CHEMISTRY AND PHARMACOLOGY OF POLYPEPTIDE TOXINS IN SNAKE VENOMS[1,2]

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Increasing interest in animal toxins, especially those from snake venoms, has generated many significant contributions to the literature on this field. During the past five years three major international symposia on animal toxins have been held (1–3), and numerous reviews pertaining to snake venoms have appeared in several recent publications (4–7), including one in the Annual Review of Pharmacology (8).

The present review will concentrate on the chemistry and pharmacological properties of polypeptide toxins purified from snake venoms. Although some of the neurotoxins such as β-bungarotoxin and crotoxin, whose molecular weights exceed 10,000, may not be called polypeptide toxins, they are included in this review for the sake of convenience, especially from the pharmacological point of view. For information on toxic proteins of large molecular size such as hemorrhagins, coagulant and anticoagulant principles, the reader is referred to the recent reviews of Rosenfeld et al (9), Jiménez-Porras (10), Tu (11), and Ohsaka (12).

CHEMISTRY OF POLYPEPTIDE TOXINS FROM ELAPID AND HYDROPHID VENOMS

Venoms of snakes belonging to the families *Elapidae* (cobra, krait, coral snake, mamba, death adder, copperhead, tiger snake, black snake, taipan, etc.) and *Hydrophidae* (many species of sea snakes) are extremely toxic and produce flaccid paralysis and respiratory failure in animals. These effects have been attributed to the so-called "neurotoxins" (NTs) contained in these venoms (cf. 8, 13–18). Besides NTs, cobra and some other elapid

[1] Abbreviations used in this review are: NT (neurotoxin); CT (cardiotoxin); DLF (direct lytic factor); ACh (acetylcholine); N-M (neuromuscular); EPP (endplate potential); MEPP (miniature endplate potential); pHi (isoelectric point at pH); CD (circular dichroism); ORD (optical rotatory dispersion).

[2] The author's research cited in this article was supported by the U. S. Army Research and Development Command Research Grant DM-MD-49-193-64-G108, DA-MD-49-193-66-G182, DA-CRD-AFE-S92-544-67-G89, DA-CRD-AFE-S92-544-69-G138, DA-CRD-AFE-S92-544-71-G174, and by the National Science Council, Republic of China.

venoms contain strongly basic polypeptides which are responsible for various pathophysiological effects caused by these venoms. They have been variously named as cardiotoxin (19, 20), the direct lytic factor (DLF) (21, 22), cobramines (23, 24), cytotoxin (25, 26), and toxin γ (27, 28). There is ample evidence to indicate that they are either the same substance or at least a family of homologous polypeptide toxins (see section on the Cardiotoxin Group).

Cobra and Sea Snake Neurotoxins

Among the best chemically and pharmacologically characterized snake venom constituents are the cobra and sea snake NTs. They are remarkably similar in their chemical structures and identical in their mode of action, and therefore may be regarded as "isotoxins." In many cases, more than one NT has been isolated from the same venom (20, 23, 29–34), and a difference in NT composition of the same species venom from different sources has also been reported (31, 32).

The elapid and hydrophid NTs so far isolated are all basic polypeptides with pHis above 9.0 (29–38), remarkably heat-stable in acid medium (32, 39–41), and most of them are dialyzable (35, 42, 43). Many of them have been purified as nonenzymatic, as homogeneous by three or more criteria for purity (29–39), or as crystalline preparations (30, 38, 39, 44).

Amino acid composition.—Cobra and sea snake NTs can be grouped into two families of homologous polypeptides according to the number of their amino acid residues. NTs belonging to the first group are composed of 61 or 62 residues of 15 or 16 amino acids in a single peptide chain cross-linked by four disulfide bridges, whereas those belonging to the second group consist of 71 residues of 17 or 18 amino acids with five disulfide bridges (Table 1). All of the sea snake NTs so far isolated belong to the first group (30, 32, 37, 38, 44). While the principal NTs found in the majority of cobra species are also composed of 61–62 amino acid residues (33, 35, 36, 45, 46), toxin α from *Naja nivea* venom (33), toxin III from *N. haje* venom (31), and the principal NTs from the venoms of *N. naja naja* and *N. naja siamensis* (34) have recently been shown to consist of 71 residues. Three minor NTs consisting of 61–62 residues are also found in the *N. naja siamensis* venom and one of them appears to be nearly identical to cobrotoxin of *N. naja atra* (34). It is surprising to find that the amino acid composition of the principal NTs of Indian (*N. n. n.*) and Thailand (*N. n. s.*) cobras is quite different from that of cobrotoxin of Formosan cobra (*N. n. a.*) (45). The classification of Formosan cobra as a subspecies of *N. naja* may not be justified.

Cobra NTs belonging to the first group (Type I) are devoid of alanine, phenylalanine, and mostly also of methionine (31, 35, 36, 45, 46). Sea snake NTs are also devoid of one (38) or mostly two (30, 32, 37, 44) out of these three hydrophobic amino acids. In contrast, the second group NTs (Type II) contain two or three residues of alanine and three residues of phenylalanine (31, 33, 34). Another striking difference between the first and second

group NTs can be found in their content of glutamic acid and valine. While the first group NTs are rich in glutamic acid but poor in valine, the second group NTs are just the opposite. The abundance of these hydrophobic amino acids in the second group NTs appears to account for the irreversibility of their neuromuscular (N-M) blocking action.

On the other hand, all of these NTs are rich in basic amino acids, the number of total residues of lysine and arginine ranging from 7 to 13, and are also rich in aspartic acids, mostly in the amide form, a property accounting for their strong basicity. The content of threonine and serine is also high, the total aliphatic OH-groups ranging from 8 to 14. Another common feature is the presence of one residue of tryptophan and one or two residues each of tyrosine, histidine, and leucine. Some cobra NTs are even devoid of histidine (31) or leucine (31, 33). The C-terminus of the first group NTs is mostly asparagine (33, 35, 36, 44–47), whereas that of the second group NTs appears to be variable (33, 34). The N-terminus of most Type I cobra NTs is leucine (33, 35, 36, 45, 46), whereas that of Type II cobra NTs is isoleucine (33, 34) and that of sea snake NTs is mostly arginine (47).

Amino acid sequence.—The primary structures of several cobra and sea snake NTs have been determined (33, 36, 45–48). All of them consist of a single peptide chain cross-linked by either four or five disulfide bridges. The position of disulfide bonds in cobrotoxin (49) and erabutoxins (50) has recently been determined. It is noteworthy that four disulfide bonds that maintain the polypeptides in their native configuration are in the same positions in both cobra and sea snake NTs. As shown in Figure 1, besides the disulfide bonds, the following 22 amino acid residues are common to eight NTs whose primary structures are known: Asn-5, Gln-6, Gln-10, Ser-8, Ser-9, Thr-13, Gly-20, Tyr-25, Lys-27, Trp-29, Asp-31, Arg-33, Gly-34, Glu-38, Arg-39, Gly-40, Gly-42, Pro-44, Val-46, Lys-47, Gly-49, and Asn-61. Among them, Tyr-25, Trp-29, and Lys-47 have been shown to be essential for the toxicity of these NTs. A striking feature of the sequences is repeated clustering of the same or similar amino acids in their molecules. The central region of the molecule from the 25th to 40th residues, containing most of the basic and aromatic amino acids in close order, has been speculated to be the "active site" of the molecule (48).

The similarity in amino acid sequence is found not only among the first group NTs but also between the first and second group NTs assuming that three residues are deleted between Cys-3 and Cys-17, four residues including two half-cystines are inserted between Trp-29 and Arg-33, and another two residues are inserted between Cys-41 and Cys-43 of the first group NTs. Thus, the locations of eight half-cystines and nine other residues (Gly-20, Tyr-25, Lys-27, Trp-29, Arg-33, Gly-34, Gly-40, Pro-44, and Asn-61) shown in Figure 1 are found to be identical in the first and second group NTs of known primary structures (33, 34). It is obvious that the 71 residue NTs have evolved from the 61–62 residue NTs.

Neurotoxins from *Bungarus* (Krait) Venom

Among various elapid venoms, except cobra venom, the venom of *Bungarus multicinctus* appears to be the only one whose toxins have been chemically and pharmacologically well characterized. Two different types of NTs have been isolated from this venom (51). One called α-bungarotoxin produces an irreversible N-M block of antidepolarizing type. The two most electropositive fractions, called β- and bungarotoxin respectively, both produce a N-M block by depressing the ACh release from the motor nerve endings. Both α- and β-bungarotoxins, purified by CM-Sephadex column chromatography followed by rechromatography on CM-cellulose column, were found to be free from any enzyme activities present in the crude venom (Lee et al, unpublished). The primary structure of α-bungarotoxin has recently been determined (52). It consists of 74 residues of 18 amino acids in a single chain cross-linked by five disulfide bridges. Striking similarities can be found between α-bungarotoxin and Type II cobra NTs, not only in their amino acid compositions (see Table 1) but also in their primary structures. Thus, if deletions of two residues at the positions 14 and 15 and of one residue at the position 22 in the molecule of α-bungarotoxin are considered, about half of the total residues, including the location of ten

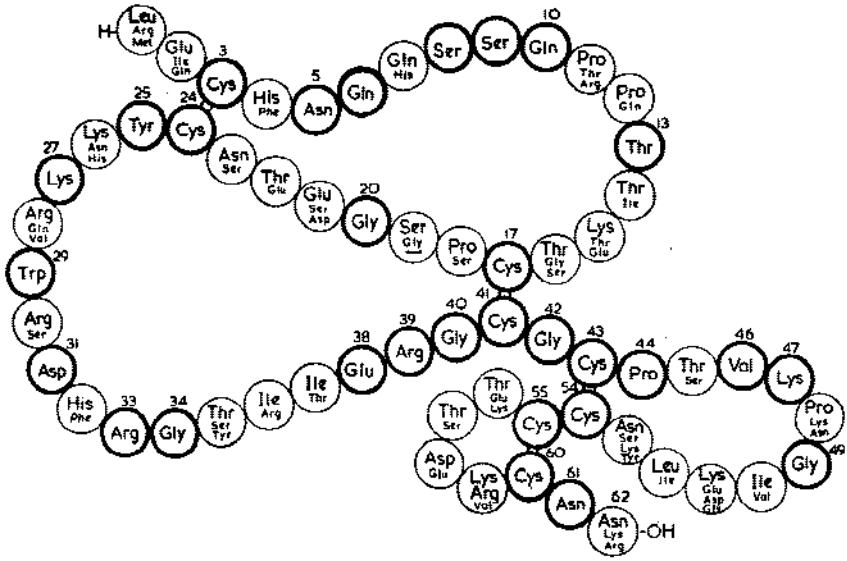

FIG. 1. Common Structure of Sea Snake and Cobra Neurotoxins (Type I). Sea snake NTs: Erabutoxins a & b from *Laticauda semifasciata* (50). Type 1 cobra NTs: Cobrotoxin from *Naja naja atra* (49), Toxin α from *N. nigricollis* (48), Toxin α from *N. haje haje* (36), Toxin δ from *N. nivea* (33), Toxin β from *N. nivea* (33), and Toxins II & IV from *Hemachatus haemachatus* (46). Heavy rings denote amino acid residues common to all of these NTs.

TABLE 1. COMPARISON OF AMINO ACID COMPOSITIONS OF ELAPID AND SEA SNAKE TOXINS

Amino acid	Sea snake neurotoxins	*Cobra neurotoxins* Type I	Type II	*Bungarotoxins* α	β	Cobra cardiotoxins (DLF, Toxin γ, etc)
Lysine	4–5	3–7	4–6	6	13	8–12
Histidine	1–2	2	0–1	2	5	0–1
Arginine	3–5	3–6	5–6	3	14	1–2
Aspartic acid	4–9	5–9	9–10	4	22	5–8
Threonine	4–8	5–9	5–9	7	12	3
Serine	6–8	2–4	3–4	6	6	2–3
Glutamic acid	7–8	5–8	1–3	5	12	0–1
Proline	3–5	2–5	6	8	8	4–6
Glycine	4–5	5–7	4–5	4	16	2
Alanine	0–1	0	2–3	5	11	1–2
Half-cystine	8	8	10	10	20	8
Valine	1–2	1–2	4–5	5	4	3–7
Methionine	0–1	0–1	0–2	1	2	2–4
Isoleucine	2–4	1–5	3–5	2	8	1–4
Leucine	1	0–2	1	2	7	5–7
Tyrosine	1	1–2	1	2	13	1–5
Phenylalanine	0–2	0	3	1	6	0–2
Tryptophan	1	1	1	1		0–1
Total residues	61–62	61–62	71	74	~179	57–62
N-terminal	Arg (mostly)	Leu (mostly)	Ile (mostly)	Ile		Leu
C-terminal	Asn (mostly)	Asn (mostly)	Variable	Gly		Asn (mostly)
Reference	(30, 32, 37, 38, 44, 50)	(31, 33, 35, 36, 45, 46)	(31, 33, 34)	(52)	(*)	(22, 74, 75, **)

(*): Narita & Lee, unpublished.
(**): Eaker & Fryklund, unpublished.

half-cystines, are identical between α-bungarotoxin and Type II cobra NTs (52). Since the mode of N-M blocking action of α-bungarotoxin is also quite similar to that of cobra NTs, α-bungarotoxin could be regarded as a variant "isotoxin" of cobra NTs. On the other hand, the amino acid composition of β-bungarotoxin (Narita & Lee, unpublished) is quite different from those of other NTs (Table 1). Its molecular weight has been estimated to be 28,500, but it might be a dimer. It is noteworthy that the circular dichroism (CD) spectrum of α-bungarotoxin (53) is similar to that of cobrotoxin (54) which contains β-structure, whereas β-bungarotoxin contains α-helical structure (53).

STRUCTURE-ACTIVITY RELATIONSHIP OF ELAPID AND SEA SNAKE NEUROTOXINS

It has been repeatedly demonstrated that the integrity of the disulfide bonds in the NT molecules is essential for their toxicity (55–57). Reduction breaks the disulfide bonds and results in complete loss of toxicity. The reduced cobrotoxin regains full toxicity upon re-oxidation (57). Cobrotoxin was first believed to contain a right-handed α-helical structure from its optical rotatory dispersion (ORD) pattern (58), but a subsequent study of its CD spectrum discloses the presence of β-structure (54). On reductive

cleavage of the disulfide bonds, cobrotoxin becomes a mixture of a large amount of random coil and a small amount of β-structure (54). The re-oxidized cobrotoxin, however, shows essentially the same ORD and CD patterns as those of the native toxin (57). All these findings suggest that the disulfide bonds are essential for holding the polypeptides in their active conformation.

Studies on chemical modification of these NTs with group specific reagents have revealed that basic amino acids, especially lysine residues, are essential for their biological activity (56, 59, 60). Probably, as in the case of *d*-tubocurarine, at least two basic groups with positive charge held at a certain distance in the molecules may be responsible for their N-M blocking action. The conclusion that lysine residues are unessential for toxicity of sea snake NTs (32) appears to be incorrect, since O-methylisourea used in this study converts the ε-amino group of lysine residue into the guanidinium group, which is still basic and therefore capable of binding with the anionic site of cholinergic receptor. Tyr-25 (60) and Trp-29 (32, 37, 38, 61, 62) have also been found to be important for toxicity, probably for maintaining the polypeptides in their active conformation. It is noteworthy that Tyr-25, Lys-27, Trp-29, and Arg-33 shown in Figure 1 are common not only to all cobra and sea snake NTs but also to α-bungarotoxin (52). This region of the molecules has been suggested to be the "active site" of NTs (48). Lys-47, an essential residue in cobrotoxin (60), is also common to most NTs except toxins 3 and 4 from *N. n. naja* venom, in which it is replaced by arginine (Eaker, personal communication). On the other hand, Tyr-35 in cobrotoxin (60) and His-26 in erabutoxin b (63) have been shown to be unessential for toxicity. Of special interest are the findings that both tryptophan (32, 62) and free amino groups (64) are unessential for antigenicity, although essential for toxicity.

While the N-M blocking action of Type I cobra NTs is slowly reversible (65, 66), that of α-bungarotoxin (51, 67–69) and toxin 3 from *N. n. siamensis* venom (70) is rather irreversible; α-Bungarotoxin and Type II cobra NTs differ from NTs with 61–62 residues in having more residues of hydrophobic amino acids, such as valine, alanine, and phenylalanine, and fewer residues of glutamic acid (Table 1). Such differences in their amino acid compositions appear to account for the difference in reversibility of their action.

Cardiotoxin Group from Cobra Venoms

The history of the isolation of cardiotoxin (19, 20), the direct lytic factor (DLF) (21, 22), cobramines (24), cytotoxin (26), and toxin γ (27) has been recently reviewed (18). They are all strongly basic polypeptides (pHi above 12) isolated from various cobra venoms. As previously assumed by some authors (13, 23), they turned out to be identical to each other, or may at least be grouped as a family of homologous polypeptide toxins (10, 18, 71, 72). Cardiotoxin (CT) is the most basic and most abundant constituent of cobra venoms, amounting to 25–55% on a dry weight basis (20, 24,

TABLE 2. AMINO ACID COMPOSITION OF CARDIOTOXIN AND ITS ANALOGS

Amino acid	*N. naja atra* Cardiotoxin	*N. naja* Cobramine B	*N. naja* CM-XI	*N. naja* CM-XII	*N. naja* F8	*N. nigricollis* F14 (Toxin γ)	*H. haemachatus* DLF	*H. haemachatus* F12B
Lysine	9	8	8–9	9	8	9	10	12
Histidine	0	0	0	0	0	0	1	1
Arginine	2	2	2	2	2	2	1	1
Aspartic acid	6	5	7	7–8	8	6	6	6
Threonine	3	3	3	3	3	3	3	3
Serine	2	2	2	2	3	2	3	3
Glutamic acid	0	0	1	0	0	1	1	1
Proline	5	4	5	5	4	6	5	5
Glycine	2	2	2	2	2	2	2	2
Alanine	2	2	2	2	2	2	1	1
Half-cystine	8	6	8	8	8	8	8	8
Valine	7	6	6	7	4	3	4	4
Methionine	2	2	2	2	3	4	2	3
Isoleucine	1	1	2	1	4	3	2	2
Leucine	6	5	6	6	6	5	6	7
Tyrosine	3	3	5	4	2	2	1	1
Phenylalanine	2	1	0	1	1	1	1	1
Tryptophan	0	0	0	0	0	1	0	0
Total	60	52	61–62	61–62	60	60	57	61
N-terminal	Leu		Leu	Leu	Leu	Leu	Leu	Leu
C-terminal	Asn				Asn	Asn	Ser	Asn
Reference	(75)	(24)	(74)		(*)	(*)	(22)	(*)

(*): Eaker & Fryklund, unpublished.

71, 73, 74). It is heat stable at acid pHs but not at alkaline pHs (24). Like NTs more than one CT has been isolated from the same venom (20, 24, 73, 74). Some of them have been purified as nonenzymatic, homogeneous or crystalline preparations (22, 24, 74, 75). The molecular weight was found to be about 6000–7000 by sedimentation equilibrium measurements (22, 24, 74, 75). A molecular weight of 10,500 was reported for cytotoxin (26), probably due to contamination with phospholipase A or other component. A stable binding of these basic polypeptides and acidic proteins of larger molecular size in the crude venom appears to account for earlier reports to ascribe depolarizing and neurotoxic actions to phospholipase A and the conflicting reports on the molecular weight of polypeptide toxins (13, 78–80).

Amino acid composition.—Toxins of the CT group are composed of about 60 (57–62) residues of 15–17 amino acids in a single peptide chain cross-linked by four disulfide bonds (Table 2). Cobramine B was apparently underestimated as being composed of 52 residues (24). Comparison of their pHi's shows close correspondence of cobramine A with fraction CM-XI and cobramine B with CM-XII purified from the same cobra (*N. naja*) venom (74). Toxin γ (27) is probably identical with CT (Fr. 14) of *N. nigricollis* venom (Boquet, personal communication). The amino acid compositions of CTs differ substantially from those of cobra NTs in the following aspects: (*a*) high content of lysine (8–12 residues) and surprisingly low content of arginine (1–2 residues); (*b*) higher contents of valine and leu-

cine and lower contents of threonine, serine, and glycine; (*c*) the absence of one to three amino acids (tryptophan, histidine, and glutamic acid) different from those missing in NTs with 61–62 residues (methionine, alanine, and phenylalanine). All these features are probably significant in determining the conformation required for the biological activity of CTs.

Amino acid sequence.—The primary structures of CTs from the venoms of *N. naja atra* (75), *N. naja* of Cambodian origin, *N. nigricollis,* and *Hemachatus haemachatus* (Eaker & Fryklund, unpublished) have recently been determined. Despite the dissimilarity in their amino acid compositions, some resemblance can be found in the amino acid sequences between CTs and NTs. Comparison of the primary structure of CT (75) with that of cobrotoxin (45) isolated from the same snake (*N. naja atra*) venom revealed that 20 residues including eight half cystines in the two toxins are identical, while 20 residues can be explained by one base change and 18 by two base changes in their genetic codons, assuming that three residues are deleted between Cys-3 and Cys-17 and two residues are inserted between Cys-41 and Cys-43 in the cobrotoxin molecule (75). In this respect, the primary structure of CT is rather close to that of Type II cobra NTs. These findings suggest that CTs might have evolved from cobra NTs despite the dissimilarity in their mode of action.

The amino acid sequences of CTs from the venoms of *N. naja* and *N. nigricollis* and DLF from *H. haemachatus* venom are surprisingly homologous to CT from *N. naji atra* venom. Only 13 out of 60 residues are different either between CTs of *N. naja atra* and *N. naja* of Cambodian origin or between CTs of *N. naja atra* and *N. nigricollis.* Even between DLF of *H. haemachatus* and CT of *N. naja* or *N. naja atra* there are only 17 differences, assuming histidine is inserted between the 3rd and 4th residues in the molecule of CTs.

Phylogenetic Relationships Among Polypeptide Toxins of Snakes

The elucidation of chemical structures of polypeptide toxins from sea and elapid snakes has made it possible to speculate on the phylogenetic relationships among these toxins. A possible evolutional tree of these toxins is shown in Figure 2.

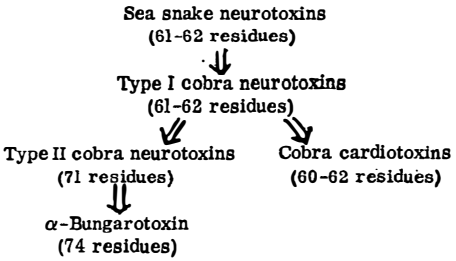

FIG. 2. A possible evolutional tree of polypeptide toxins of sea and elapid snakes.

MODE OF ACTION OF ELAPID AND HYDROPHID NEUROTOXINS

The mode of action of elapid and sea-snake NTs has recently been reviewed (16, 17). These NTs can be classified into two groups according to their mode of action. The first group of NTs produces N-M block of the antidepolarizing type similar to *d*-tubocurarine. Cobra NTs (65, 66, 67, 70, 84–86), sea-snake NTs (30, 85–88), α-bungarotoxin (51, 67–69), and probably most of the other elapid NTs (89, 90) belong to this group. The second group, as exemplified by β-bungarotoxin, produces a N-M block by acting presynaptically on the motor nerve endings, leaving the sensitivity of the endplate to ACh unaffected (51, 67). The black snake (*Walterinnesia aegyptia*) toxin has been reported to produce a N-M block of the toad gastrocnemius muscle without affecting the sensitivity of the muscle to ACh (91), but a recent study using several muscle preparations revealed that the venom of the same species from Israel produces a N-M block of the antidepolarizing type (90).

Neurotoxins Belonging to the First Group

Mode of N-M blocking action.—It is well established that cobra NTs, α-bungarotoxin, and sea snake NTs all produce an anti-depolarizing N-M block by acting on the post-junctional membrane of the motor endplate (cf. 8, 13, 16–18). These NTs produce competitive inhibition of ACh response in the frog's rectus abdominis without affecting KCl response (30, 51, 65). The ACh response of both the isolated chick biventer cervicis (51, 65) and the cat gastrocnemius muscle in situ (Lee & Tsai, unpublished) is abolished before complete N-M block by these NTs. The response to micro-iontophoretically applied ACh or carbachol in the isolated frog sartorius is also blocked by these NTs (69, 70). The NTs depress endplate potentials (EPPs) as well as miniature EPPs (MEPPs) without affecting conduction in the nerve terminal and passive electrical properties of the muscle membrane (67, 70, 84). Unlike crude venoms, these purified NTs do not inhibit ACh release from nerve endings (51, 84). All these findings indicate that the mode of N-M blocking action of these NTs is essentially identical with that of *d*-tubocurarine.

Binding to cholinergic receptors.—Autoradiographic studies have demonstrated that α-bungarotoxin, cobra NT and erabutoxin b, all labeled with ^{131}I, accumulate on the motor endplate zone of the mouse or rat diaphragm (92–95). Both α-bungarotoxin and cobra NT have also been shown to bind specifically to the cholinergic receptors in the electric tissues of *Electrophorus electricus* and *Torpedo* (68, 69, 96–100). Pretreatment with *d*-tubocurarine, ACh, carbachol, or choline protects receptors against the binding of toxin (65, 67–69, 96). Because of its specific and irreversible nature of re-

ceptor binding, α-bungarotoxin has recently been used for characterization and isolation of cholinergic receptors (68, 69, 101). These studies revealed that the cholinergic receptor and the acetylcholinesterase molecules are distinct from each other, although the number of receptor sites is approximately equal to the number of acetylcholinesterase catalytic sites in the tissue.

Reversibility.—Unlike the effect of crude venoms, the N-M blockade by purified NTs from the venoms of *N. naja atra* and *N. nigricollis* can be reversed by either neostigmine or repeated washing (65, 66). In contrast, α-bungarotoxin (51, 67), as well as the NT(T_3) from *N. naja siamensis* venom (70), blocks N-M transmission irreversibly. Direct comparison of several NTs from different species reveals that α-bungarotoxin has the highest degree of irreversibility followed by the NT from *N. naja naja* venom, while both erabutoxin b and cobrotoxin are slowly reversible, suggesting that the richness in hydrophobic amino acids in their molecules may account for the irreversibility (Lee & Chang, unpublished).

Neurotoxins Belonging to the Second Group

As exemplified by β-bungarotoxin, the second group of NTs acts exclusively on the presynaptic site. In isolated preparations, neither ACh response nor the amplitude of MEPPS is reduced by β-bungarotoxin (51, 67). The reduction of ACh release is usually preceded by a facilitated release of transmitter and an increased frequency of MEPPs (67). In the frog sartorius muscle, β-bungarotoxin provokes fasciculation, which is blocked by *d*-tubocurarine (Chang, Huang & Lee, unpublished). Quantum contents of EPPs are finally decreased to the level of MEPPs and then complete failure of nerve impulse-induced release of transmitter occurs before disappearance of MEPPs (67). As in the magnesium-induced paralysis, the amplitude of EPPs on repetitive stimulation is rather sustained, suggesting the failure of release mechanism. Either high magnesium or low calcium delays, whereas a higher rate of stimulation accelerates the action of β-bungarotoxin (67). Ineffectiveness of choline as an antagonist (51) and unaltered uptake of choline in the β-bungarotoxin-treated rat diaphragm (102) exclude the possible involvement of a hemicholinium-like action.

Electron microscopic study has shown that β-bungarotoxin induces increased profiles of opened synaptic vesicles at the axolemma, accompanied with a decreased number of synaptic vesicles, and subsequently, almost complete depletion of the vesicles in the axon terminal (103). A similar finding has been described with black widow spider venom which produces a N-M block of presynaptic origin and displays little post-synaptic action (104). Surprisingly, however, a depression of ACh response was observed with this venom in the cat gastrocnemius muscle preparation in situ (105). In contrast, no appreciable change of ACh response was observed with β-bun-

garotoxin using the same muscle preparation (Lee & Tsai, unpublished). The differences between the actions of β-bungarotoxin and those of botulinum toxin have been discussed (17).

Selectivity of the Site of Action

Unlike the crude venoms, the purified NTs of both groups are devoid of any appreciable effects on the circulatory system except for the secondary changes due to respiratory paralysis (Lee & Tsai, unpublished). These NTs do not affect cardiac and smooth muscles, indicating that they have no affinity to muscarinic receptors (18, 51, 96, 106). Unlike botulinum toxin (107), β-bungarotoxin does not affect cholinergic nerve endings other than the motor nerve (51). While cobra venom produces ganglionic blockade, cobra NT exerts no appreciable effect on the ganglionic transmission (108). The fact that *Enhydrina schistosa* venom abolished the contraction of nictitating membrane of the cat produced by i.a. injection of ACh into the superior cervical ganglion (109) may be due to some component(s) other than NT, since the ACh response was found to be unaffected by erabutoxin b or by cobra NT (F. L. Lee & C. Y. Lee, unpublished).

Central Effects

Controversy over the central effects of cobra venom and its purified toxins has been fully discussed (18). Since neither the crude venom nor the purified NT can penetrate into the brain in sufficient amounts to account for such central effects (92, 94), they are most probably a secondary result from peripheral effects. One possibility has been the suggestion that hypoxia is the cause of the EEG changes (110). Cobra NT shows a lower lethality by direct central application than by i.v. injection in cats, and the respiratory paralysis is peripheral in origin even after central application of the toxin (18). Application of a neurotoxic fraction prepared from *N. naja* venom to the exposed cerebral cortex of the rat produces a long-lasting convulsant effect (111). Whether this effect is caused by the NT itself or by phospholipase A which may be present in the neurotoxic fraction remains to be clarified.

PHARMACOLOGICAL PROPERTIES OF THE CARDIOTOXIN GROUP

CTs affect various kinds of cells, both excitable and nonexcitable, causing irreversible depolarization of the cell membrane and consequently impairing both the function and structure of cells (18, 20). DLF, cobramines, toxin γ, and cobra cytotoxin are all pharmacologically indistinguishable from CT, although there might be some minor differences among them, mostly quantitatively (71, 72).

Lethality.—The lethal potency of CT in mice given i.p. is only one-twentieth of that of cobra NT, the latter being about six times more lethal than the crude venom (18, 20, 23). If assayed in cats given i.v., however, the

lethal potency of CT is about one half of that of cobra NT (18), as the cat is highly resistant to the N-M blocking action of the latter (112). If given s.c., the lethality of CT is very low because of its slow absorption from the s.c. site (94). Phospholipase A enhances the lethality of CT (71, Lee, unpublished).

Action on skeletal muscle.—CT causes contracture followed by paralysis of the skeletal muscle (20, 113), probably a consequence of irreversible depolarization of the cell membrane (20, 67, 84). These properties were previously attributed either to phospholipase A (79) or to cobra NT (80), but are most probably due to the coexistence of CT (84). The depolarizing effect of CT is enhanced by phospholipase A at a concentration which by itself does not depolarize the membrane (84). After deprival of calcium with EDTA, no contracture is produced, although the depolarizing effect remains unaffected (20). On the other hand, high calcium inhibits the contracture (113) as well as the depolarizing effect (Chang, Lee & Wei, unpublished). The contracture is also prevented by polyanions (gangliosides, RNA and heparin) (72), but not by procaine or neoantergan (113). Also, unlike the scorpion toxin (114, 115), CT does not provoke twitching and fibrillation of skeletal muscle.

Action on peripheral nerve.—The axonal conduction of peripheral nerves is blocked by CT (DLF) at high concentrations (116). This effect is greatly potentiated by phospholipase A which by itself does not affect axonal conduction (Chang, Lee & Wei, unpublished). CT combined with phospholipase A is considered the factor in cobra venom responsible for inducing conduction block. The terminal nerve spike is also abolished by CT (84).

Action on smooth muscle.—CT induces a contraction of the guinea-pig ileum at a concentration higher than that of phospholipase A (18, 20). This effect is partially inhibited by atropine, procaine, or morphine, suggesting the involvement of nervous elements of the gut, in addition to its direct action on muscle membrane. The response of the gut to nicotine as well as to transmural electrical stimulations is first enhanced and then depressed by CT at higher concentrations. In the muscle depolarized by KCl no response to CT is observed (Lee & Wei, unpublished). CT causes vasoconstriction of the rabbit ear vessels which is partially antagonized by phenoxybenzamine (20).

Action on the heart.—CT causes augmentation of contraction at low concentrations and systolic arrest at high concentrations in isolated heart preparations (20, 117). Although a digitalis-like action has been suggested (117), CT acts on the heart by an entirely different mechanism (cf. 18). The ventricular fibrillation provoked by CT (toxin γ) in the rat is com-

pletely antagonized by the injection of $CaCl_2$. CT induces automaticity in the rat isolated left atrium and reduces irreversibly the transmembrane potential of guinea-pig ventricular muscle (119).

Circulatory effects.—CT causes an initial rise in systemic arterial blood pressure followed by a progressive decline leading to cardiac arrest (20, 121). While the pulmonary artery pressure is markedly increased by crude cobra venom as well as by the phospholipase A fraction, it is increased only slightly by CT (121). Cardiac output as well as stroke volume is decreased, ventricular contractile force depressed, and total peripheral resistance increased by CT (20, 121).

Action on ganglionic transmission.—CT as well as cobra venom, if administered directly into the arterial supply of the superior cervical ganglion of the cat, produces a complete ganglionic block after a transient phase of stimulation, probably due to irreversible depolarization of the ganglion cells (108).

Local irritant action.—CT provokes congestion and chemosis of the rabbit conjunctiva and edema of the rat paw (20). It causes myolysis and inflammatory reaction but no hemorrhage at the site of injection (Lee et al, unpublished). The hemorrhagic effect observed on canine lung surface caused by cobra venom is dependent on its content of a basic polypeptide, most probably identical with DLF (122–124). Heparin forms an inactive complex with the hemorrhagic factors of cobra venom (123–125). The local irritant action as well as the cytotoxic effect of CT (72) may account for the local tissue damage observed in cobra bite (126).

Direct hemolytic effect.—The direct lytic factor (DLF) which can lyse washed erythrocytes of several animal species (21, 22, 127) has been identified with CT (71, 72). CT is, by itself, only weakly hemolytic, but acts synergistically with phospholipase A (21, 71, 72, 128–130). Erythrocytes of different animal species show striking variations in their susceptibility to hemolysis by CT (72, 128). The precise mode of action of CT on the red blood cells as well as its synergistic effect with phospholipase A is not known. Since the action of CT depends on the integrity of disulfide bonds (130, 131), it was suggested that the synergistic effect is due to an alteration of the membrane structure caused by interaction of CT with SH groups of membrane constituents, and that the combination of basic charge with disulfide bonds is a general structural feature of peptides that enable phospholipase A to cause hemolysis (131, 132). Contradictory to this hypothesis is the finding that cobra NT, a basic polypeptide with disulfide bonds, does not enable phospholipase A to cause hemolysis (Lee & Wei, unpublished), whereas mellitin, being devoid of disulfide bonds, does cause potentiated hemolysis with phospholipase A (131, 133). Similarly, gramici-

din S and several synthetic basic copolymers with lipophilic groups, despite the absence of disulfide bonds, do facilitate phospholipase A to cleave phospholipids of red cell ghosts (134). It was, therefore, suggested that the basicity of natural and synthetic polypeptides, by promoting electrostatic attraction, is held responsible for their attachment to the membrane, whereas the lipophilic side chains are involved in the facilitation of the approach of the phospholipase to the phospholipid substrate situated inside the membrane (134).

Cytotoxic effects.—A cytotoxic factor (cytotoxin) showing preferential cytotoxicity to Yoshida sarcoma cells both in vitro and in vivo has been isolated from Indian cobra (*N. naja*) venom (25, 26). The claim that the cytotoxin is different from DLF or CT (26) was not substantiated; it does cause direct hemolysis of erythrocytes from the guinea-pig, dog, and cat, contracture of skeletal muscle and systolic arrest of isolated frog heart (Lee et al, unpublished). CT from *N. naja atra* venom also exhibits cytotoxic effects not only on HeLa and KB cells (72) but also on Yoshida sarcoma cells in vitro (Hayashi, personal communication). However, CT is ineffective in preventing the growth of Yoshida sarcoma in vivo (Lee & Lin, unpublished). The claim that cytotoxin has phospholipase C activity (26, 134a) is unconfirmed (72, 74). Phospholipase C from *Clostridium welchii* does not exhibit cytopathic effects on HeLa cells (72). Cytotoxin combines initially with surface membrane components of the susceptible cells, producing swelling of the cells, followed by shrinkage of the membrane, which is accompanied by release of cytoplasmic RNA and protein (135). Competitive experiments using phospholipids suggested combination of cytotoxin with the more acidic phospholipid components of lipoproteins present in the plasma membrane of susceptible cells (135).

Biochemical effects.—CTs (cobramines A and B) from *N. naja* venom inhibit iodide accumulation by thyroid slices, amino acid and 3-*O*-methyl-D-glucose transport by small intestine, and the uptake of para-aminohippurate and amino acids by kidney cortex slices (23, 136–138). Although accumulation of these substances is thought to be linked to cation concentration or movement, Na^+ efflux from human erythrocytes or the inhibition of this process by ouabain is unaffected by cobramines (137). However, the Na^+ pump of the isolated toad bladder is inhibited by cobramine B, as is the resting potential difference (Mendoza, unpublished). The inhibitory effect on the accumulation of anions has a rapid onset, is temperature-dependent, and is prevented by polyanions or antivenom, but it is reversed with great difficulty, presumably because of very firm binding to tissue (138). The major portion of the inhibition of I^- accumulation by thyroid slices is due to increased I^- efflux from the tissue. This appears to be a result of a general increase in the permeability of the cell membrane, since the cell also becomes "leaky" toward K^+ (136, 138).

Toxin γ (CT from *N. nigricollis* venom) inhibits oxygen consumption of and amino acid uptake by KB cells. It causes a sharp decrease of nucleotide triphosphates of the cells. These effects are antagonized by calcium (139).

Anticholinesterase activity.—The cholinesterase inactivating factor in cobra venom (140–142) has recently been identified with CT (143). This anticholinesterase activity is inhibited not only by $MgCl_2$, $CaCl_2$ or univalent cations (141, 143) but also by polyanions. While the contracture inducing activity of CT is abolished after reduction of disulfide bonds, its anticholinesterase activity remains unaffected (143). Polylysine as well as protamine mimics the anticholinesterase activity of CT, but neither lysine nor arginine possesses this activity (143). All these findings suggest that CT inactivates the cholinesterase by ionic binding of its basic groups with the anionic sites of cholinesterase molecules and that inorganic cations interfere with their interaction. Because of such an interference, no anticholinesterase effects could be observed with CT in any biological system (141).

NEUROTOXINS FROM CROTALID AND VIPERID VENOMS

Most of the crotalid and viperid venoms are characterized by their actions on the local tissue (myonecrosis, hemorrhage, etc), blood coagulation and circulatory system, but usually do not cause neurotoxicity such as muscular paralysis due to N-M blocking action (cf. 8–12, 14, 16). Nevertheless, venoms from several crotalid (144–147) and from at least one viperid species, *Echis carinata* (148, 149), exhibit a weak N-M blocking action demonstrable only, in most instances, in experiments on amphibia. The South American rattlesnake, *Crotalus durissus terrificus,* constitutes an exception, secreting markedly neurotoxic, nonhemorrhagic, nonmyonecrotic venom (150–154).

Toxins from *Crotalus durissus terrificus* Venom

The chemistry and pharmacological properties of this venom have been recently reviewed (10, 16). Two regional varieties can be identified; one type is "crotamine-positive" and the other is "crotamine-negative" (150, 151). Crotoxin (153) is responsible for the N-M block elicited by the crude venom of either type (152, 154).

Crotoxin.—Crotoxin, which showed both neurotoxic and indirect hemolytic properties (153), has a molecular weight of 30,000, and a pHi of 4.7, and behaved as a homogeneous protein (155, 156), has been proven to be a molecular complex of an acidic and a basic protein (157–161). Whereas the acidic protein lacks the hemolytic and neurotoxic activity of crotoxin and the basic protein shows only the high indirect hemolytic activity of crotoxin, a mixture of the two components shows restoration of the high neurotoxicity of crotoxin (159–161). Crotactin, separated from crotoxin (162,

163), also appears to be a combination of these two components (161). The hemolytic activity of the basic component is greatly depressed by the acidic one (160).

Crotoxin induces flaccid paralysis in most animal species (152). The N-M block in mammalian muscles is of the nondepolarizing type, predominantly or exclusively post-junctional (154). In the denervated rat hemi-diaphragm, ACh is antagonized by doses of crotoxin smaller than those necessary to block N-M transmission in the rat phrenic nerve-diaphragm preparation (154). On the other hand, its activity in antagonizing carbachol in the frog sartorius muscle is rather small (164). In the latter muscle, crotoxin produces spontaneous contractions prior to complete N-M blockade. A reduction of MEPP frequency always precedes the onset of large potentials that are followed by an explosive burst of MEPPs. The quantal content of transmitter released by an impulse is rapidly reduced by crotoxin, though a small increase occurs prior to block (164). It is suggested that crotoxin may act in both pre- and post-synaptic sites but is more active presynaptically in amphibian muscles and more postsynaptically in mammalian muscles (Brazil, personal communication).

Crotamine.—Crotamine is a polypeptide toxin, strongly basic (pHi 10.3), with a molecular weight of about 5500 (165–167). It is composed of 46 residues of 15 common amino acids including four half-cystines, but devoid of threonine, alanine, and valine (167). Crotamine resembles cobra CT in its very high lysine (11 residues) and low arginine (2 residues) contents. The N-terminus is tyrosine and the C-terminus glycine (167).

Crotamine induces a contracture of skeletal muscle in mice and rats (85, 150, 167a, 167b) but no effects in chicks (167a). On diaphragm of the rat it elicits an immediate contracture followed by spontaneous and irregular contractions and tachyphylaxis (85, 167b). This effect is inhibited by tetrodotoxin, Ca^{++}, Mg^{++}, and K^{+}, but only partly by chronic denervation or previous curarization (85, 150, 167b). The toxin sensitizes the frog rectus adominis to K^{+} and to a much lesser degree to ACh (85, 167b). The mode of action has not been fully elucidated, but a change in Ca^{++} or Na^{++} permeability on the muscle fiber membrane has been postulated (167a, 167b). The difference between the mode of action of crotamine and that of veratrine has been discussed (16, 85, 167a, 167c).

Convulxin.—Convulxin is another NT isolated from some *C. d. terrificus* venoms (168–170). It is an acidic (pHi 6), nondialysable protein which is devoid of both phospholipase A and proteolytic activities. It causes a brief period of apnea, loss of equilibrium, convulsions, nystagmus, salivation, and violent intestinal contractions in unanesthetized cats and dogs after i.v. administration. The apnea appears to be reflex in origin, arising from stimulation of receptors in lungs. Little is known of the mechanisms and sites of its convulsive and other effects.

Gyroxin.—Gyroxin is another NT separated from the crotoxin complex (171). It is also a nondialyzable, acidic protein. It produces a typical neurological syndrome in mice, which consists of a cataleptic posture followed by whirling movements. This syndrome is similar to that produced by a labyrinth lesion, but its mechanism of action is unknown.

Neurotoxins from Other Crotalid Venoms

A survey of North American rattlesnake venoms revealed the presence of small proteins with neurotoxic activity in only 6 out of 27 venoms (172–174). The purified toxins from the venoms of *Crotalus adamanteus* (173), *C. h. horridus, C. h. atricaudatus,* and *C. v. viridis* (174) are characterized by having small molecular weights, pHi above 10.8, and pharmacological activities both in vivo and in vitro resembling those of crotamine. Some similarities in their amino acid composition to that of crotamine are also found. Of special interest is the finding that the toxins from *C. adamanteus* and *C. h. atricaudatus* produce myocardial damage in mice, leading to elevation of serum enzyme levels (173, 175).

Neurotoxins from Viperid Venoms

Although neurotoxicity is displayed by venoms from several viperid species (176, 177), viperotoxin isolated from *Vipera palestinae* venom (178) appears to be the only NT so far purified from viperid venoms. Viperotoxin is a nondialyzable basic protein that contains 108 amino acid residues in a single chain cross-linked by three disulfide bridges. This toxin is unique among the snake venom NTs in causing circulatory rather than respiratory failure. It has been suggested that viperotoxin acts primarily on medullary vasopressor centers leading to lethal circulatory disturbances (179, 180). It remains to be proven, however, that such a basic protein can pass through the blood-brain barrier in sufficient quantity to produce central effects.

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