both plasmin and thrombin are capable of activating complement (Bokisch, *et al.*, 1969; Donaldson, 1968), it is possible that the reduction was due to activation of the complement system by snake venom. Despite several common symptoms shared by the two groups of patients renal failure was observed only in Russell's viper bite.

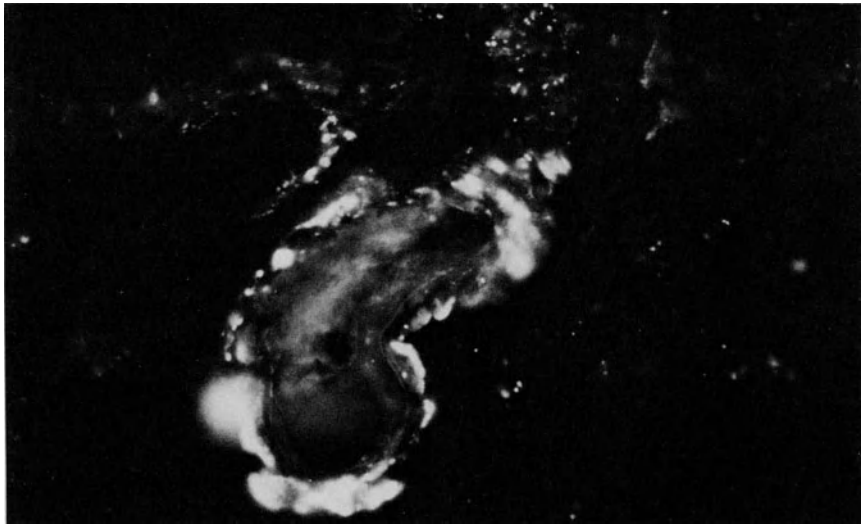

Fig. 6. Granular deposition of β1C globulin in the wall of an interlobular artery and in the peritubular capillaries (fluorescent antihuman β1C, 352 ×).

Renal Pathology

Tubulo-Interstitial Changes. In all Russell's viper cases tubular necrosis was observed. The interstitial lesions consisted of edema and cellular infiltration. The findings were in accord with those in the previous reports. In green pit viper cases these changes were not observed.

Glomerular Changes. In animal experiments glomerulitis with fibrin deposit has been demonstrated when the kidney was exposed to viper venom (Jerushalmy *et al.*, 1970). In experiments in rabbits with the venom of *C. adamanteus*, Pearce (1909) found glomerular changes of both hemorrhagic and exudative natures. The hemorrhagic lesion confined mostly to the glomerular tuft, while the exudative lesion involved the capsular space. He believed that the venom gave a direct injurious effect to the endothelial cells. Such effect was previously described by Flexner and Noguchi (1902). While the glomerular changes might be due to the toxic effect of the venom, it might also represent a reaction to fibrin deposit due to intravascular coagulation.

Although glomerulonephritis and nephrotic syndromes have been described in snakebite, this has not been our experience. In our patients the glomerular changes, in general, were not remarkable. There was only mild mesangial proliferation. Fibrin deposit was not observed despite the evidence of intravascular coagulation. In dog experiments with crotoxin, Hadler and Brazil (1966) observed glomerular congestion and deposit of PAS-positive material between

capillary loops during the first 4 days. The lesions disappeared after the fourth day. Failure to observe the glomerular changes in our cases might be due to the delay in performing renal biopsy, since the biopsy was performed 9–26 days after the bite. Of interest is the deposit of β1C globulin in the mesangial area in all cases. Immunoglobulins could not be detected.

Necrotizing arteritis was observed only in cases bitten by Russell's vipers. Because of the deep location of the arterial lesion in the renal cortex this could have been missed in the previous biopsies (Sitprija *et al.*, 1973). There was β1C deposit in the arterial wall. No immunoglobulin or fibrin deposit was noted in the lesion. Arteritis was not present in green pit viper bite. The arterioles and capillaries showed no changes. However, there was deposition of β1C globulin in the arteriolar wall. Findings in our cases differ from those observed by Efrati and Reif (1953) and McKay *et al.* (1970), who described capillary and arteriolar changes characterized by endothelial swelling and tearing of the wall with hemorrhage.

Deposition of a complement in the arterial wall without immunoglobulins along with the drop in serum complement suggests the nonimmunologic activation of the complement system through the alternate pathway (Bruninga, 1971; Ruddy *et al.*, 1972), and the viper venom could be the activator.

The mechanism responsible for renal failure in Russell's viper bite is not well understood. Since there were bleeding, intravascular hemolysis, disseminated intravascular coagulation, and arteritis, renal failure could have been caused by multiple factors. However, Raap and Kaiser (1966) have shown that snake venom has a direct cytotoxic effect on the renal tubular cells. In a study on crotoxin, Hadler and Brazil (1966) attributed renal tubular lesions partly to the effect of lysolecithin formed by the activity of phospholipase. It is thus possible that renal failure might also be explained by the direct nephrotoxic effect of the venom. Under similar clinical and laboratory settings the presence of renal failure in Russell's viper bite and its absence in green pit viper bite suggest nephrotoxicity of Russell's viper venom.

Management. The management of renal failure in snake bite does not differ from that due to other causes. Dialysis is indicated when uremia ensues. Recovery is usually complete unless there is cortical necrosis. Because of the high molecular weight of the venom (Efrati and Reif, 1953), and the rapid tissue fixation, dialysis would not remove the venom from the circulation and would not improve the clinical symptoms except uremia. However, in sea-snake poisoning dramatic improvement of muscular symptoms by hemodialysis has been observed (Sitprija *et al.*, 1971). The improvement was believed to be due to the correction of hyperkalemia which aggravated the myotoxic effect of sea snake venom.

From the pooled data (Table 1), including our earlier experience, antivenom therapy does not seem to prevent renal failure. This could reflect the delay in its

institution. Conventional treatment to prevent renal failure should be emphasized. This can be accomplished by prompt fluid load and diuretic therapy in any patient bitten by a snake known to cause renal failure. Alkalinization of urine should be attempted when there is hemoglobinuria or myglobinuria. Heparin should be given when there is disseminated intravascular coagulation with active bleeding.

SUMMARY

With the use of the immunofluorescent technique, renal biopsy study was made in eight patients. Three patients were bitten by Russell's vipers, and the other five bitten by green pit vipers. Gross hematuria was a common renal manifestation, but renal failure was observed only in Russell's viper cases. In green pit viper bite the glomerular function was normal. There was intravascular hemolysis of varying degrees. Of interest was the decrease in serum β1C globulin. In Russell's viper cases, apart from tubular necrosis, arteritis was observed in medium size arteries. The glomeruli showed mild mesangial proliferation. There was no change in the arteriole. By immunofluorescence, deposition of β1C globulin was demonstrated in the arterial and arteriolar walls and in the glomerular mesangium. Immunoglobulins and fibrin could not be detected. In green pit viper cases no histological changes were observed except for mild mesangial proliferation. However, deposition of β1C globulin in the arteriolar wall and glomeruli was demonstrable.

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Anticoagulation in Hemorrhagic Snake Venom Poisoning

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INTRODUCTION

High mortality in hemorrhagic snake venom poisoning continues to be an important problem in medicine. Nearly two centuries ago, hemorrhagic snake venoms were classified as coagulant and anticoagulant in their toxic action. In recent years, two major mechanisms causing the coagulation disorders of snake venom poisoning have been suggested. In crotalid venom, the typical coagulant effect has been attributed to its thrombinlike action (Macfarlane, 1961, 1967; Williams and Esnouf, 1962; Meaume, 1966), while in viperid venom, the prototype of Russell's viper venom, an activation of factor X followed by conversion of prothrombin to thrombin in the presence of factor V, platelet, and Ca^2 is reported to be responsible for the toxic hemorrhagic manifestations (Macfarlane, 1961, 1967; Williams and Esnouf, 1962; Esnouf and Williams, 1962; Nahas *et al.*, 1964; Meaume, 1966; Schiffman *et al.*, 1969). In either case, however, the pathogenesis of the hemorrhagic manifestation would be one of defibrination syndromes resulting in intravascular coagulation and a massive consumption of fibrinogen. Such a pathological state logically calls for a specific therapeutic approach to prevent intravascular clotting and rapid fibrinogen replacement in the treatment of the disorder.

The present study was undertaken to determine the predominant nature of the coagulation defect in the hemorrhagic snake bite in northern India, around Delhi, and also to evaluate the role of anticoagulation and fibrinogen replacement in their management.

MATERIALS AND METHODS

Clinical Materials. Twenty cases admitted to Safdarjang Hospital, New Delhi, which has a long history of treating snake bite, presenting characteristic fang marks and hemorrhagic manifestations, were included in the study.

Investigations. In all cases, the following hematological investigations were carried out by the standard methods (Dacie and Lewis, 1968): (1) hemoglobin estimation (g/per 100 ml); (2) bleeding time (Ivy method); (3) coagulation time (Lee and White); (4) one stage prothrombin time (Quick); (5) partial thromboplastin time with kaolin; (6) thrombin time with bovine thrombin (Parke and Davis); (7) plasma fibrinogen estimation (Ratnoff and Menzie, 1951); and (8) Platelet count (Rees-Ecker). In the anticoagulated patients, the follow-up coagulation studies were carried out after *in vitro* protamine sulfate neutralization of the circulating heparin.

Treatment Group. All patients on admission were given the recommended dose of 40-60-ml polyvalent antivenin, obtained from Central Research Institute Kasauli. For further management the patients were divided into two groups.

Control Group. Four patients were treated with whole blood transfusions with symptomatic treatment only.

Trial Group. Sixteen patients were anticoagulated with rapid intravenous heparinization with 15,000 units, followed by 5000-10,000 units every six hours. In fourteen cases (trial group A) the patients were given 300-600 ml of intravenous fibrinogen (300 mg per 100 ml), with supportive blood transfusion only in cases with severe blood loss. In the remaining two cases (trial group B), the patients received no fibrinogen but only whole blood transfusions.

Follow-Up Studies. Daily clinical and hematological examinations were carried out in all cases, until the termination of the illness.

RESULTS

Clinical Presentations

Interval between the Bite and the Appearance of Toxic Symptoms. A mean interval of 15 hr with a range of 2-24 hr was observed. This interval is significantly longer than that observed in the neurotoxic cases (Banerjee *et al.*, 1972).

Symptomatology. The incidence of varied symptomatology observed in twenty cases is shown in Table 1. All patients presented prolonged bleeding from the fang marks. In 70.0% of the cases, echymosis, purpura, and large hematoma appeared in different parts of the body. Painful cutaneous hemorrhagic manifestations of purpura fulminans or purpura gangrenosa type was observed in 25.% of the cases. Hematuria of microscopical or frank nature was observed in 80.0% of the cases. Of the other systemic presentations, gastrointestinal bleeding, hemoptysis, cerebral hemorrhage, and acute renal failure were important.

Table 1. Clinical Presentations

Symptomatology	Number of cases	Percentage
Bleeding from bite	20	100.00
Ecchymosis, Hematoma, Purpura	14	70.00
Purpura fulminans	5	25.00
Hematuria	16	80.00
Hemetemesis	2	10.00
Melaena	2	10.00
Hemoptysis	1	5.00
Cerebral hemorrhage	1	5.00
Acute renal failure	2	10.00

Hematological Changes before Treatment

Anemia. A severe to mild degree of anemia with hemoglobin ranging from 4-13 g/100 ml was observed. The anemia was characteristically of a posthemorrhagic type (Table 2).

Platelet Count. In 95.0% of the cases (19 out of 20), there was no significant reduction in the platelet count. The values ranged from 120,000 to 300,000 per cm^3. In only one case (case 4), an initial thrombocytopaenia of 90,000 per cm^3 was observed.

Bleeding Time. A normal bleeding time of less than 4 min 30 sec was observed in all the cases.

Coagulation Studies. (a) clotting time (CT)—in all the cases (100.0 per cent) blood was incoagulable up to one hour; (b) one stage prothrombin time (PT)—in 85.0% of the cases (17 cases out of 20), PT was incoagulable up to 5 min, in

Table 2. Hematological Results before Treatment

Investigation	Results in percentage of cases[a]		
	Prolonged	Normal	Decreased
Hemoglobin	–	–	100 (4-13 g/100 ml)
Clotting time	100 (20)	–	–
Prothrombin time	100 (20)	–	–
P.T.T.K.	100 (20)	–	–
Thrombin time	100 (20)	–	–
Plasma fibrinogen	–	–	100 (20) (70-130 mg/100 ml)
Bleeding time	–	100 (20)	–
Platelet count	–	100 (20)	5 (1)

[a]Number of cases in parentheses.

the remaining three cases PT was prolonged to 50-260 sec with a mean of 75 sec, the control being 17 ± 2 sec.; (c) partial thromboplastin time with Kaolin (PTTK)—it was incoagulable up to 5 min in 85.0% of the cases (17 cases), in the remaining three cases it was prolonged to a mean of 175 sec, the control value being 60 ± 7 sec; (d) thrombin time (TT)—all patients presented prolonged thrombin time, in 85.0% of the cases (17 cases), it was incoagulable up to 5 min, in the remaining 3 cases, it was prolonged to 60, 50, and 68 sec, the mean control value being 18 ± 2 sec; (e) plasma fibrinogen—all cases showed marked hypofibrinogenemia, with a mean value of 94.3 mg/100 ml and a range of 70-130 mg/100 ml.

Hematological Changes with Treatment. These results are shown in Table 3 and 4. In the control group (Fig. 1), the coagulation defects in the two surviving cases (cases 1 and 2), were corrected on the 13th and 11th day, respectively. Of the two fatal cases, in case 3, a state of hypofibrinogenemia and prolonged thrombin time continued till death. In case 4, the patient showed a partial correction of the coagulation defect with reduction in the thrombin time and a rise in the plasma fibrinogen concentration up to 185 mg% on the 5th day of the treatment; however, the patient subsequently showed a relapsed hypofibrinogenemia and hypocoagulability till death.

In the trial group A (Fig. 2), all of the 14 cases showed a rapid correction of the coagulation defects, with a steady rise in the plasma fibrinogen level attaining the normal values in 2-5 days, with a mean of 4 days. In trial group B (Fig. 3), on the other hand, in both the cases the state of hypocoagulability and a severe hypofibrinogenemia persisted till the terminal hemorrhagic episodes on the 5th and 3rd day.

Table 3. Serial Thrombin Time Response in Control and Trial Groups of Cases

Treatment group	Days[b]										
	1	2	3	4	5	6	7	8	9	11	13
Control, 4 cases											
Case 1	NC[a]	NC	NC	NC	NC	80	72	50	40	25	19
Case 2	NC	NC	85	70	55	45	40	35	20	20	18
Case 3	NC	NC	NC	NC	NC						
Case 4	68	50	45	–	22	30	35	45	60		
Trial, 16 cases											
Heparin and fibrinogen, mean of 14 cases	NC	35.6	23	16.2	16.3	–	–	–	–	–	–
Heparin and whole blood											
Case 3	NC	NC	NC	120	110						
Case 4	NC	NC	NC								

[a] Incoagulable up to 5 min.
[b] Thrombin time, in seconds.

Table 4. Serial Plasma Fibrinogen Changes in Control and Trial Groups of Treatment

Treatment group	Days[a]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Control, 4 cases													
Case 1	95	90	110	110	115	135	150	145	150	–	180	–	250
Case 2	85	90	92	90	95	110	140	–	155	–	250	–	325
Case 3	70	70	85	90	100								
Case 4	110	125	150	155	185	160	145	130	95				
Trial, 16 cases													
Heparin and fibrinogen, mean of 14 cases	94.3	128	193.3	255	280.5	320.0	–	–	–	–	–	–	–
Heparin and whole blood													
Case 3	94	85	90	85	105								
Case 4	70	70	85										

[a] Plasma fibrinogen, in mg/100 ml.

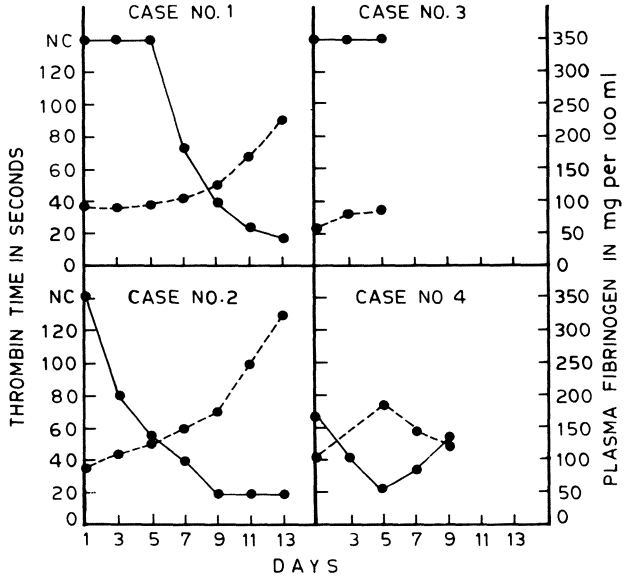

Fig. 1. Thrombin time and plasma fibrinogen response to whole blood transfusion only (control group) showing slow reduction in thrombin time and rise in plasma fibrinogen in two surviving cases (cases 1 and 2); case 4 showed an initial reduction of thrombin time with elevation of plasma fibrinogen followed by a relapsed state of terminal defibrination; case 3 showed no response till death.

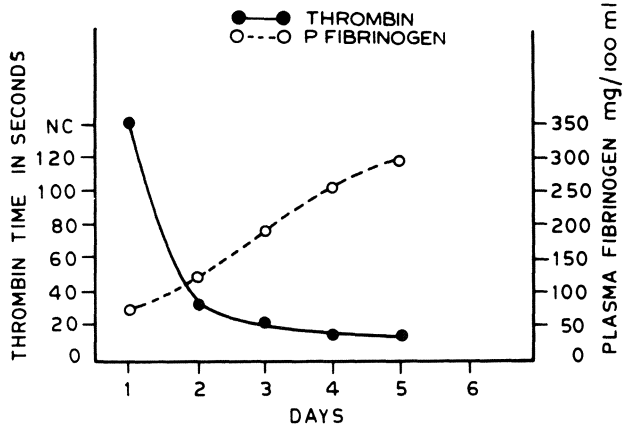

Fig. 2. Thrombin time and plasma fibrinogen response (mean values of 14 cases) to rapid heparinization with fibrinogen replacement therapy (trial group A) showing a rapid correction of defibrination state.

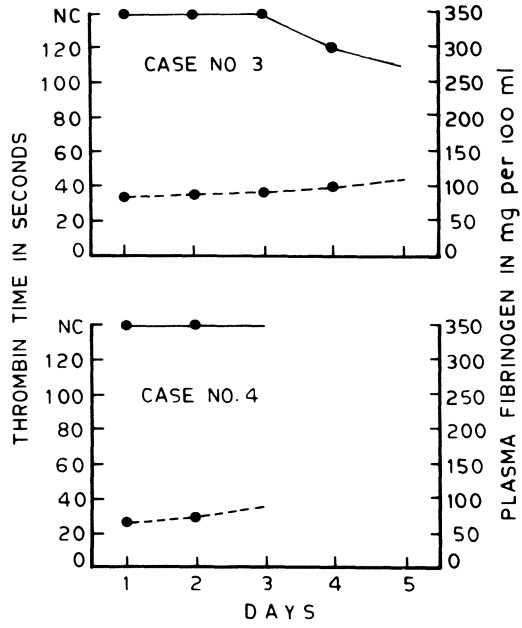

Fig. 3. Thrombin time and plasma fibrinogen response to rapid heparinization with supportive whole blood transfusion only (trial group B), showing inadequate control of the defibrination state in both the fatal cases.

Therapeutic Results

Survival Rate. In the control group, two out of four cases survived. In the trial group A, 100% survival was observed in the fourteen cases that were treated with anticoagulation and fibrinogen transfusion. In trial group B, two cases treated with anticoagulation and whole blood transfusion ended fatally.

Speed of Recovery. In the control group, complete recovery in the two surviving cases were noticed on 13th and 11th day. In trial group A, clinical and hematological recovery was observed to be significantly faster, with a mean recovery period of 4 days and a range of 2-5 days.

Cause of Death. In the control group, both of the fatal cases died of acute renal failure. In trial group B, the cause of death was gastrointestinal and cerebral hemorrhage on the 5th and 3rd day. The fatal hemorrhage in both the cases was attributed to a state of hypofibrinogenemia due to inadequate fibrinogen replacement by the whole blood transfusion, which was evident from the persistent low plasma fibrinogen level (85 and 105 mg/100 ml) till the end (Table 5).

Table 5. Therapeutic Results

Results	Control group	Trial group A	Trial group B
Survival	50% (2)[a]	100% (14)[a]	–
Recovery period	13 and 11 days	Mean 4 days	–
Cause of death	Acute renal failure	–	Hypofibrinogenemic hemorrhage

[a]Number of cases in parentheses.

DISCUSSION

The problem of blood coagulation disorders in snake venom poisoning has been extensively studied in the past. However, treatment of the condition has continued to be polyvalent antiserum administration and whole blood transfusions without specific reference to the nature of the coagulation defect or the pathogenesis of the toxicity. In our experience, the hospital mortality of hemorrhagic snake venom poisoning has been as high as 50%-60% with the conventional treatment. The aim of the present study was, therefore, to explore the possibility of a more specific therapeutic approach in their management.

The hemorrhagic manifestations observed in the present series are well known in viper venom poisoning. However, the incidence of severely painful and coalescent purpura fulminans, accompanied by predominant renal involvement, suggested a clinical resemblance to the reported hemorrhagic state of the Schwartzman phenomenon (Mckay and Wahle, 1955; Shumway and Miller, 1957; Kunzar and Aalam, 1964; Kibel and Bernard, 1964; Sharp, 1964).

The principal coagulation defect in the present series, was found to be a state of defibrination with a prolonged thrombin time and a marked reduction in the plasma fibrinogen concentration. In presence of these defects, the prolongation of the other coagulation parameters, such as clotting time, prothrombin time, and PTTK, were considered to be of secondary nature.

A defibrination syndrome is usually associated with thrombocytopaenia which was, however, conspicuously absent in the present series. The absence of significant thrombocytopaenia would suggest that the offending toxin is likely to be thrombinlike in its action, rather than like the factor X accelerator, which consumes platelet in prothrombin conversion to thrombin. Such thrombin-like toxin is known to be restricted to crotalid venoms. The pathogenesis in such cases, therefore is one of thrombin-induced defibrination syndromes with possibly a hemorrhagic state of Schwartzman phenomenon type (Schwartzman, 1937).

The role of heparinization in such a hemorrhagic state is now well established. There is evidence that the beneficial role of heparin is not only due to

its direct inhibitory action on clotting but its prevention of hemorrhagic fibrinolytic activity and renal cortical necrosis of Schwartzman phenomenon (Kane *et al.*, 1953; Rodriguez-Erdman, 1964a,b). The rationale of supportive fibrinogen transfusion lies in the rapid replacement of the depleted plasma fibrinogen pool and a correction of hypofibrinogenemia.

The therapeutic advantage of heparinization with fibrinogen transfusion has been well borne out in the present study by the progressive hematological recovery observed in all fourteen cases treated in our trial group A. The effectiveness of the treatment is not only evident in 100% survival of the treated cases but also in a significant reduction in the period of morbidity by a faster pace of recovery.

The importance of rapid fibrinogen replacement rather than whole blood as the supportive adjunct to heparinization is also appreciated from the fatal results of the two cases in the trial group B.

The role of heparinization in preventing renal complications in the disorder is suggested by the absence of any incidence of renal failure in the trial groups while both the fatal cases in the control group succumbed to acute renal failure.

The results of the present study therefore suggest that the predominant nature of the hemorrhagic snake venom poisoning in our part of the country is one of defibrination syndromes with resemblance to the hemorrhagic state of Schwartzman phenomenon, induced by a thrombinlike toxin of crotalid vipers. The study has also confirmed the therapeutic benefit of rapid heparinization with fibrinogen transfusion as an adjunct to the current antivenin treatment in such cases. The therapy improves the survival rate, hastens recovery, and prevents possible fatalities from renal complications.

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The Action of *Tityus trinitatis* Venom on the Canine Pancreas

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INTRODUCTION

Acute pancreatitis following the sting of the scorpion of Trinidad, *Tityus trinitatis* has been described (Waterman, 1938; Poon-King, 1963; Bartholomew, 1970). Whereas excessive salivary secretion is well documented as one of the common sequelae of scorpion stings, as far as we are aware no reports have ever been published on the secretory response of the pancreas to scorpion venom. This paper records for the first time the pancreatic exocrine response to scorpion venom, in this case that of *T. trinitatis* from Trinidad.

MATERIALS AND METHODS

Experiments were done on fasting mongrel dogs, and anaesthesia was induced with epontol (Bayer) and continued with chloralose. Laparotomy was performed through a small upper midline incision with careful technique to minimize handling of the pancreas. A cannula was inserted for a short distance into the pancreatic duct near its termination without opening the duodenum. The cannula was then secured and the laparotomy incision closed. A venous catheter was introduced into the femoral vein and advanced towards the inferior vena cava.

The pancreatic juice was collected in weighed tubes under oil. Amylase was measured by the Phadebas method (Ceska *et al.*, 1969) and expressed in international units (I.U.). Lipase activity was measured on an emulsified olive oil substrate and expressed in I.U. Trypsinogen in juice was activated by purified canine enterokinase. The tryptic activity was measured by the BAPNA method of Erlanger *et al.* (1961) and expressed as μg of trypsin.

Crude venom of the scorpion, *T. trinitatis,* was obtained by electrical milking and stored as dry powder at -20°C. The dried venom was dissolved in normal saline immediately before use and the solution was administered slowly through the venous catheter.

RESULTS

Doses of venom smaller than 0.4 mg/kg body weight produced no significant flow of pancreatic juice, while a dose of 3.5 mg/kg resulted in immediate ventricular arrythmia and death soon afterwards. Figure 1 shows the response to repeated injections of venom in a 10-kg cannulated dog in increasing doses at 15-min intervals. A significant rise in volume of juice and output of enzymes was seen after 45 min. A slight rise in serum amylase was also observed. From this experience, 1 mg/kg body weight venom was chosen as a dose which should produce an adequate pancreatic response without inducing terminal cardiac effects. Figure 2 shows the serum effect of repeated injections of 1 mg/kg body weight at 2-hr intervals in two dogs without pancreatic cannulation. In both cases there is a slow elevation of serum amylase but the response of serum lipase is not a sustained rise.

The response of dogs with pancreatic cannula to 1 mg/kg body weight venom was then studied. Two examples are shown which show definite pancreatic responses though differing in detail. Salivary secretion occurred soon after the initial injections. Figure 3 shows the pancreatic volume response in a dog which had no basal secretion. Secretion was not marked following the first injection, but subsequent injections at two-hour intervals provoked significant responses. The output of pancreatic enzymes in this dog was likewise small following the first injection but the second injection gave marked peak outputs which were further stimulated by a third injection (Fig. 4). The serum enzymes of this animal behaved like those in noncannulated animals, amylase showing a slow rise and lipase a sharp rise and a fall pattern (Fig. 5).

Figure 6 shows the volume and serum enzyme response to repeated injections in another dog. The volume response was more moderate. Again there was a gentle serum amylase elevation and the lipase response showed a similar undulant pattern as before. The enzyme output in the pancreatic juice showed clear responses following each injection (Fig. 7).

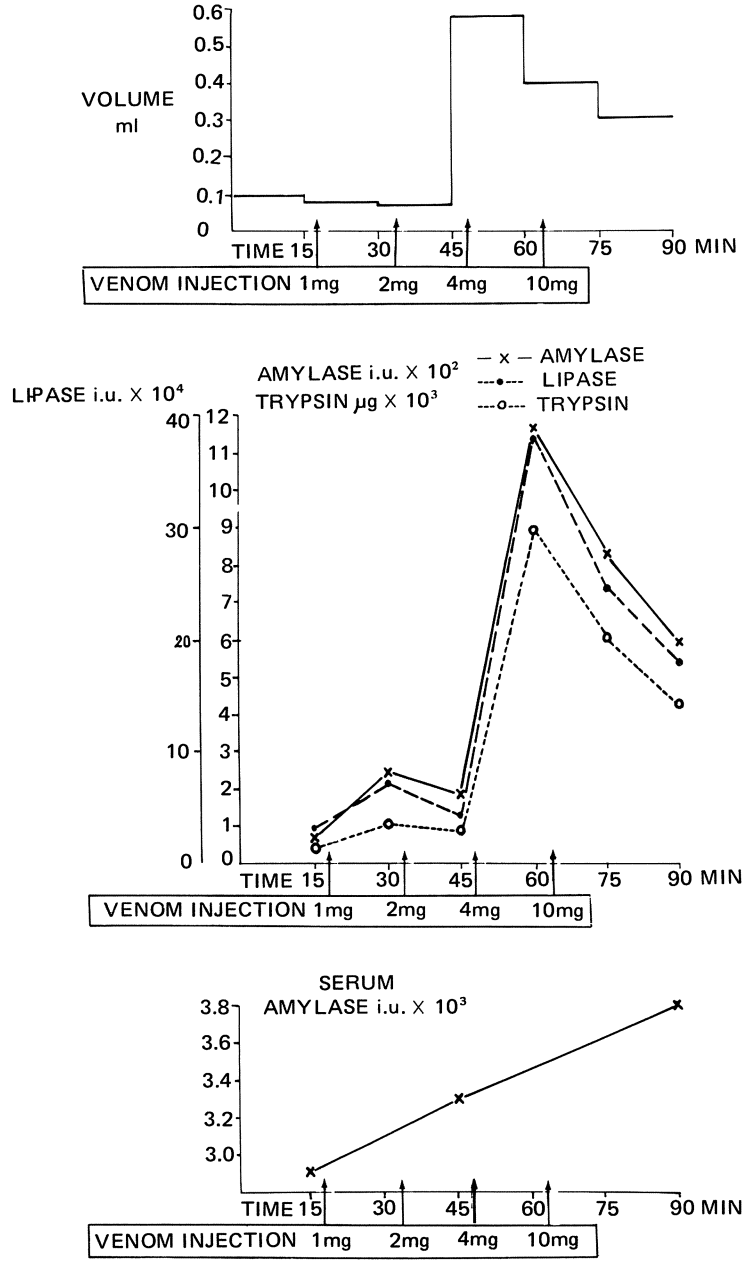

Fig. 1. Pancreatic volume, enzyme response, and serum amylase response to increasing doses of venom in a cannulated dog.

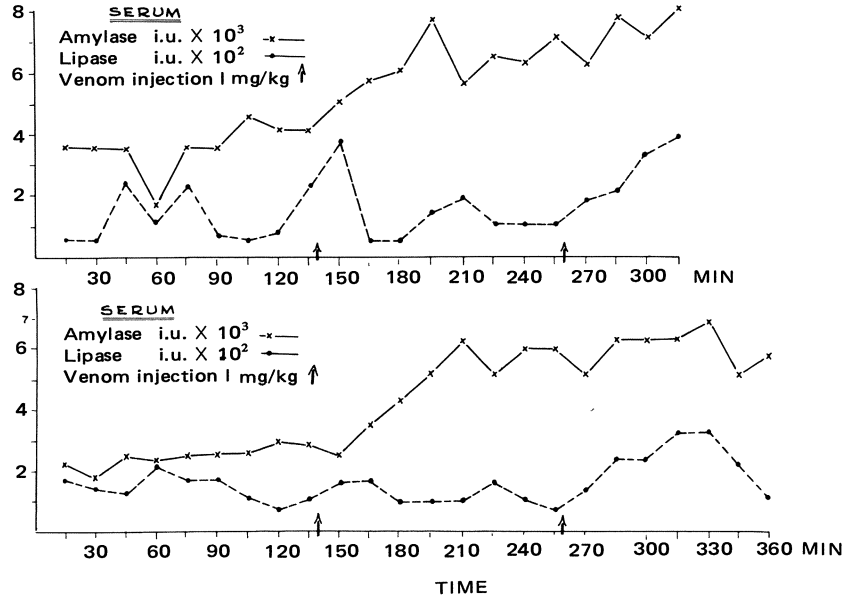

Fig. 2. The serum anzyme response in two dogs without cannulation. 1 mg/kg body weight venom at 2-hr intervals.

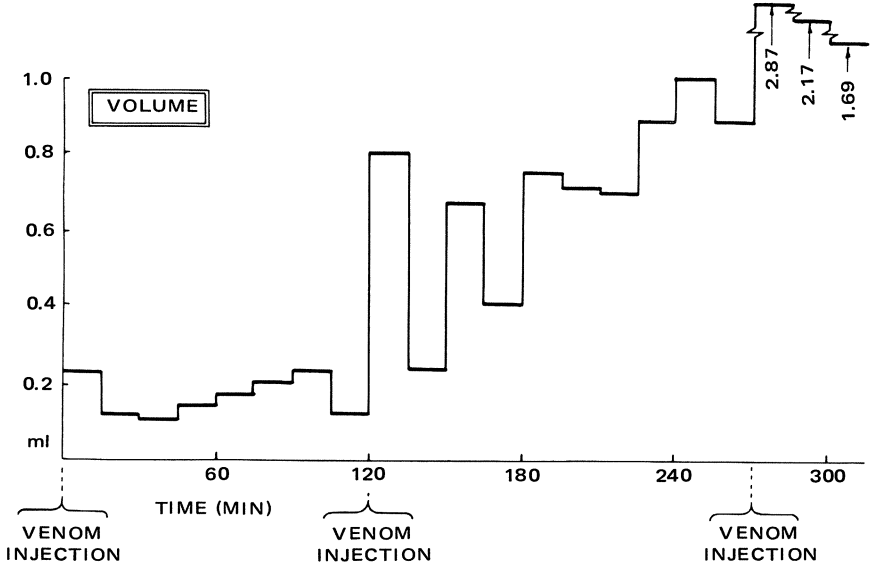

Fig. 3. Pancreatic volume response to 1-mg/kg body weight venom (dog 1).

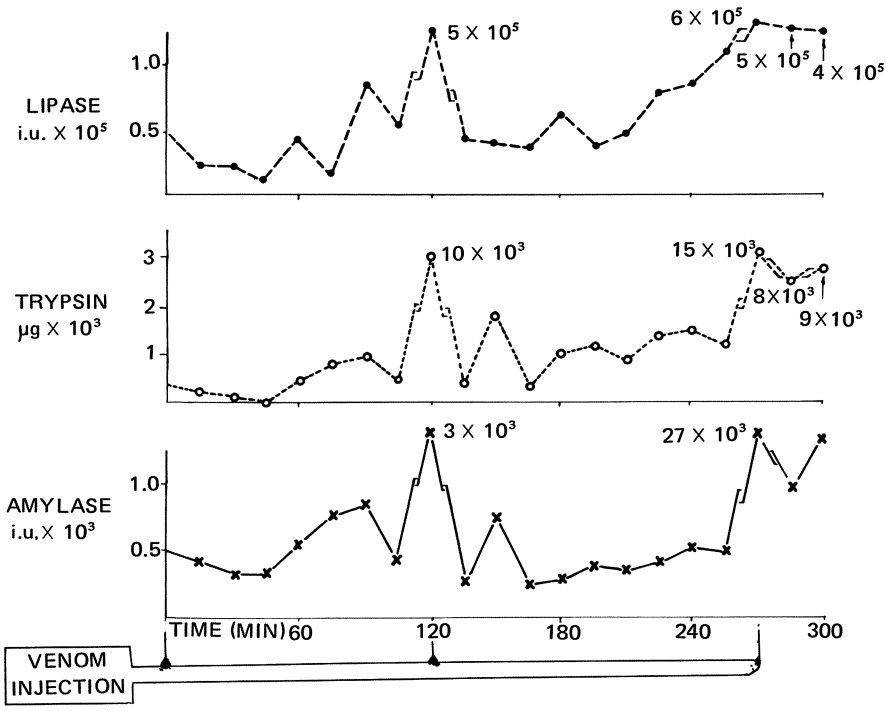

Fig. 4. Pancreatic enzyme response to 1-mg/kg body weight venom (dog 1).

DISCUSSION

From these results we conclude that the venom of *T. trinitatis* evokes a definite exocrine pancreatic response of a juice rich in enzymes similar to that evoked by pancreozymin and caerulein. The fact that the serum enzymes rise even in dogs with no pancreatic cannula in place suggests that the venom may also have a vascular permeability effect.

Contractions of the isolated smooth muscle caused by scorpion venom (*T. serrulatus*) have been claimed to be due to the release of an acetylcholinelike substance (Diniz and Goncalves, 1956). Diniz and Torres (1968) incubated fragments of guinea pig ileum with *Tityus* venom and tested the release of acetylcholine according to Paton (1957). In the flasks containing venom, a smooth muscle contracting substance with pharmacological and some chemical properties similar to acetylcholine accumulated. They concluded that the contracting effect of the venom on the guinea pig ileum was indirect and mediated

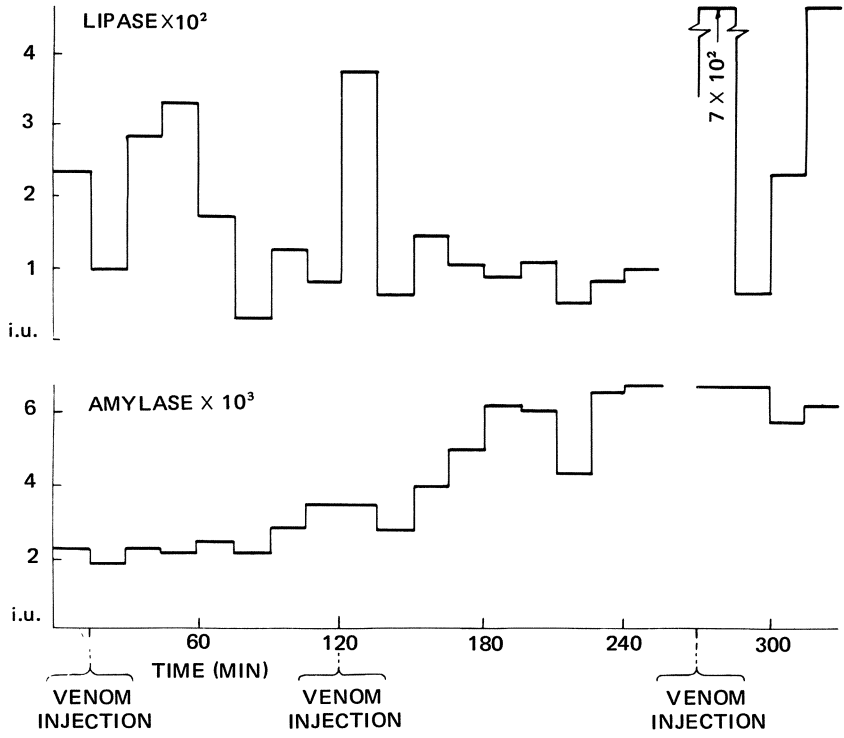

Fig. 5. Serum enzyme response to 1-mg/kg body weight venom (dog 1).

through acetylcholine. Diniz *et al.* (1966) suggested that the effect of scorpion venom on the guinea pig ileum was due to the activation of the parasympathetic postganglionic nerves.

Recently it has been shown that certain peptides, such as caerulein, cholecystokinin octapeptide, gastrin-1, and pentagastrin, stimulate the ganglionic cells of the myenteric plexuses which in turn release acetylcholine from the nerve terminals (Vizi *et al.*, 1972). Parasympathetic ganglia and nerve fibres are frequently seen in the interstitium of the pancreas (Richins, 1945); Bencosme, 1959; Ekholm, *et al.* 1962) and it is our opinion that the venom acts probably on the parasympathetic postganglionic terminal nerve endings in the gland releasing acetylcholine and discharging a juice rich in enzymes.

ACKNOWLEDGMENTS

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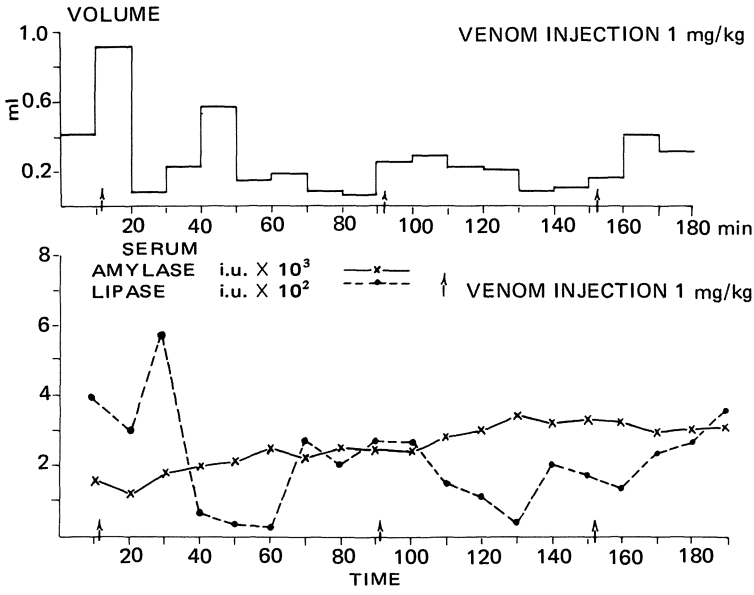

Fig. 6. Pancreatic volume and serum enzyme response to 1-mg/kg body weight venom (dog 2).

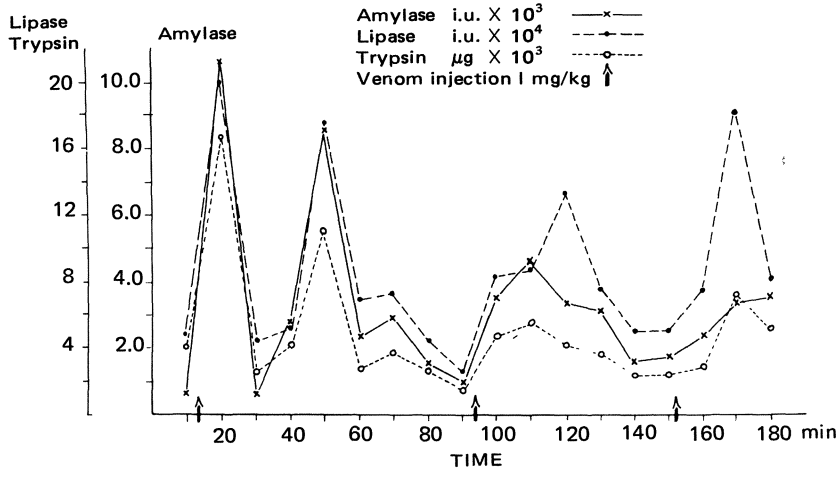

Fig. 7. Pancreatic enzyme response to 1-mg/kg body weight venom (dog 2).

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Ecology and Distribution of Some Sea Snakes in Peninsular Malaysia

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INTRODUCTION

The sea snakes belong to a single family, *Hydrophiidae*. This is a very distinct group of snakes, the great majority of which live entirely in the sea. They have a number of distinctive morphological features including reduction or complete degeneration of the ventral shields and a laterally flattened tail like the blade of an oar, the latter being an obvious adaption for swimming. This feature distinguishes them from those water snakes inhabiting tidal waters, whose tails taper to a point and are at most only slightly flattened.

In Peninsular Malaysia, more than 20 species belonging to 12 genera are known to inhabit the Malaysian waters. Tweedie (1961) grouped these into two subfamilies, *Laticaudinae* comprising *Laticauda colubrina* and *Aipysurus eydouxi*, and *Hydrophiinae* comprising all other species. With the exception of *Hydrophis*, all genera of sea snakes in local waters are represented by a single species.

Sea snakes in Peninsular Malaysia cause occasional hazards to fishermen and bathers. A number of fatal cases of sea snake bite have been reported from Penang and other coastal towns (Reid *et al.*, 1963; Lim and Abu Bakar, 1970; Sawai and Koba, 1971; Sawai *et al.*, 1972). To date very little is known of this group of snakes, and the present paper will reveal ecological and distributional patterns of some of these snakes.

RESULTS

Distributions. A total of 10 species of sea snakes were collected and studied, and their distributional patterns are shown in Fig. 1. Some of these species were more commonly found in the west coast than in the east coast of Peninsular Malaysia (Table 1). Nine species of sea snakes were collected on the west coast, but only four from the east coast. Of these, *Enhydrina schistosa* is the most com-

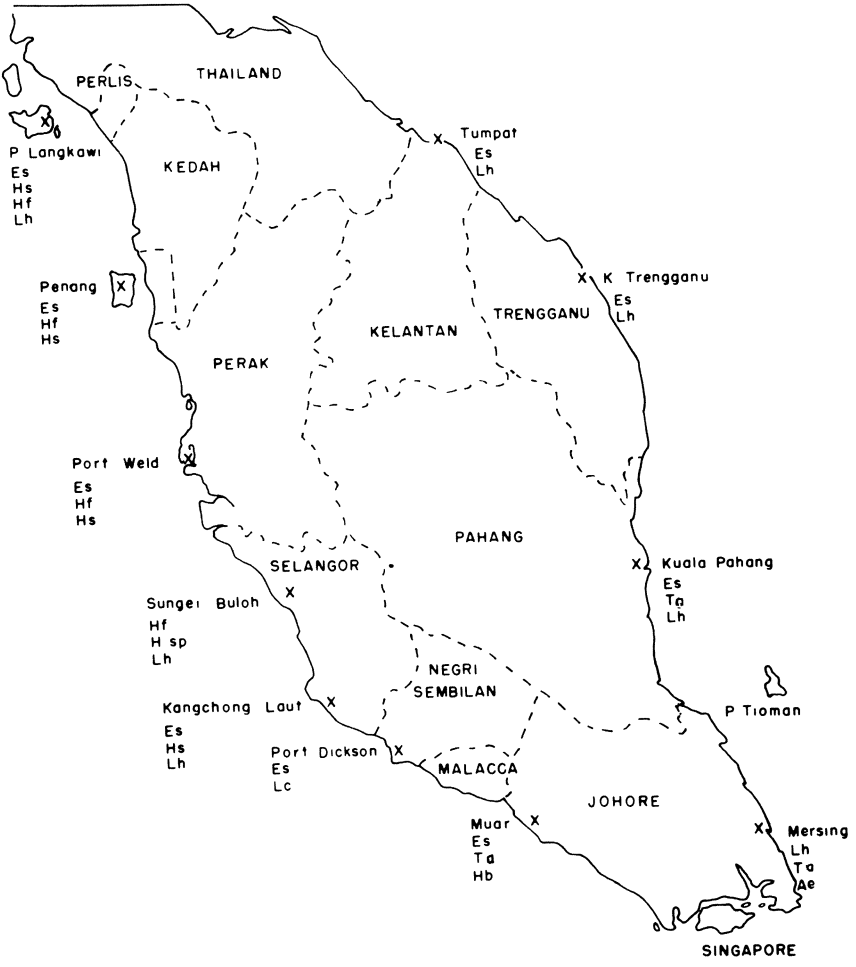

Fig. 1. Map showing collecting sites of some of the sea snakes in peninsular Malaysia. Abbreviations: Es, *Enhydrina schistosa*; Ta, *Thalassophis anomalus*; Hb, *Hydrophis brookei*; Hs, *Hydrophis spiralis*; Hf, *Hydrophis fasciatus*; H.sp, *Hydrophis* spp.; Hc, *Hydrophis cyanocinctus*; Ae, *Aipysurus eydouxi*; Lc, *Laticauda colubrina*; Lh, *Lapemis hardwickei*.

Table 1. Distribution Pattern of Some Sea Snakes in Peninsular Malaysia

Localities	Species of snakes[a]									
West coast										
Johore	Es	Ta	Hb							
Malacca	Es		Hb							
Negri Sembilan	Es			Lc						
Selangor	Es				Lh	Hs	H.sp.			Hc
Perak	Es					Hs		Hf		
Penang						Hs		Hf		Hc
Kedah/Perlis					Lh	Hs		Hf		
East coast										
Kelantan	Es				Lh					
Tregganu	Es				Lh					
Pahang	Es	Ta			Lh					
Johore		Ta			Lh				Ae	

[a]Abbreviations: Es, *Enhydrina schistosa*; Ta, *Thalassophis anomalus*; Hb, *Hydrophis brookei*; Hs, *Hydrophis spiralis*; Hf, *Hydrophis faciatus*; H.sp, *Hydrophis* spp.; Hc, *Hydrophis cyanocinctus*; Ae, *Aipysurus eydouxi*; Lc, *Laticauda colubrina*; Lh, *Lapemis hardwickei*.

mon and abundant, occurring throughout the shore lines of the country. *Lapemis hardwickei* is fairly common, but it is more commonly found on the east coast than the west coast. Of the family *Laticaudinae*, only *L. colubrina* was found in Port Dickson on the west coast, whereas *A. eudouxi* was observed in Mersing of the east coast.

Habitats. The habitats of most of the sea snakes are found in shallow coastal waters and in river mouths (Tweedie, 1961), which was confirmed in our recent observations of some of these sea snakes (Table 2). *E. schistosa*, *H. brookei*,

Table 2. Habitats of Some Sea Snakes in Peninsular Malaysia

Species	Shallow coastal waters and river mouths	Rocky places near shores	Deep sea more than 10 miles away from shores
Hydrophinae			
Enhydrina schistosa	+		+
Hydrophis brookei	+		+
Hydrophis spiralis			+
Hydrophis fasciatus			+
Hydrophis cyanocinctus			+
Hydrophis spp.			+
Thalassophis anomalus	+		+
Lapemis hardwickei	+		+
Laticaudinae			
Laticauda colubrina		+	
Aipysurus eydouxi			+

Thalassophis anomalus, and *L. hardwickei* are commonly encountered in shallow coastal waters as well as in deeper waters, but the most common of these is *E. schistosa* which is also found in river mouths. *H. spiralis*, *H. cyanocinctus*, and *Hydrophiinae* spp. were collected only from deep sea waters more than 10–15 miles from the shores. *A. eydouxi* was collected as far as 50 miles from the shores. *L. colubrina* is commonly found in rocky places by the shore lines or on rocky islands not far away from the shore.

DISCUSSION

The preliminary data regarding sea snakes in our recent studies indicate that they are widely distributed throughout the coastal waters of Peninsular Malaysia. The fact that only a few species of sea snakes were collected does not necessarily mean that most of the others are not found. Lack of information on the other species could be due to the application of inadequate techniques for collecting them. It could be due to the particular seasonal periods when some species are more abundant than others. The study also revealed that *E. schistosa* is the most common of all the sea snakes found in the coastal regions. Although the number of species found was greater on the west coast than the east coast, data are insufficient to ascertain (a) that other species of sea snakes are as abundant as *E. schistosa*, and (b) that there is some marked difference in species distribution between the west and east coasts of Peninsular Malaysia.

It is interesting to note that of the sea snakes studied, the most common species, *E. schistosa*, was commonly found in shallow coastal water and in river mouths, although they were also collected in deeper waters along with three other species, *H. brookei*, *T. anomalus*, and *L. hardwickei*. Those snakes appear frequently in shallow waters, which may probably be due to the fact that they must have access to both the surface to breathe and the bottom to seek their food, and that they are not adapted to deep diving. Tweedie (1961) mentioned that *Pelamis platurus*, having been found in oceanic waters, is a truly pelagic animal. The fact that we observed *A. eydouxi*, a member of the subfamily, *Laticaudinae*, 50 miles away from the shores could indicate that its habit is similar to that of *P. platurus*. Another member of this family, *L. colubrina*, is amphibious in habit. The latter species is always found in rocky places as it seeks such shelters to breed, laying its eggs in rock crevices and under stones. This is the only snake known to be oviperous, the rest are viviparous (Tweedie, 1961).

Knowledge gained on the ecology and distribution of some of the sea snakes in the present study would give an indication of involvement of particular species of sea snakes in sea snake bite incidence in a particular area. It is often difficult for the victims to identify the sea snake involved in the bite, but it is quite

possible to postulate the species from the area where the incident occurred. For example, if snake bites occur among bathers at sea coasts, it is quite probable that the snake responsible would be either one of those species that are frequently found in shallow waters or river mouths. If fishermen are involved while at sea, it is quite probably that some deep water species of snake may be responsible. Although antivenin is available for sea snake bites, it is essential that the identification of the species is made so that it may help in the more effective treatment of such cases.

Very little is known about sea snakes in general, but during the present decade, when exploitation of the sea for more food resources is being made due to the increasing human population and shortage of food on land, hazards of sea snake bites may eventually increase and can be of economic as well as public health importance. Further studies, therefore, are required to know more about the ecology, distribution, and medical importance of such a highly specialized group of snakes in Peninsular Malaysia.

SUMMARY

A total of 10 species of sea snakes were collected. Nine species were collected in the west coast, whereas only four from the east coast. *E. schistosa* is the most common and abundant species occurring throughout the country. On the other hand, *L. hardwickei* is fairly common on the east coast, and *A. eydouxi* was also observed on the east coast. *E. schistosa, H. brockei, Thalassophis anomalus*, and *L. hardwickei* are commonly encountered in shallow waters as well as in deeper waters. *E. schistosa* is found in river mouths. *H. spiralis*, *H. cyanocinctus*, and *Hydrophis* spp. were found only in deep sea waters 10–15 miles from the shores. *A. eydouxi* was collected as far as 50 miles from the shores. *L. colubrina* is common in rocky places along the shore.

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Submicroscopic Organization of the Venom Gland of *Vespa orientalis*

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INTRODUCTION

Arthropod chemical defenses are better understood now than before. Venoms in general are poisonous chemical compounds which many animals secrete and transfer into their victims either by stinging or biting. This definition excludes a variety of repugnatorial, corrosive, and irritating toxins produced by a variety of insects, e.g. certain beetles and ants, and which are essentially defensive in nature (Beard, 1963), in contrast to the venoms which necessarily have to be injected into the victims primarily for offensive and perhaps also for defensive purposes (Roth and Eisner, 1962).

In situ studies pertaining to venom synthesis in insects are rather scarce primarily because, so far, interest has been centered on the chemical nature of the venoms and their pharmacological effects on a variety of animals. Further, insect venoms are immensely complex mixtures obviously arising from different histological sources (Beard, 1963). Therefore, studies on the gross morphology and cytoarchitectural details of the glands coupled with functional interpretation of the morphological data perhaps are prerequisites for full understanding of cellular processes involved in the venom secretion (Eisner *et al.*, 1964).

In most cases active molecules of insect venoms or toxins comprise an astonishingly high concentration of formidable poisons (Eisner *et al.*, 1964). How do insect gland cells produce such corrosively poisonous substances without poisoning themselves?

Not much work by electron microscopy has yet been accomplished on the poison apparatus of *Vespa orientalis*, the Indian hornet.

MATERIAL AND METHODS

The material for electron microscopic studies was fixed in ice-cold phosphate-buffered 4% glutaraldehyde for 2 hr, subsequently washed for 48 hr with repeated changes of ice-cold buffer to which was added 0.22 M sucrose, postfixed in OsO_4 for 4 hr at 4°C (Millonig, 1962), dehydrated quickly in graded ethyl alcohol containing uranyl acetate, and embedded in Epon (Luft, 1961). The ultrathin silver sections (800–1200 Å) were further stained in uranyl acetate in 50% ethyl alcohol followed by lead citrate (Reynolds, 1963).

OBSERVATIONS

The poison apparatus in *V. orientalis* comprises a sizable pear-shaped poison vesicle which opens at the base of the sting by means of a duct into which the duct of Dufour's (alkaline) gland also merges. Anteriorly from this poison vesicle originate two long, slender tortuous glandular tubules. These tubules float freely in the body cavity of the animal and constitute the acid or the venom gland of *Vespa* (Fig. 1a).

The venom gland of *V. orientalis* is a simple tubule of an acinar type comprising wedge-shaped secretory cells arranged around the cuticulerized lumen, the central duct (Fig. 1b). Joining the apical end of each gland cell is a small ductule with the help of which the individual cells drain off their products into the central duct. These efferent ductules, each of which joins the central duct with the apical end of individual secretory cells, penetrate or pierce deeply into the gland cell cytoplasm and branch so as to permeate the apical regions of the gland cells (Figs. 1b and c). These ductules serve as one way conduits allowing venom discharge from the cells into the central duct. The intracellular and the efferent ductules as well as the central duct are lined by dense cuticular material which can be differentiated into two concentric layers, epicuticle and endocuticle. The cuticle is enveloped all through by an underlying nucleated epidermal layer of undifferentiated epithelial squamous cells, barely visible under an optical microscope (Figs. 1c, 2, 3, 9, and 10). The duct system comprising the central duct, the efferent, and intracellular ductules is intimal, cuticle-lined infolding of the body wall.

The cytoplasm of the glandular cells comprises a homogeneously dense granular matrix of moderate electron density, and is interspersed with (i) almost electron-transparent or translucent secretory vesicles, and (ii) more or less homo-

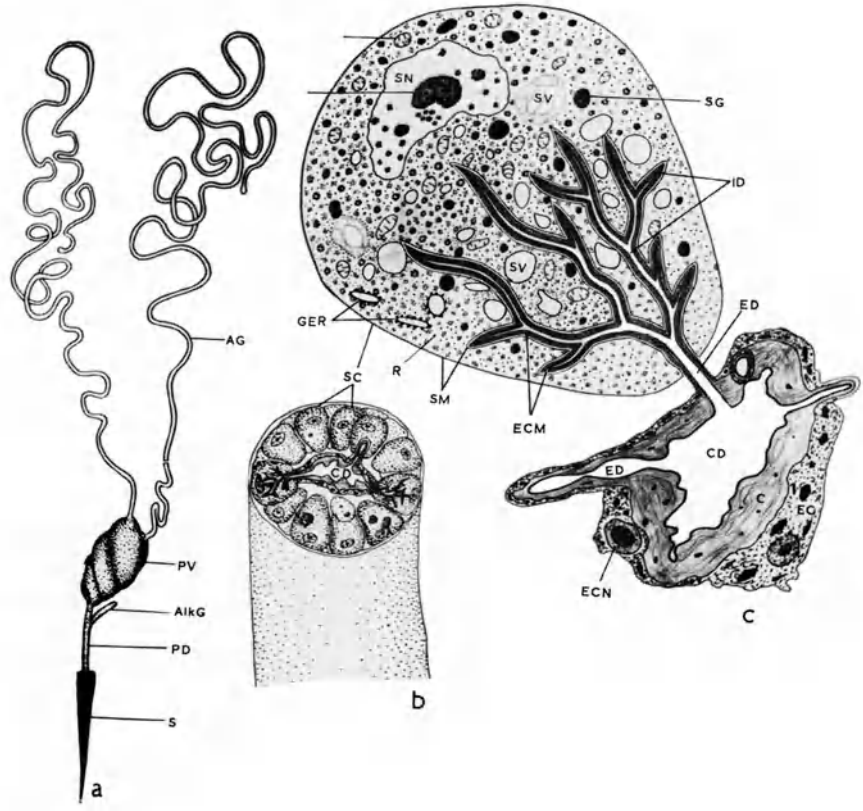

Fig. 1. Simplified reference diagram of (a) the venom apparatus, (b) transverse section of the venom gland, and (c) a transverse cut of the central duct, the efferent ductule, and one of the secretory cells. Abbreviations: AG, acid gland; AlkG, alkaline gland; C, cuticle; CD, central duct; EC, epithelial cell; ECM, epithelial cell membrane; ECN, epithelial cell nucleus; Ed, efferent ductule; GER, granular endoplasmic reticulum; ID, "intracellular ductule," M, mitochondrion; MT, microtubule; NE, nuclear envelope; NI, nuclear involution or invagination; NM, nuclear membrane; NP, nuclear pore; PD, poison duct; PV, poison vesicle; R, ribosome; S, stinger; SC, secretory cell; SG, secretory globule; SM, secretory cell membrane; SN, secretory cell nucleus; SV, secretory vesicle.

geneously electron-dense secretory globules of various sizes (Figs. 4–7 and 9–11). The large-sized vesicles seem to arise from the coalescence of the smaller ones rather than by growth of the latter (Figs. 9 and 10). Randomly dispersed, more or less homogeneously electron-dense bodies of variable sizes, the secretory globules, are homologous with cytochemically complex secretory globules of optical microscopy (Kanwar and Sethi, 1971).

The mitochondria are generally ovoid and abundant though small and

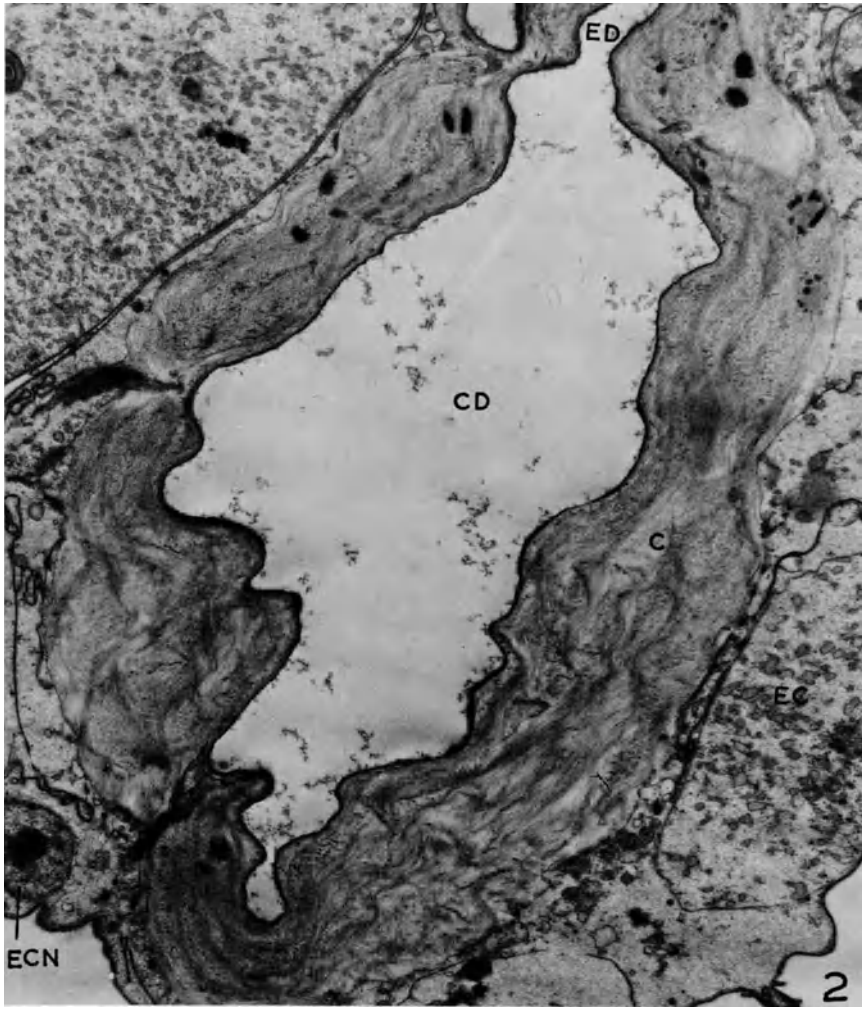

Fig. 2. A traversely sectioned central duct into which are opening efferent ductules. Note the thick cuticle lining the duct and the ductules surrounded by squamous epithelial cells (14,200 ×). See caption to Fig. 1 for definition of symbols.

cristae deficient (Figs. 4–7). Mitochondrial concentration in vicinity of the nuclear membrane is of common occurrence (Figs. 4 and 7).

The secretory cells are highly basophilic. The cytoplasm reveals elaborate and uniformly dispersed vesiculated granular ER, and also an abundance of loose ribosomes (Figs. 4–11). Granule-studded membranous profiles are only rarely seen.

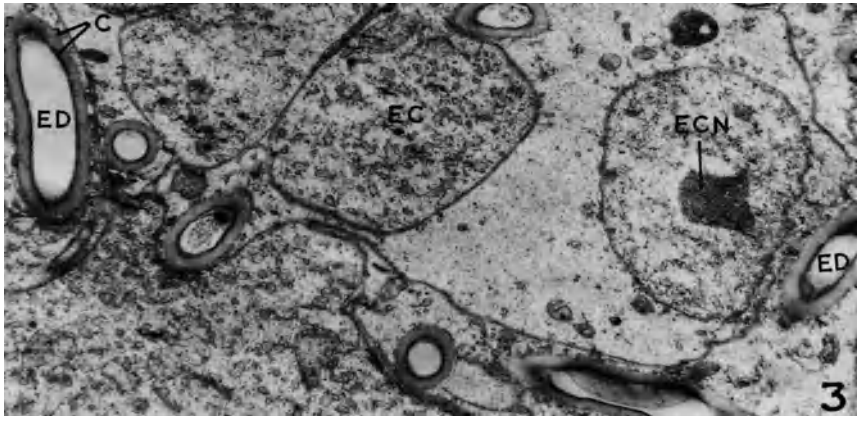

Fig. 3. A sectioned efferent ductules and the surrounding epidermal epithelial squamous cells. Note two concentric layers of the cuticle, the epicuticle and endocuticle (14,200×). See caption to Fig. 1 for definition of symbols.

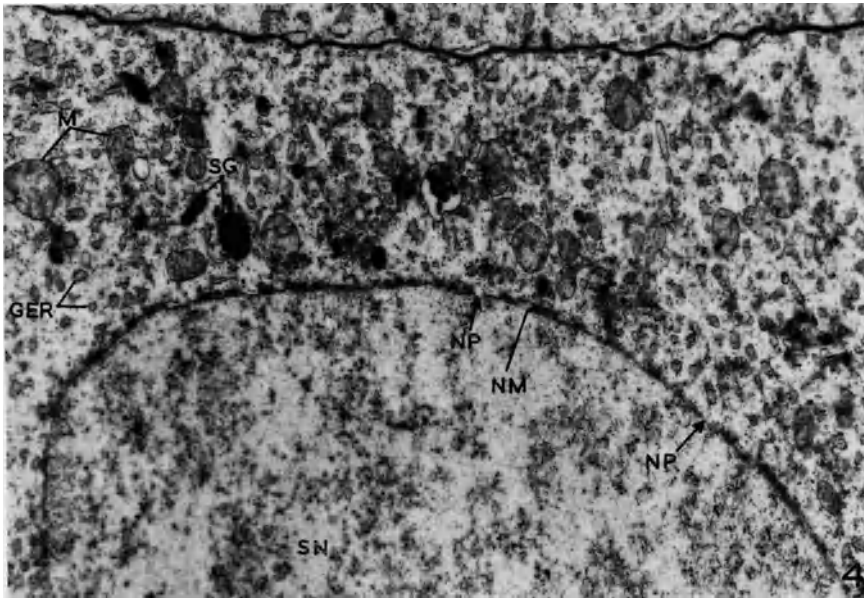

Fig. 4. Secretory cell showing granular ER elements, small ovoid mitochondria and homogeneously electron dense secretory granules or globules. Nuclear membrane is smooth and reveal distinct nuclear pores (12,500×). See caption to Fig. 1 for definition of symbols.

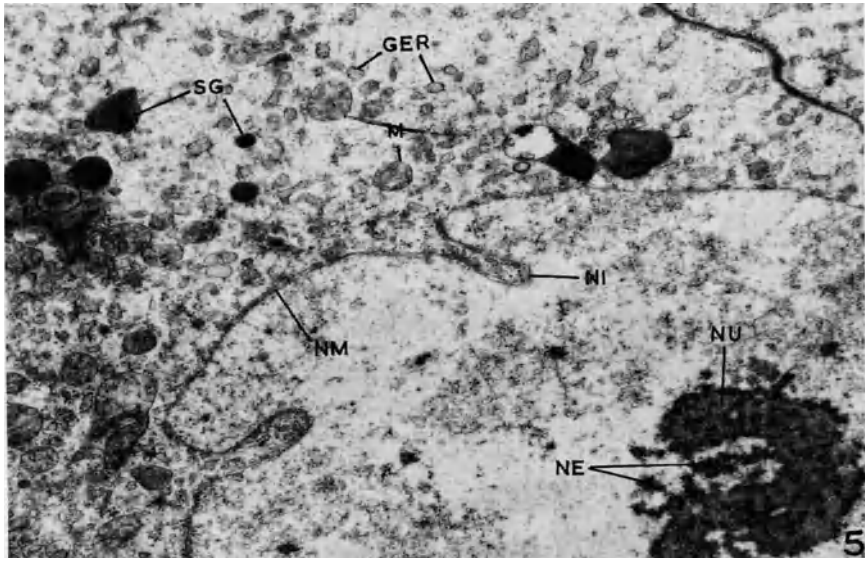

Fig. 5. Nuclear membrane is no longer smooth, and nucleolar hypertrophy is clearly discernible. The cytoplasm shows granular ER, secretory globules and mitochondria (12,200×). See caption to Fig. 1 for definition of symbols.

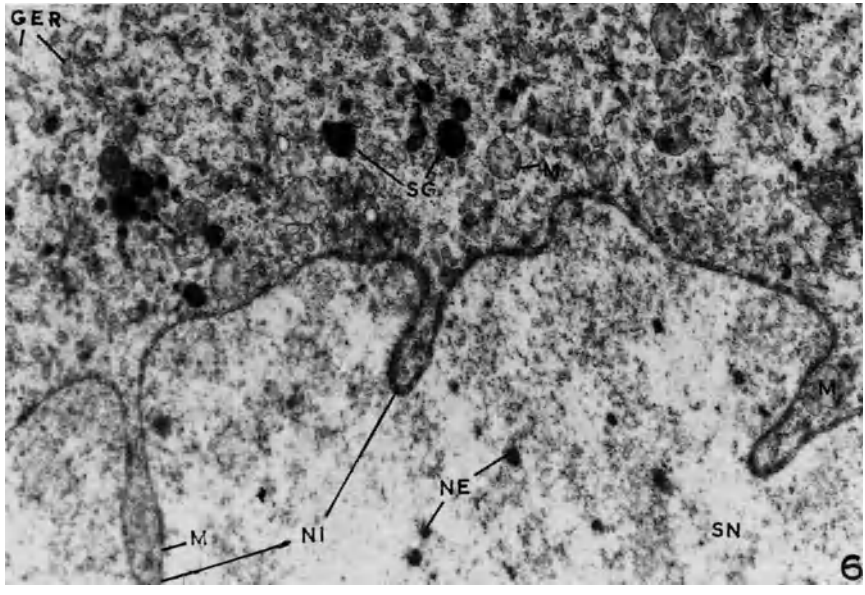

Fig. 6. Nuclear involutions are more prominent. Mitochondria are seen deep in nuclear invaginations. In the cytoplasm are seen ER elements, secretion globules and mitochondria (12,200×). See caption to Fig. 1 for definition of symbols.

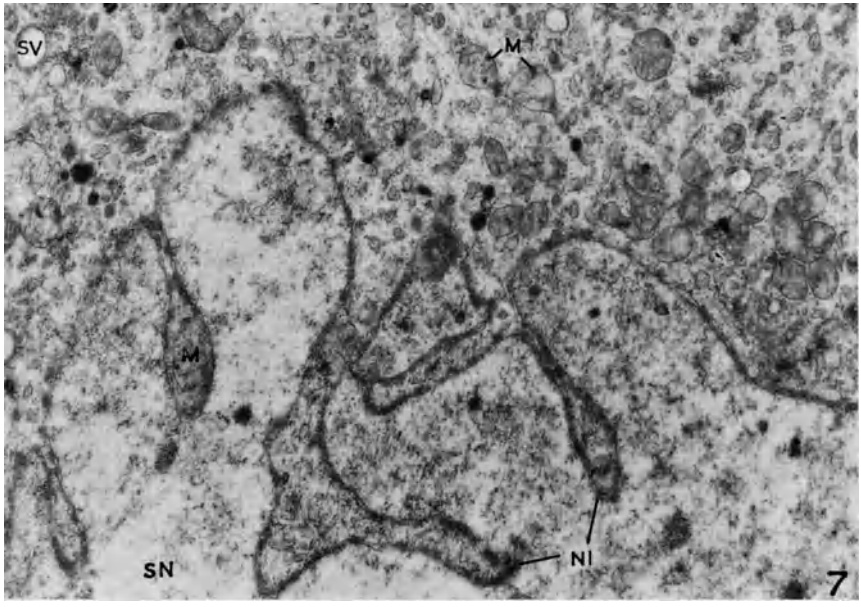

Fig. 7. A secretory cell showing heavy nuclear infolding. Mitochondrial presence in nuclear involutions is clearly seen. Mitochondria in the cytoplasm are concentrated in close proximity of the nuclear membrane (12,200×). See caption to Fig. 1 for definition of symbols.

Tubular or vesiculated nongranular ER components are scarce. Stacked Golgi cisternae are wanting.

Randomly placed and hence variedly sectioned microtubules are frequently encountered in the venom gland cells (Fig. 11). The microtubules do not show any special association with any cellular component.

The nuclear envelope is thrown into prominent karyothecal involutions (Figs. 5-7), which most often are loaded with mitochondria, and penetrate deep into the nucleoplasm (Figs. 5-7). These cells also present intense nucleolar activity as evidenced by (i) highly irregular and electron-opaque nucleolar peripheries (Figs. 5 and 8), and (ii) continuous shedding of sizable electron-dense emissions which disperse in nucleoplasm (Figs. 5, 8, and 9) and perhaps cross into the cytoplasm.

DISCUSSION

Amongst interesting adaptations of certain insect gland cells, particularly those engaged in synthesizing venoms and toxins, is the presence of a network of very fine branching ductules in the cytoplasm (Beams and Anderson, 1961). The

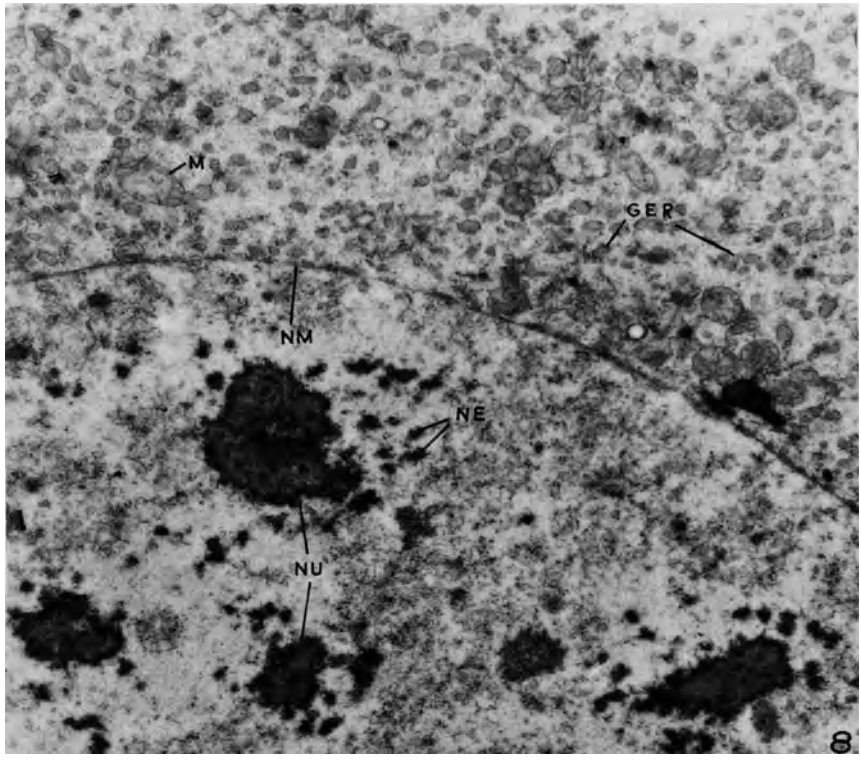

Fig. 8. A secretory cell showing intense nucleolar hypertrophy (11,150X). See caption to Fig. 1 for definition of symbols.

term "intracellular ductules or canals," which is so often used to describe this system of "drain pipes" is in fact a misnomer inasmuch as even the finer branches of these ductules lying deep in the secretory cell cytoplasm are intimal and ensheathed with cytoplasmic lining of the invaginated epidermal squamous cells (Figs. 9 and 10), the plasma membrane of which is closely opposed by the involuted plasma membrane of the gland cell (Beams and Anderson, 1961). The branching system of cuticularized ductules embedded in the cytoplasm of the gland cells is, therefore, in reality extracellular and not intracellular as popularly described. This arrangement results in increased polarized secretory surface area, a condition conducive to a more efficient functioning of these cells (Beams and Anderson, 1961). Further, the intracellular ductules greatly reduce the distance involved in the transport or diffusion of the toxic secretions synthesized within the cell cytoplasm, thereby eliminating chances of possible autointoxication (Beams and Anderson, 1961). The intracellular duct system perhaps

compensates for the conspicuous absence of the regular ER channels which are vitally implicated in the transference of secretory products intracellularly (Fawcett, 1966).

The submicroscopic organization of the venom gland cells is reminiscent of cells engaged in active synthesis. This is evidenced by:

1. Nuclear contour is most often highly irregular and in certain cases during peak activity the nuclear envelope is thrown into prominent karyothecal, mitochondria-loaded, involutions penetrating deep into the nucleoplasm. Nuclear lobulation results in substantial increase in the functional surface area of the nuclear envelope (Bernhard, 1969).

2. Intense nucleolar activity as manifested by (a) nucleolar fragmentation or budding (Fig. 8), (b) highly irregular and electron-opaque nucleolar periph-

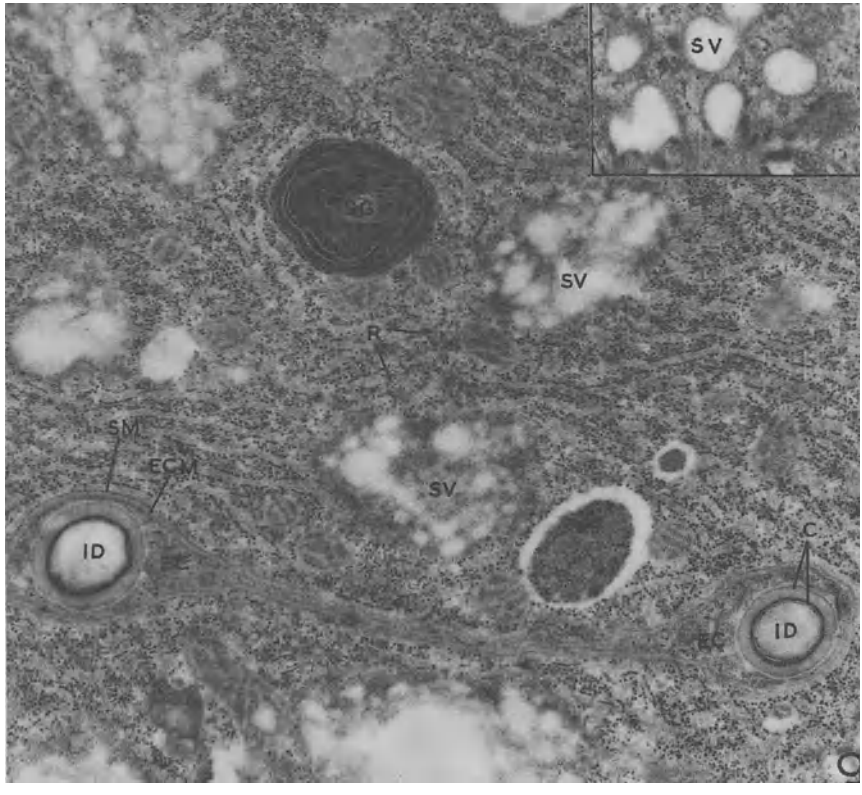

Fig. 9. Secretory cell cytoplasm showing abundance in loose ribosomes. Coalescence of smaller vesicles giving rise to larger bodies is seen. Two intracellular ductules are surrounded by a common sheath of epithelial cell cytoplasm. Epicuticle and endocuticle concentric layers are clearly visible (20,700X). See caption to Fig. 1 for definition of symbols.

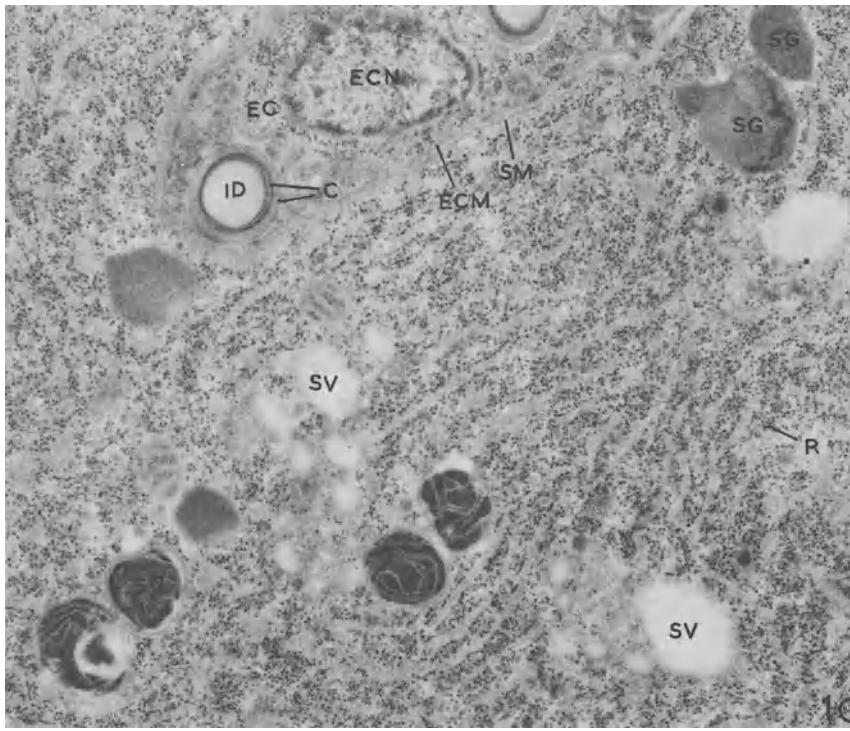

Fig. 10. Secretory cell cytoplasm showing loose ribosomes, secretory globules and vesicles. An intracellular ductule is seen ensheathed by cytoplasm of the epithelial cell (21,800×). See caption to Fig. 1 for definition of symbols.

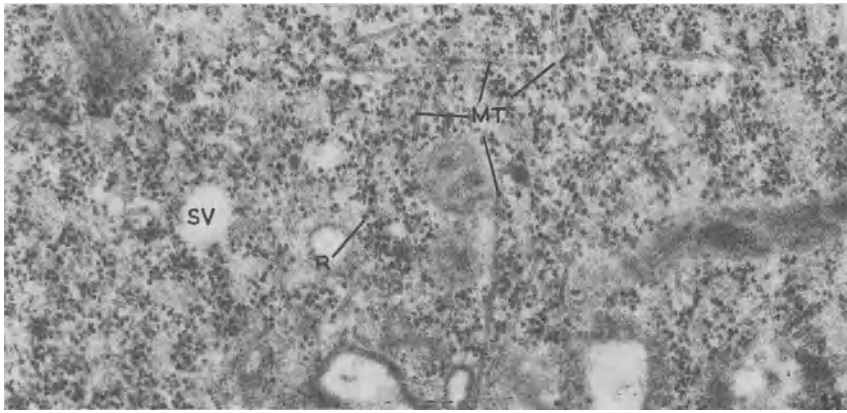

Fig. 11. Secretory cell cytoplasm. In addition to ribosomes and secretory vesicles there are seen microtubules (34,000×). See caption to Fig. 1 for definition of symbols.

eries, and (c) continuous shedding of sizable electron-dense emissions which disperse in nucleoplasm and perhaps cross into the cytoplasm where bodies conforming in structure, size, and electron opacity to nucleolar emissions in the nucleoplasm have frequently been seen.

3. Heavy perforation of the nuclear envelope is evident (Stevens and André, 1969).

4. Preponderance of ribosomes (Fawcett, 1966) and also of mitochondria in the cytoplasm; mitochondrial presence in the cytoplasmic involutions penetrating deep in nucleoplasm are related to rapid exchanges of energetic intermediates, metabolites, and basic constituents from nucleus to cytoplasm and vice versa (Moses, 1964; Stevens and André, 1969).

The cytoplasm of the gland cells comprises a homogeneously dense granular matrix of moderate electron density and is interspersed with electron-transparent vesicles of various sizes. These vesicles are homologous with the hyaline vacuoles of optical microscopy (Kanwar and Sethi, 1967) and represent the sites of water-diffusible (Kanwar and Sethi, 1971) and heat-labile enzymic components of the insect venom which reportedly is rich in hyaluronidase, phospholipases, hydroxytryptamine, low molecular weight proteins like histamine and free amino acids, and acetylcholine, all of which are leached out during aqueous treatment, although their presence in insect venom has been established biochemically (Kaiser and Michael, 1958; Eisner and Meinwald, 1966; Goldman *et al.*, 1960; cf. Beard, 1963).

In the cell cytoplasm are also encountered randomly dispersed homogeneous but varyingly electron-dense bodies of variable sizes. These are homologous with the cytochemically complex secretory globules which are predominantly composed of lipids, an appreciable amount of polysaccharides, and traces of proteins (Kanwar and Sethi, 1971). These bodies seem to correspond with the "dense bodies" of Eisner *et al.* (1963).

As observed also by Eisner *et al.* (1964), not all glandular cells in any particular section appear in a state of functional synchrony, since marked morphological variations have been observed among neighboring and even adjoining cells, viz., (i) widely varying number, size and position of the secretory globules and vacuoles in the cytoplasm, (ii) differential nuclear placement (basal, central or apical), (iii) marked variations in the nuclear contour and nucleolar morphology, and finally (iv) differential ribosomal concentration and disposition in the cytoplasm.

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Tetrodotoxin: Rapid Release of a High Concentration from Skin of the Atlantic Puffer, *Spheroides nephelus*

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INTRODUCTION

Blowfish (fugu) poisoning has for generations been the most important single type of food poisoning in Japan, where peak reported annual incidence of fatalities was 470 in 1947 (Halstead, 1967). Many deaths due to blowfish poisoning occurred in the Pacific theater of operations during World War II (Halstead and Lively, 1954). In Dade County, Florida, U.S.A., 3 deaths have been attributed to ingestion of *Spheroides* species (Benson, 1956; Benson and Davis, personal correspondence). Thus, while many species of blowfish have been assayed for toxin, most assays were directed toward screening of fish which might cause human poisoning. Sampling of meat, roe, and liver was usually emphasized, and most studies showed liver or ovaries to contain the highest toxin concentrations.

Toxin assays of *Spheroides* species in southeastern and eastern central Florida have been published. Assay of skin from fish commercially collected and extracted with 2-ml distilled water/g of skin showed an LD_{50} of 0.14 ml/20 g when injected i.p. (intraperitoneally) in mice (Larson *et al.*, 1959). Assuming even distribution in skin and supernatant, this is approximately ($\sim$) 21.4 LD_{50}/g of fish skin. In a later study (Lalone *et al.*, 1963), fish were collected by the authors, placed on ice, frozen, and dissected (no further details given concerning handling); skin extracts prepared as described above gave an LD_{50} of 0.105 ml/20 g when injected i.p. in mice ($\sim$28.6 LD_{50}/g of fish skin).

Almost all other assays of blowfish, mostly Pacific, have shown greater toxicity in ovary and liver than in skin. In all but 1 of 14 species tested by Tani and tabulated by Kao (1966), this held true, with liver- or ovary-skin toxin ratios up to 100:1. In contrast, Tani found the μg tetrodotoxin/g fresh tissue values in *S. pardalis* to be; ovary 200; liver 1000; skin 100 (Kao, 1966), while for the same species, Macomber (1956) states that the major portion of the toxin is in the skin and may be removed by solvent wash. Could this reflect variables in handling? Eger (1963) found that both mucous secretions and extracts of the skin of the puffer, *Arothron hispidus*, yielded large amounts of tetrodotoxin. Thompson (1968) states that certain poisonous puffers are ichthyocrinotoxic. No assays for tetrodotoxin which I have seen indicate any use of controls in the methods of handling intact fish specimens. They were collected from commercial sources or captured by the investigator or his staff by undisclosed means (Lalone *et al.*, 1963), or by use of rotenone, spear, dynamite, or dipnet (Halstead and Schall, 1955); usually placed in ice and/or frozen thereafter, and dissection carried out without further details.

The chemical structure and mechanism of action of tetrodotoxin on a cellular level are now known; it is much used as a tool in neurophysiological research, inter alios (Kao, 1966; Goldman and Blaustein, 1966; Moore *et al.*, 1967; Kusano *et al.*, 1967; Albuquerque and Grampp, 1968; Tasaki *et al.*, 1968) and medical uses have been suggested (Down, 1970). Attention might now be directed toward finding better ways to produce and harvest the toxin. Also, the biogenesis of tetrodotoxin remains unknown, and usefulness of the toxin to the fish has not been demonstrated or ruled out. Adequately controlled methods of capture and handling are important if we are to develop reliable assays as an aid to solving these and other problems.

MATERIALS AND METHODS

Collection of Fish. Since no method involving less trauma or delay is available, blowfish were collected from shallows by means of a crab dipnet with an 8-ft handle, from the Banana River (a marine estuary) at Milford Point (for parts I–IV) and from the Indian River (a similar marine estuary) at the Bennett Causeway, at Merritt Island, Florida, and subsequent work performed at a residence on Milford Point (parts I–IV), and at Holmes Cove, Cape May Court House, New Jersey (part V).

Parts I–III were carried out on 10–14 September 1965 using fish netted on 10–11 September, when water was calm, fish by the dozens actively feeding, and no dead or disabled puffers were seen. Gonads of fish collected were ripening. On each day, 1–2 dozen fish were netted in 1 hr and placed in the same empty collecting bucket, then immediately processed. Part IV was carried out on

27 December 1965 using fish netted on 17 December, at which time wind action had caused fouling of the shallows with masses of decomposing seaweed and large numbers of dead blowfish were seen. Two fish were netted, one normally active and the other very lethargic and barely able to swim. Each was removed from the net by hand over which a small polyethylene bag had been inverted; each bag was tightly closed, and processing begun within minutes. Both fish were sexually immature males. For Part V, 20 apparently normal blowfish were netted on 6 May 1972, when water was calm and clear.

Handling of Specimens and Preparation of Extracts. Dilute HCl was prepared using 1.5-ml concentrated HCl plus 98.5-ml distilled water. It was used as a rinse and diluent, the amount added to each sample being equal in ml to the weight in g of tissue. Supernatant was drained off and brought to the desired pH by the addition of concentrated NaOH before further dilution (pH 6–7 was used except where pH 3 is specified in part I). At this point (parts I–III and part V) the supernatant was designated as 1 : 2 dilution, since the toxin originally present in each g of tissue was contained in 1 ml of tissue plus 1 ml dilute HCl. Supernatants which appeared turbid were spun in a hand centrifuge for 2–5 min until relatively clear. Distilled water was used for further dilutions of 1 : 4, 1 : 8, and so on. In part IV, each undiluted rinse was considered "full strength," not 1 : 2, and when diluted with an equal volume of distilled water was designated 1 : 2, since the toxin remaining in the skin was assayed in successive rinses and the results of all rinse assays added to the total. In parts I and II, fish were so handled as to minimize toxin loss onto cutting board, hands, and knife, but possible toxin loss to these and to bucket and tap water was ignored, while in part III, loss to all of these except tap water was ignored.

For parts I-V, handling of specimens and preparations of extracts varied as follows:

Part I. A fish (sex not determined) was taken from the collecting bucket on each of two days before the others, many still living were placed in a refrigerator. Following a 2–3-sec rinse in running tapwater to remove sea water, these two fish were weighed. Each was skinned with a knife and bare hands, the skin cut into small pieces and placed in a beaker which next received the fish then a dilute HCl rinse, and passed through a meat grinder. Each beaker was brought to 100°C and simmered 5 min, then refrigerated for more than 4 hr before the supernatant was decanted.

Part II. Within 1 hr of netting the last fish, six fish were given a 2–3-sec tapwater rinse, then skinned using cutting board, knife, and hands. Three of these skins were then minced with the knife while the other three were left whole. Minced skins were placed in one beaker, whole skins in another, and to each was added dilute HCl before stirring and refrigeration. Part of the supernatant was decanted for assay at 15 hr, the beakers were again stirred, and the rest decanted at 22 hr of refrigeration. This supernatant was designated 1 : 2 dilution. Heat was

Fig. 1. Effects of tetrodotoxin (~LD_{50}) upon *Fundulus heteroclitus.* Three light-adapted killifish over a light background, 2–5 min after i.p. injection: left anterior sector beginning to darken (top); left anterior and right posterior sector darkened (bottom); all four sectors dark, midsection still light (center) just prior to onset of other toxic signs. (Credit: Photo by Lauro Boccioletti.)

not used in its preparation. The same skinned fish were then pooled, and a separate beaker used to receive (a) ovaries, (b) male liver, and (c) female liver. Cells of each were disrupted in an A. H. Thomas no. 4288-E pyrex glass tissue grinder, and material plus dilute HCl tube rinsings returned to separate beakers, simmered 30 min, and cooled. The contents of each beaker were centrifuged for

5 min but the male liver preparation still yielded only turbid supernatant, and the female liver no distinct supernatant. Ovary and male liver supernatants were collected and refrigerated.

Part III. Five fish were removed from the refrigerated bucket within 2 hr of netting the last fish, with three (1 male, 2 female) given the same 2–3-sec tap-water rinse, while the other two (both male) were not rinsed. Thereafter, the rinsed male and females and unrinsed fish were kept separate, skinned, and the skins cut in ~1 cm^2 pieces using knife, cutting board, and hands, as in parts I and II. After addition of dilute HCl, each batch of skins was simmered ½ hr, cooled, and supernatant (designated 1 : 2 dilution) decanted.

Part IV. Each fish was removed from the polyethylene bag with forceps, placed in a mortar and skinned by means of dissecting scissors and forceps without hand contact, and then weighed. The skin of each fish plus dilute HCl of equal volume used to rinse bag and mortar was placed in a beaker, stirred, and refrigerated for 10 days. Supernatant was decanted and saved, then 5 successive 10-ml aliquots of distilled water added, stirred, drained off, and saved. These volumes were:

Supernatant decanted after	Designated as rinse number	Normal	Lethargic
Incubation in dil. HCl	1	4.3 ml	5.0 ml
First aliquot (dist H_2O)	2	7.5 ml	8.3 ml
Incubation + 1st aliquot	1 + 2 (combined)	11.8 ml	13.3 ml
2nd + 3d aliquot	3 + 4 "	21.6 ml	21.7 ml
Fourth aliquot	5	10.0 ml	10.0 ml
Fifth aliquot	6	10.0 ml	10.0 ml

Each sample from each skin was centrifuged free of suspended matter and adjusted to pH 7.

Part V. Odd numbered ones of the 20 netted blowfish were, by means of surgical clamps and scissors, taken from the net and skinned alive without contacting the collector's hands or other surfaces, and these ten skins were placed in one container. Even numbered ones were placed in a small bucket and processed within 2 hr in the same manner as the rinsed specimens in part III, except that the skins were left whole and raw and placed in a second container. These two pooled samples were kept frozen for air transport to and storage at the New Jersey location until 15 July 1974, when they were thawed and extracts prepared. Following the addition of 1 ml dilute HCl/g of skin, each sample of 10 whole skins was stirred for only 5 min before the supernatant was decanted, adjusted to pH 6, and centrifuged; however, before this was done, 1 ml of liquid was removed by pipette from each bottle of thawed skins, centrifuged, and the supernatant stored. Assays of all samples were done on 16 July 1974.

Test Animals. For parts I–IV, white mice were fed a Purina diet and segregated by sex one or more months before use. Most were 6–12 months old and weighed more than (>) 30 g when used. One ml volumes were i.p. injected.

For part V, killifish (*F. heteroclitus*) were trapped on 13 July 1974 from Holmes Cove, Cape May County, New Jersey. Maintenance in seawater and injection techniques are given elsewhere (McLaughlin and Down, 1969). Size range was 2–4 g. They were placed over a light background for ½ hr before injection. Air and water temperatures during assay were 27–29°C. Times of earliest visible melanophore reactions were recorded, as were the succession of sectoral changes. Death times represent irreversible cessation of visible opercular movements. All killifish were drained of excess water and weighed immediately.

LD Definition. Because of a limited supply of mice and an absence of *F. heteroclitus* in the area at which the blowfish were collected, no attempt was made to establish LD_{50}'s except in part V. Lethal dose (LD) as used in parts I–IV is defined as the least amount of material which caused death of all animals into which it was injected. Considering the steep dose-response relation of tetrodotoxin (Kao, 1966), enough serial dilutions were made and enough mice injected to assure that this LD, in μg/kg, is no more than 50% greater than the LD_{50} would have been for any individual determination, and no more than 25% greater than the LD_{50} for any one tissue assay. The recording of mean death times of test animals for each dilution of each extract gives further basis for comparison of results within this study. The mouse unit (M.U.), herein referred to is based upon a 10-min death time. Most death times observed in this study were less than 10 min; thus, the LD, as used in this study, represents a less precise and generally greater amount of tetrodotoxin than the LD_{50} or the M.U.

RESULTS

Whole Fish Assays. Fish 1 weighed 60 g; fish 2, 70 g. For fish 1 the LD for average 28-g mice was a 1.0-ml solution derived from 50 mg of fish (see Table 1) Thus, fish 1 contained 1200 LD for 28-g mice, equivalent to ~1680 LD for 20-g mice. For fish 2, the LD for average 26-g mice was a solution from 27 mg of fish (see Table 1); thus, fish 2 contained ~3276 LD for 20-g mice. Shorter average death times for pH 3 than for pH 7 solution were observed only when injected in less than the LD.

Fish Organ Assays (Skins Rinsed). Toxicity of ovary and male liver extracts were determined for comparison with rinsed skin toxicity (see Table 2). Ovaries (ripening) were from two fish and totalled 10 g. Owing to the turbidity and small yield of liver supernatant, the relatively low toxicity found was probably not reliable. The LD for 33-g (average) mice of 1-ml solution derived from 62 mg ovary (i.e., 16 LD/g) is equivalent to 26 LD for 20-g mice per g of ovary.

Table 1. The Toxicity of Extracts from Rinsed Whole Blowfish (*Spheroides nephelus*)

	Fish 1							Fish 2				
Toxin dilution	1:2	1:8	1:16	1:20	1:20[a]	1:24	1.24[a]	1:16	1:20	1·32	1:36	1:40
Number of mice injected	1	2	2	10	5	7	7	1	3	3	4	5
Number of survivors	0	0	0	0	0	2	2	0	0	0	0	3
Average weight, g, (nonsurvivors)	30	25	28	28	30	28	28	25	23	24	26	22
Mean death times, min	4.5	5.2	7.7	10.5	11.0	14.8	8.2	5.2	4.7	8.1	7.0	22.8

[a]pH 3: all other solutions were pH 6–7.

For rinsed skins, two variables were introduced; (1) supernatant from minced and whole skins was assayed (2) after contact with tissue for 15 hr and again after 22 hr (see Table 2). Surprisingly, the yield was higher for whole than for minced skins, with the toxin concentration from whole skin supernatant being no greater after 22 hr than after 15 hr: therefore, 22 hr minced-skin supernatant was not tested. Combining the results for whole skin regardless of incubation time (8 mice used), the LD for 26-g mice of a 1.0-ml solution derived from 31 mg of whole skins (i.e., 32 LD/g) is equivalent to ~42 LD for 20-g mice per g of skin, vs 26 for ripe (sexually mature) ovary.

Rinsed vs Unrinsed Skin Assays. Without otherwise altering the methods of fish collection or supernatant preparation, and without guarding against loss of toxin into collecting bucket, hands or utensils, the LD in 35-g mice of 1-ml solution derived from 16 mg *unrinsed* skin (i.e., 64 LD/g) is equivalent to ~112 LD for 20-g mice per g of skin, vs values of 47 and 28 LD's for 20-g mice per g of skin in the one male and two female *rinsed* skins, respectively (see Table 3).

Assays of Skin Protected from Toxin Loss during Collection and Extraction. Skins of the "normal" and "lethargic" blowfish weighed 12.3 and 14.3 g, respectively. Definition of each "rinse" and volumes of rinses were as described in Methods. Dilutions (in distilled water) of each of these full-strength rinses are given in Table 4 as 1 ml of full strength rinse to total ml of solution. Results given in Table 4 show approximate LD's for the mice used. Only one mouse was used for each dilution of each rinse for each sample, except for the 1:60 dilution of rinse 1 + 2, for which two mice per sample were used; therefore, values are only approximate. To determine the approximate minimum number of LD's for 20-g mice for each rinse, the reciprocal of the highest dilution which proved lethal × weight of mouse used ÷ 20 was calculated (see Table 4). To estimate the amount of toxin remaining in skin, the same concentration present in rinse 6 was

Table 2. The Toxicity of Ovary, Liver, and Rinsed Skin Extracts of the Blowfish (*Spheroides nephelus*)

	Ovary					Liver, male	Skin, minced, 15 hr		Skin, whole, 15 hr			Skin, whole, 22 hr	
Toxin dilution	1:4	1:8	1:16	1:20	1:24	1:2	1:16	1:32	1:16	1:32	1:48	1:32	1:48
Number of mice injected	1	2	4	5	4	4	1	4	1	4	2	4	4
Number of survivors	0	0	0	3	4	1	0	4	0	1	2	0	4
Average weight, g, (nonsurvivors)	30	30	33	29	–	22	30	–	33	26	–	26	–
Mean death times, min	3.2	4.5	7.5	11.2	–	31.7	6.7	–	5.7	7.5	–	8.8	–

Table 3. The Toxicity of Rinsed vs Unrinsed Skins of *Spheroides nephelus* Prepared without Regard to Possible Toxin Loss

	Rinsed								Unrinsed, male				
	Male					Female							
Toxin dilution	1:8	1:16	1:28	1:32	1:64	1:16	1:20	1:32	1:8	1:32	1:64	1:80	1:96
Number of mice injected	1	5	3	6	2	2	3	2	2	2	4	5	3
Number of survivors	0	0	0	2	2	0	2	2	0	0	0	3	2
Average weight, g (nonsurvivors)	30	36	36	34	–	35	34	–	31	34	35	30	21
Mean death times, min	4.5	4.7	6.7	7.5	–	9.0	9.7	–	2.5	4.3	8.2	7.0	21.6

Table 4. The Toxicity of Skin Extracts of *Spheroides nephelus* Protected from Toxin Loss during Collection and Extraction

Rinse number	Normal blowfish				Lethargic blowfish			
	Highest lethal dilution	Mouse weight, g	Rinse volume, ml	Minimum number of LD's	Highest lethal dilution	Mouse weight, g	Rinse volume, ml	Minimum number of LD's
1 and 2	1:40	29	11.8	684	1:60	33	13.3	1317
3 and 4	1:10	30	21.6	324	1:20	34	21.7	749
5	1:7.5	37	10.0	139	1:15	34	10.0	255
6	1:4	35	10.0	70	1:6	31	10.0	93

multiplied by the original weight of each skin to the nearest g. The total approximate toxicity, in LD for 20-g mice, was 1301 and 2544 for skin from the normal and lethargic fish, respectively, or ~106 and ~178 LD/g, respectively.

Pooled Skin Samples (A) Protected and (B) Not Protected from Toxin Loss during Collection and Extraction, Assayed in *F. heteroclitus*. Killifish were so selected that, for each trial, there was an approximately even gradation of size from the smallest animal (just over 2 g) to the largest (4 g or under). Injections were first made into 3 killifish per dilution for each extract, up to that dilution which caused sectoral darkening (Fig. 1) in all specimens, but from which 1 or 2 animals survived. Each dilution so selected was then assayed in 6 killifish of graded size. If 2–4 killifish survived, that dilution was assayed in another 6 killifish, and if the LD_{50} was not thereby established, the procedure was repeated for an intermediate dilution.

Using dilutions of the pH-adjusted dilute HCl extracts, 0.1-ml solution derived from 1.25-mg unprotected skins killed 2 of 6 killifish of 3.5 g, and 4 of 6 killifish of 3.1-g average weight, giving an LD_{50} for unprotected skins of 0.38 mg/g, or 380 mg/kg: 0.1-ml solution derived from 0.42 mg protected skins killed 3 of 6 killifish of 3.0 g and 3 of 6 killifish of 3.3-g average weight, giving an LD_{50} for protected skins of 0.13 mg/g, or 130 mg/kg. Assays of the 1 ml of liquid removed by pipette from each bottle of thawed skins prior to addition of dilute HCl were performed in like manner and gave comparable results.

DISCUSSION

Most species within the order Tetraodontiformes are easily captured, so there is little justification for physically or chemically injuring the fish in the process of capture and thus introducing possible error in subsequent toxin assay. Many species are easily netted (personal experience). Halstead (1967) reports that a night light can attract *S. annulatus* to the surface by the hundreds. Even when captured without trauma, it is difficult to prevent possible contamination of gills

and meat surface by toxin so readily released from skin, while swallowing by the fish either into the saclike ventral diverticulum of the stomach in the attempt to inflate or in true swallowing may result in contamination of viscera with toxin from skin. Relatively high toxin levels in liver and intestine would suggest that blowfish acquire rather than manufacture their toxin (Halstead and Bunker, 1954), but it is possible that high toxin levels found in the intestine may be due to swallowed skin toxin. Halstead (1967) reports that in studying gut contents of poisonous fish, Dawson *et al.* found more algae than any other type of food, with *Lyngbya* in a large majority of samples. Habekost *et al.* (1955) reported finding toxic substances in aqueous extracts obtained from a number of tropical and temperate marine algae. Assay for toxicity of marine algae species predominant in an area in which *S. nephelus* are toxic has given negative results (Larson and Humphries, 1969). Blowfish used in this study and in the three referenced works by Larson and colleagues were thought to be *Spheroides maculatus* until it was established by Shipp and Yerger (1969) that they represent a separate species.

Cameron and Endean (1973) state, "although tetrodotoxin or something akin to it occurs in the skin of many puffer fishes, the presumed glandular source of the toxin has been described in the epidermis of only one species." However, consideration should be given to the possibilities that tetrodotoxin is (1) produced by the skin (or elsewhere in the animal), with or without need for specific precursor(s), (2) useful to tetraodons for devastation of large potentially voracious predators, (3) present in other organs than skin only by overflow from skin, or (4) found in relatively high concentration in the liver because that is where it is detoxified. Relatively high liver concentration might be expected whether the toxin is acquired or produced intra- or extrahepatic. The countless spicules which give the skin of *S. nephelus* a sandpaperlike roughness may function to abrade the alimentary mucosa of predators, to further speed absorption of released toxin. This study did not determine whether or not the observed high levels of skin toxin were present before the blowfish were subjected to extreme stress. This should be determined not only because results would bear upon the above possibilities, but also to determine whether or not toxin could be harvested repeatedly from captive animals.

Variability from fish to fish in total or separate organ toxicity is a well documented fact. Regarding Tani's studies, Kao (1966) states that the toxicity of all tissues tested from male fish is significantly lower than that of the corresponding female tissue, and that in females the concentration of toxin in liver and in ovary (even per gram) increases markedly as the fish enter spawning season. According to Halstead (1967), tests in Brazil by Fonseca of *S. testudineus* showed that the toxicity level varied with each specimen, and the liver decreased in toxicity during the reproductive period whereas gonadal toxicity increased. That such high toxicity from skins protected from toxin loss during collection and extraction

was found in this study using males collected out of spawning season (part IV) indicates that for fish with ripening gonads assayed in parts I-III more toxin may have been lost from the skins during collection and handling using routine methods than remained in the entire fish. Differences in toxicity between fish 1 and 2 in part I and in skin toxicity between the "normal" and "lethargic" fish in part IV probablv reflect variability in toxin level of fish prior to capture rather than artifacts from handling. In part II, the higher yield of toxin from whole vs minced skins may have been due to variations in toxicity of the three fish used for one sample vs the three fish used in the other, but probably was due to greater toxin loss onto utensils during mincing.

In part IV, the small volumes of the first rinses resulted from evaporation during the ten-day refrigerated incubation, and slight decrease in recovery of rinse after addition of the first aliquot probably due to incomplete drainage. Thus, rinse 1 (supernatant) for each skin equals $\sim 1/4$ the total volume of skin plus supernatant, and assuming toxin concentration was uniform between tissue and supernatant, should have contained $\sim 1/4$ the total toxin. Rinse 2 equals $\sim 1/3$ the total volume of skin plus solution, and should have contained $\sim 1/3$ of the remaining $3/4$ of the toxin, or $\sim 1/4$ of the total toxin. Rinses 1 and 2 for each skin should have, and did in fact contain very near this expected $1/2$ of the total toxin. Calculations of total toxicities in parts I-III were based on the assumption that toxin concentration was approximately uniform between tissue and supernatant. This was also confirmed by part V, which showed that stirring whole skins in an equal volume of dilute HCl at room temperature for only 5 min equally partitioned the toxin between skin and supernatant.

Toxin concentration in the "lethargic" fish in part IV was greater than that for any other sample assayed. This may have been unrelated to behavior, but tons of seaweed decomposing in the shallows probably decreased the oxygen tension of the water, and fish highest in tetrodotoxin might succumb first to hypoxia. Or, disease in the lethargic fish could have been caused by toxins or infection from microorganisms flourishing in the decomposing vegetation, resulting in decreased detoxification/excretion of tetrodotoxin, which could then have accumulated, and may have reached disproportionate toxic levels in organs other than skin. Unfortunately, organs other than skin were not assayed for animals in part IV, nor have hypoxia-prone vs hypoxia-resistant blowfish been assayed for tetrodotoxin.

Tetrodotoxin and saxitoxin are the only organic agents known to interfere with initial transient Na^+ flux without concurrent effect upon final steady K^+ flux (Kao 1966). Evidence that increased extracellular K^+ probably opposes passive inward movement of Na^+, outward movement of K^+ has been reviewed (Down, 1972). In earlier work (Down, 1972) drugs of every major classification and many inorganic salts were systemically administered to *F. heteroclitus*; sectoral darkening regularly resulted only from tetrodotoxin, saxitoxin, and hyper-

tonic K^+, and it was concluded that this uniquely patterned pigment-motor system reaction is dependent upon the $Na^+ > K^+$ blocking effect. Sectoral darkening observed in part V upon i.p. injection of > 0.5 LD_{50} was the same as in earlier work using purified tetrodotoxin (Down, 1972). Thus, the toxic principal in skin extracts assayed throughout the present work is probably tetrodotoxin; otherwise, it probably represents a third substance in this new, Na^+ blocking, group of compounds. Toxin values greater than 3X those reported by others for skin of *S. nephelus* from the same general area and ~3X those for unrinsed (part III) and protected (part V) skins, when compared with rinsed and unprotected controls, illustrate the unexpectedly high toxin concentrations in, and the rapidity with which they can be lost from, *S. nephelus* skin.

The mouse unit (M.U.) used at Stanford for tetrodotoxin/tarichatoxin testing is defined as the amount needed to kill a 20-g mouse in 10 min, and one such M.U. = 143-ng pure toxin (7000 M.U./mg) (Kao, 1966). The susceptibility of several different strains of mice showed no substantial variability within the resolution of their method. Kao (1966) states, "... the minimal lethal dose, given i.p. to 20 g mice is 8 μg/kg; with 12 to 14 μg/kg all the mice used in the assay procedure are killed... The LD_{50} estimated graphically is 10 μg/kg in mice." The minimum dose of i.p. tetrodotoxin lethal to all *F. heteroclitus* (weighing not over 6 g) injected has been determined to be ~0.005 μg/g (5 μg/kg) (McLaughlin and Down, 1969). If, as indicated by Kao's figures, the LD_{50} is ~75% of the amount which is lethal for all animals injected, then the LD_{50} in *F. heteroclitus* is ~3.75 μg/kg. Thus, in part V unprotected skins contained ~9.3 μg/g, or ~0.1 mg per whole fish skin, while protected skins contained ~28.8 μg/g, or ~0.3 mg per whole fish skin vs more than 46 μg/g for the skin of the lethargic fish in part IV. But the sensitivity to crude tissue extracts is lower than to purified tetrodotoxin. For this reason and because of factors as discussed in Materials and Methods, conversion of results in this study to microgram of toxin, except as lower limits, is not advised.

SUMMARY

The concentration of toxin found in *S. nephelus* skin was more dependent upon the degree of precautions taken against inadvertant toxin loss, from capture through extraction, than it was upon such factors as (1) season of collection (before, during, or after spawning season), (2) year of collection, (3) calm, favorable vs lethal water conditions, (4) apparent state of health of specimen, (5) estuary from which collected, (6) one vs 10 skins per sample, (7) whether processed immediately or after two-plus yrs frozen storage, (8) whether skins were minced or left whole, or (9) whether dilute HCl eluent was decanted after 5 min or more than 20 hr, with or without heating.

Pigment-motor system response of *F. heteroclitus* to skin extracts indicates that the toxin involved is probably tetrodotoxin, and it therefore seems likely that tetrodotoxin, while also present in viscera, is an ichthyocrinotoxin.

Blowfish to be used for toxin assay should be captured quickly without physical or chemical trauma, taken directly from net or other collecting device by the use of surgical clamps and skinned immediately without contacting the hands of the collector or any other surface, or each fish should be transferred directly into a separate dry plastic bag, skin removed within minutes (before the fish can swallow mucous secretions or other liquid contacting the skin), all surfaces contacted by the outside of the skin rinsed with 2–3 aliquots of dilute HCl totalling, in milliliters, 1 or 2 times the weight in grams of skin, and rinses added to the skin sample. Using the first method, accurate measurement of total animal and differential organ toxicity is possible, while using the second, meat and gills may be subject to toxin contamination from skin and skin subject to some toxin loss, but contamination of other organs is minimized.

Few land animals release overpowering amounts of potent chemicals into the fluid environment for defense or as a side-product of death throes or of living (notable exceptions, man and skunk), but for marine organisms it may be more common than we imagine. Allowances for this fact should be made in *all* marine collecting. Only rarely should the marine biological scientist delegate collecting of his material to others.

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Histopathological Changes in the Liver of Fishes Resulting from Exposure to Dieldrin and Lindane

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INTRODUCTION

Laug *et al.* (1950) has argued that histopathological changes could be detected in the liver of rats at concentrations of 5 ppm of DDT. Mathur (1962a,b, 1965, 1972) argued that lesions could be detected in the liver of fish even at very low dosages of BHC (10 ppm), lindane (5 ppm), and dieldrin (5 ppm), but at higher dosages these were fairly marked.

EXPERIMENTAL METHODS

For histopathological studies, tissues were removed from the affected liver of the following fish: *Channa punctatus*, *Heteropneustes fossilis*, *Puntius sophore*, and *Trichogaster fasciatus*. No pathological changes similar to those of experimental livers were noted in the controls (Fig. 1).

The liver was fixed in 10% formalin, Zenker-Helly, Bouin's fluid, and Carnoy fixatives. The sections were cut at 6-8-μ thick and stained with Mallory triple, hematoxylin, and eosin.

RESULTS

The histopathological changes caused by dieldrin and lindane in the liver of fishes are described below.

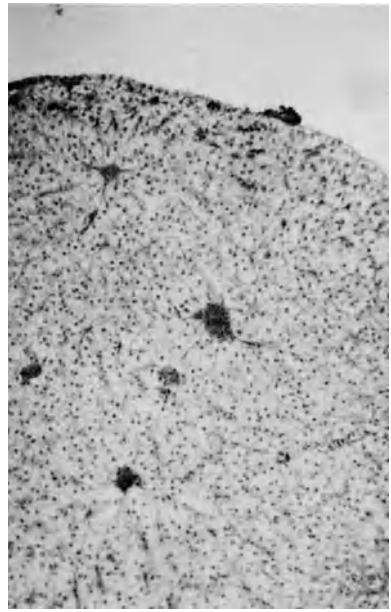

Fig. 1. Photomicrograph of T.S. of normal fish liver cells, showing the usual pattern of cell size and cytoplasmic granules (control slide), with 10× objective and 10× eyepiece.

Lindane

Channa punctatus. The hepatic cells were moderately vacuolated, having small and large vacuoles. In a small area, there was slight atrophy of cells in the peripheral and central regions. The common pathological findings reveal variable changes in the liver such as necrosis or atrophy or a combination of both. The central hepatic cells were stained less deeply than the peripheral ones. The most common lesion was degeneration of the liver cells of variable characters. There was little alteration of the nuclei. The original polygonal shape of the liver cells was deformed and the whole area of cells looked like a complicated mess. The peripheral cells were more dense (Fig. 2).

Heteropneustes fossilis. The affected liver cells were found scattered. At higher dosages, margination was characteristic. Margination tendency of cells was characteristically associated with cell hypertrophy. There was necrosis of hepatic cells. The hepatic cells were moderately vacuolated. The center of the cells appeared empty due to loss of cytoplasm (Fig. 3). The change in parenchymatous cells consisted of margination of the cytoplasmic granules. At places in peripheral cells, there was much accumulation of stain, and these cells were more dense. At a few places the cells completely degenerated.

Trichogaster fasciatus. The hepatic cells were markedly vacuolated, the vacuoles being small and large. Due to degeneration of cytoplasm, the center of the cells became hollow. At places there was also degeneration of hepatic cells.

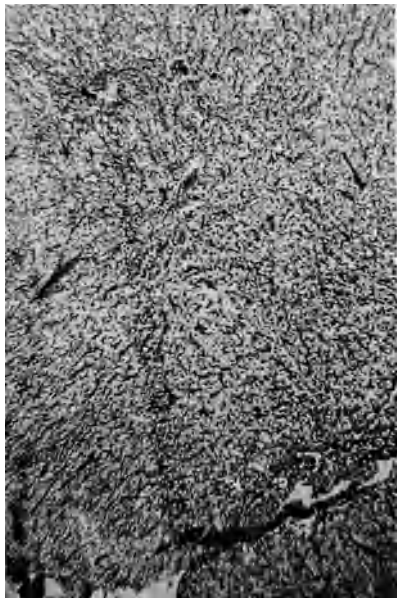

Fig. 2. Photomicrograph of T.S. of the liver of *Channa punctatus* intoxicated with lindane, with 10X objective and 10X eyepiece.

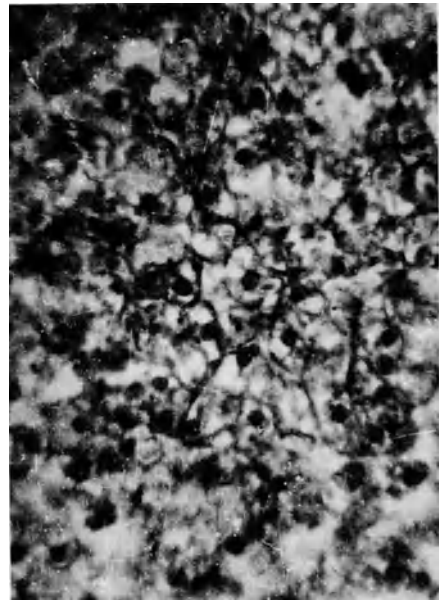

Fig. 3. Photomicrograph of T.S. of the liver of *Heteropneustes fossilis* intoxicated with lindane, with 45X objective and 10X eyepiece.

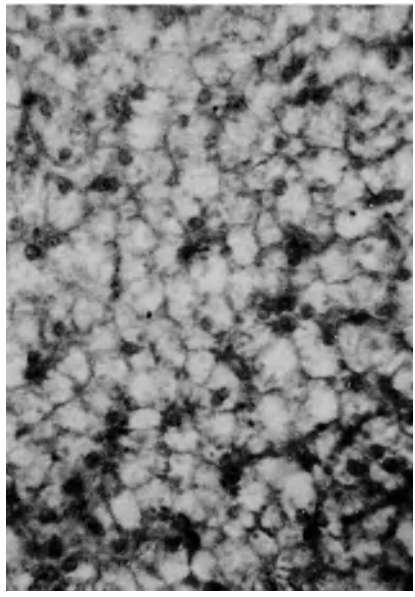

Fig. 4. Photomicrograph of T.S. of the liver of *Trichogaster fasciatus* intoxicated with lindane, with 45X objective and 10X eyepiece.

The liver cells revealed well-marked cytoplasmic alterations. Occasional liver cell abnormalities such as margination and hypertrophy were found. The lesions were more marked in the central area. Few peripheral cells showed variations in sizes, many being shrunken (Fig. 4).

Puntius sophore. The hepatic cells showed marked degeneration and at places the cells had disappeared completely. Necrosis was well pronounced. The chief alteration was hypertrophy of the cells.

Dieldrin

Channa punctatus. The hepatic cells were vacuolated. The cytoplasm of the cells disappeared and due to its absence the cells became hollow and appeared like vacuoles. The most remarkable lesion was degeneration of the liver cells of variable nature, but generally vacuolar degeneration of cytoplasm of the liver cells. The lesion occurred more often in the central lobular area. Necrosis was localized but at places it was well marked. The nucleus was slightly displaced from its original position. The chief alteration was hypertrophy of the cells. In severely affected cases, the center of the cells appeared empty and such changes did not occur at very low dosages (Fig. 5).

Heteropneustes fossilis. The affected liver cells were seen scattered, when the changes were pronounced. There was clumping of the cytoplasmic granules.

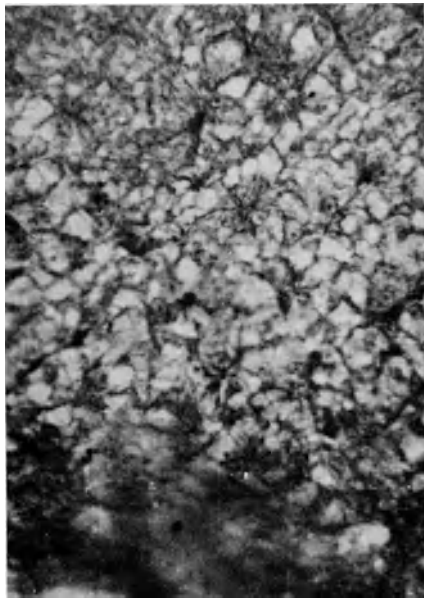

Fig. 5. Photomicrograph of T.S. of the liver of *Channa punctatus* intoxicated with dieldrin, with 45X objective and 10X eyepiece.

Fig. 6. Photomicrograph of T.S. of the liver of *Heteropneustes fossilis* intoxicated with dieldrin, with 10X objective and 10X eyepiece.

The hepatic cells in the center had slight atrophy. Necrosis was localized. The lesion occurred in the central rather than in the peripheral area and the main lesion was degeneration of liver cells; the vacuolation was not marked. There was no disintegration of the cytoplasm of the cells in the form of vacuoles (Fig. 6).

Trichogaster fasciatus. The hepatic cells were moderately vacuolated. The chief lesion was degeneration of the cytoplasm of the cells in the form of vacuoles. In severe cases, the center of the cells appeared empty. At few places localized necrosis was very marked. Hypertrophy was not noticed. At places a few hepatic cells had disappeared.

Puntius sophore. The hepatic cells were slightly vacuolated, the vacuoles being very small. Intercellular spaces developed due to the loss of cells in the hepatic region. Localized necrosis was noticed in this case also. Hypertrophy and accumulation of stain in the cells were very prevalent.

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